Ecology of Organohalide-Respiring *Dehalococcoides mccartyi*: Corrinoid Cofactor-Related Community Interactions and Controls over Strain Selection

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To my angels of my life, my Grandmother Ayşe and my Mom Figen

for the unconditional love and support

"Rise to the challenge, to live your own life.
Success is not something that can be measured or
worn on a watch or hung on a wall…
[It] is the certain knowledge that you have become yourself,
the person you were meant to be from all time."
George Sheehan

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Abstract

Organohalides such as tetrachloroethene (PCE) and trichloroethene (TCE) are among the most prevalent toxic groundwater contaminants. Remediation of organohalide-contaminated sites has high priority, and efficient and cost-effective remedies are needed to prevent environment and human exposure through contaminated water. Bacterial organohalide-respiration plays a major role in organohalide detoxification. *Dehalococcoides mccartyi* (*Dhc*) are key mediators in bioremediation, since only *Dhc* strains have been documented in complete detoxification of chlorinated ethenes to benign ethene. *Dhc* depends on other microorganisms in the environment for essential growth requirements (e.g., hydrogen and vitamins). For successful implementation of the reductive dechlorination to remediate contaminated sites, microbial interactions controlling *Dhc* reductive dechlorination must be elucidated. The overall objective of this research was to address the key gaps in the scientific understanding of the controls over *Dhc* reductive dechlorination activity, including *Dhc* corrinoid-related interactions with other microorganisms. Detailed hydrogeological and microbial characterization of mixed chlorinated solvent contaminated Third Creek site (Knoxville, TN) attributed an important role to the creek sediment, where organohalide-respiring bacteria (e.g., *Dhc* and *Dehalobacter*) co-exist, for detoxification of contaminants. Different chlorinated solvent-amendments affected *Dhc* strain selection and non-dechlorinating microbial composition in enrichment cultures derived from Third Creek sediment. Corrinoid-auxotroph *Dhc* require corrinoid cofactor for the reductive dehalogenase enzyme systems. Microorganisms including *Acetobacterium*, *Clostridium*, *Geobacter*, and methanogens were identified as corrinoid-producers in the enrichment cultures. 5,6-dimethyl-benzimidazole cobamide (DMB-Cba) was the most abundant corrinoid in enrichment cultures to support *Dhc* reductive dechlorination. Different lower base-

amendments affected *Dhc* reductive dechlorination rates and extents. Lower base-amendments to enrichment cultures caused a shift from production of DMB-Cba to production of corrinoids with the amended lower bases, some of which caused lower dechlorination rates. In addition, different *Dhc* strains became abundant with different lower base-amendment in cultures, demonstrating the role of corrinoid in *Dhc* strain selection. Lastly this research demonstrated that different geochemical conditions and corresponding microbial populations determined the composition and concentration of bioavailable corrinoid pools; thus directly controlling *Dhc* reductive dechlorination activity. The findings of this research are relevant to environmental remediation practitioners and provide valuable information for improving bioremediation strategies to achieve successful contaminated-site cleanup.

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1,2-D	1,2-Dichloropropane
1,2-DCA	1,2-Dichloroethane
5-MeBza	5-Methylbenzimidazole
5-OHBza	5-Hydroxybenzimidazole
5-OMeBza	5-Methoxybenzimidazole
5,6-DiBrBza	5,6-Dibromobenzimidazole
BES	2-Bromoethane sulfonate
bvcA	vinyl chloride reductive dehalogenase gene
Bza	benzimidazole
Cba	cobamide
*c*DCE	*cis*-1,2-Dichloroethene
CERCLA	Superfund or Comprehensive Environmental Response, Compensation, and Liability Act
cfrA	trichloroethane or chloform reductive dehalogenase gene
cVOC	chlorinated volatile organic carbon
DAD	diode array detector
DCA	1,1-Dichloroethane
dcpA	dichloropropane reductive dehalogenase gene
Dhb	*Dehalobacter*
Dhc	*Dehalococcoides mccartyi*
Dhgm	*Dehalogenimonas*
DMB	5,6-Dimethyl-benzimidazole
DNA	deoxyribonucleic acid
DNAPL	dense nonaqueous phase liquid

DNase	deoxytibonuclease
dNTP	deoxyribonucleotide triphosphate
EPA	U.S. Environmental Protection Agency
GC	gas chromatograph
HPLC	high performance liquid chromatography
mg/L	milligram per liter
MNA	monitored natural attenuation
NPL	National Priorities List
OHRB	organohalide-respiring bacteria
OTU	Operational Taxonomic Unit
p-Cre	*p*-cresol
PCE	tetrachloroethene
pceA	tetrachloroethene reductive dehalogenase gene
Phe	phenol
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RDase	reductive dehalogenase
redox	reduction-oxidation
rpm	revolution per minute
sp.	species (singular)
spp.	species (plural)
TCA	1,1,1-Trichloroethane
TCE	trichloroethene
tceA	trichloroethene reductive dehalogenase gene
VC	vinyl chloride
vcrA	vinyl chloride reductive dehalogenase gene
v/v	volume per volume

1 Introduction

1.1 Significance and Motivation

Chlorinated solvents have been widely used in a variety of industrial, military, and household applications since the 1940s. Improper handling and disposal caused widespread groundwater contamination and generated difficult cleanup scenarios.[1-4] The current EPA National Priorities List (NPL) comprises 1,315 Superfund sites in the U.S. and its territories that are impacted by hazardous substances listed under CERCLA.[5] Tetrachloroethene (PCE), trichloroethene (TCE), dichloroethenes (DCEs) and vinyl chloride (VC) are risk drivers at many sites. VC was found in at least 622 of the 1,662 hazardous waste sites that have been proposed for inclusion on the NPL, and its precursors PCE, TCE and DCEs are present at least 771, 861 and 1,045 of these sites, respectively.[6] PCE is speculated as human carcinogens[7] and TCE and VC are known human carcinogen.[8, 9] Chlorinated ethenes are the primary risk drivers at many sites, thus efficient and cost-effective remedies are needed to prevent human exposure through contaminated water and vapor intrusion into dwellings.

1.2 Background

1.2.1 Bioremediation: The role of organohalide-respiring bacteria

Halogenated organic compounds are also called organohalides. Organohalide respiration is a bacterial energy-conserving respiratory process, wherein halogenated hydrocarbons are used as electron acceptors, and the removal of halogen substituent (chlorine for chlorinated hydrocarbons) from the halogenated organic molecule occurs with the addition of a hydrogen atom.[10] The removal of halogens from the halogenated hydrocarbons generally reduces or eliminates the toxicity or makes the halogenated compounds more biodegradable, which make this process very important for remediation of contaminated sites.[11]

Biodegradation plays a major role in the transformation and detoxification of chlorinated compounds, and has had documented successes when implemented as a stand-alone technology at monitored natural attenuation (MNA), biostimulation, and bioaugmentation field sites, or as a polishing step at sites where physical-chemical treatment was chosen as the primary remedy.[12-16]

Organohalide-respiring bacteria (OHRB) are microorganisms capable of conserving energy for growth via reductive dehalogenation of halogenated hydrocarbons. OHRB are of environmental importance and the key players anaerobic bioremediation because they can detoxify or transform the toxic halogenated groundwater contaminants to non-toxic forms.[16, 17] OHRB have been identified from diverse phylogenetic branches including the *Proteobacteria*, *Firmicutes* and *Chloroflexi*[18-21] (Figure 1.1). Species belonging to different genera (e.g., *Geobacter, Dehalobacter* (*Dhb*), *Desulfitobacterium*, and *Sulfurosprillum*) have been demonstrated to couple PCE reductive dechlorination to TCE or *cis*-1,2-dichloroethene (*c*DCE) for energy conservation.[16] To date, only members of the organohalide-respiring *Chloroflexi* have been demonstrated to mediate reductive dechlorination beyond the DCE level. Only strains of *Dehalococcoides mccartyi* (*Dhc*) are capable of complete reductive dechlorination chlorinated ethenes to environmentally benign ethene.[16] *Dhb* belonging to the phylum *Firmicutes* are involved in dechlorination of a range of chlorinated compounds including PCE, TCE, chlorinated ethanes[22] and chlorinated methanes.[23-25] *Dehalogenimonas* (*Dhgm*) spp. within the *Chloroflexi* have been demonstrated in dehalogenation of chlorinated alkanes[26] and recently also in reductive dechlorination of *trans*-1,2-dichloroethene (*t*DCE) to VC[27]. OHRB, including *Geobacter, Desulfuromonas*, *Dehalobacter*, *Sulfurospirillum* and *Anaeromyxobacter* are all non-obligate organohalide-respirers because of their metabolically versatile nature.[28] In contrast, presently known organohalide-respiring *Chloroflexi* (*Dhc* and *Dhgm*) are all obligate organohalide-respirers, meaning that they possess very restricted metabolism.[28, 29]

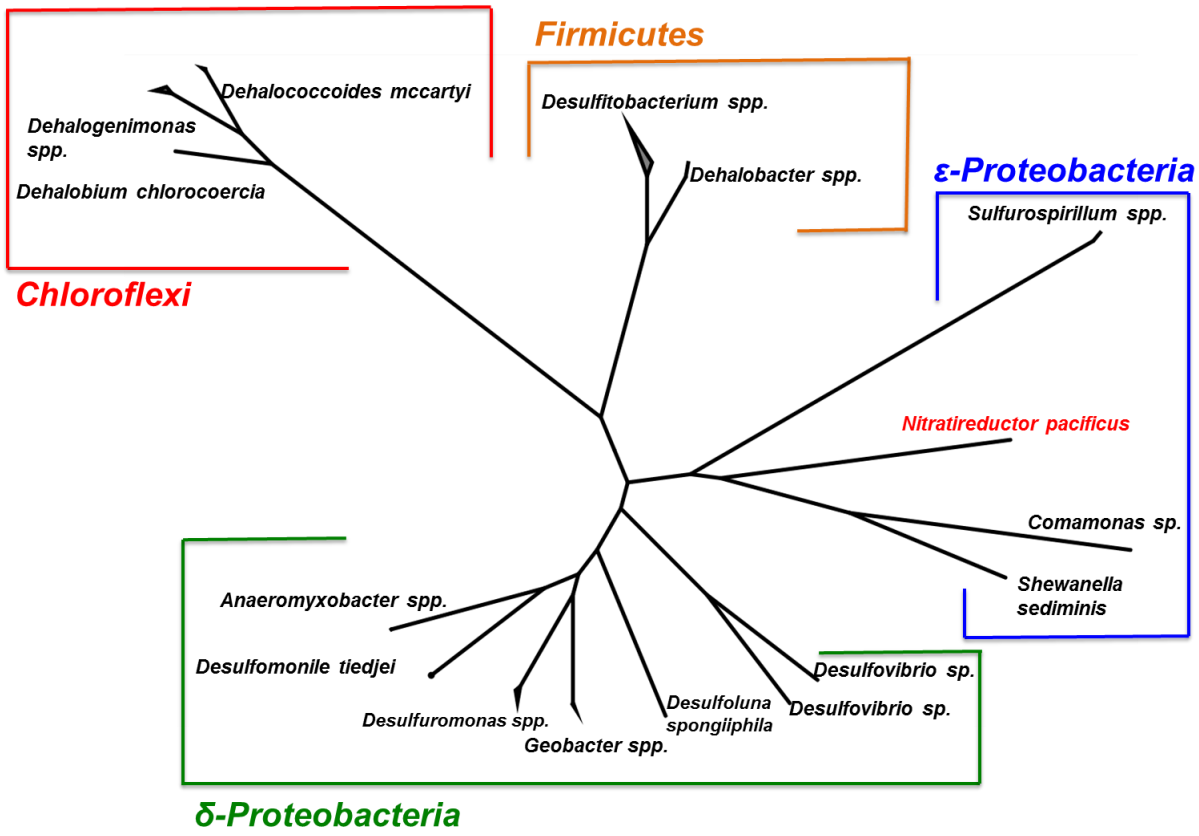

Figure 1.1. Phylogenetic tree of 16S rRNA gene sequences of the known OHRB.
Full length 16S rRNA gene sequences were obtained from NCBI and SILVA data bases, aligned by MAFF in Geneious™ Software. The phlogenetic tree was built using RAxML v7.2.8.

1.2.2 Role of aquifer-sediment transition zones for contaminant attenuation

High density chlorinated solvents form dense non-aqueous phase liquid (DNAPL) pools and ganglia that feed contaminant plumes for decades.[30] An even greater challenge is the removal of DNAPL from fractured rock formations, in part because a significant mass of chlorinated solvents might be stored in low-permeability rock matrix, and back diffusion generates a long-term contaminant source.[3, 30-33] Various *in situ* remediation technologies, including bioremediation,[16, 30] thermal[33, 34] and chemical[35] treatments have been successfully applied to porous media aquifers; however, the remediation of fractured bedrock formations remains challenging, mainly due to high flow rates in channels, difficulties in characterizing fracture networks and targeted delivery and mixing of remedial fluids.[30, 31, 33, 35-38] Recent studies have shown the relevant role of "hotspots" of anaerobic microbial activities for contaminant attenuation in hyporheic zones (i.e., aquifer-sediment transition zones).[39-41] Treatment at the fracture-sediment interface can be an alternate approach to prevent contaminant discharge from fractured bedrock formations. The role of the fracture-sediment interface for contaminant detoxification at a chlorinated solvent contaminated site, Third Creek site was addressed and results are presented in Chapter 2.

1.2.3 *Dehalococcoides mccartyi* and community interactions

The keystone bacteria involved in complete reductive dechlorination of chlorinated ethenes to environmentally benign ethene belong to the recently published species *Dhc* of the novel bacterial class *Dehalococcoidia*[21, 29, 42] *Dhc* have a unique and highly restricted lifestyle and require specific chlorinated hydrocarbons as electron acceptors, acetate as carbon source and hydrogen as electron donor to fuel their energy metabolism.[29, 43, 44] Dechlorinating-mixed cultures containing *Dhc* generally exceed pure culture performances in terms of *Dhc* growth yields and dechlorination rates.[10, 21, 45-47] The detection of *Dhc* 16S rRNA genes and reductive dehalogenase (RDase) genes, which encode the key enzymes responsible for catalyzing reductive dechlorination reactions[29, 48]

in contaminated sites does not always result in complete dechlorination of the contaminants, which could be attributed to the composition of supportive community members (e.g., corrinoid-producing).[10, 49] Some studies demonstrated that the community members sustain *Dhc* growth by maintaining a reducing environment and providing essential growth factors (e.g., H_2, corrinoid cofactor) that *Dhc* require.[45, 50-52] Relevant community members typically include fermenters, acetogenic bacteria and methanogens[46, 53-55] Non-dechlorinating members ferment organic electron donors and generate hydrogen and acetate. Another possible role of the other community members is that they might protect *Dhc* from oxidative stress.[55] These observations indicate that community members have important roles in the dechlorination process by supporting *Dhc* growth requirements. Hence, elucidating the *Dhc* interactions with other key community members, and determining the *Dhc* growth requirements are crucial for enhancing chlorinated solvent detoxification at contaminated sites.

1.2.4 *Dhc* reductive dechlorination: The central role of the corrinoid cofactor

Key to the reductive dechlorination process are RDases, which strictly require corrinoid cofactor.[29, 56-58] Figure 1.2 depicts the structure of cobalamin and several known, naturally occurring lower bases. Genome annotations of sequenced *Dhc* isolates (strains 195, VS, BAV1, CBDB1 and GT) indicated the presence of two other types of corrinoid-dependent enzyme systems (i.e., corrinoid-dependent ribonucleotide reductase and corrinoid iron sulfur proteins) in addition to RDases.[59-61] However, described *Dhc* strains lack ability for *de novo* corrinoid biosynthesis,[59, 61] and *Dhc* pure cultures require the addition of vitamin B_{12} (i.e., cyanocobalamin) to the growth medium,[62, 63] The *Dhc* genomes contain putative corrinoid salvaging and remodeling (i.e., removing and replacing the lower ligand) genes that could fulfill the requirement for special corrinoid.[59-61] *Dhc* coexist with microbial populations in various environments including groundwater, subsurface, river and marine sediments.[64] Methanogens, acetogens and fermenting microorganisms have been found to produce different corrinoids with benzimidazole, purinyl, and phenolyl as lower bases.[65-67]

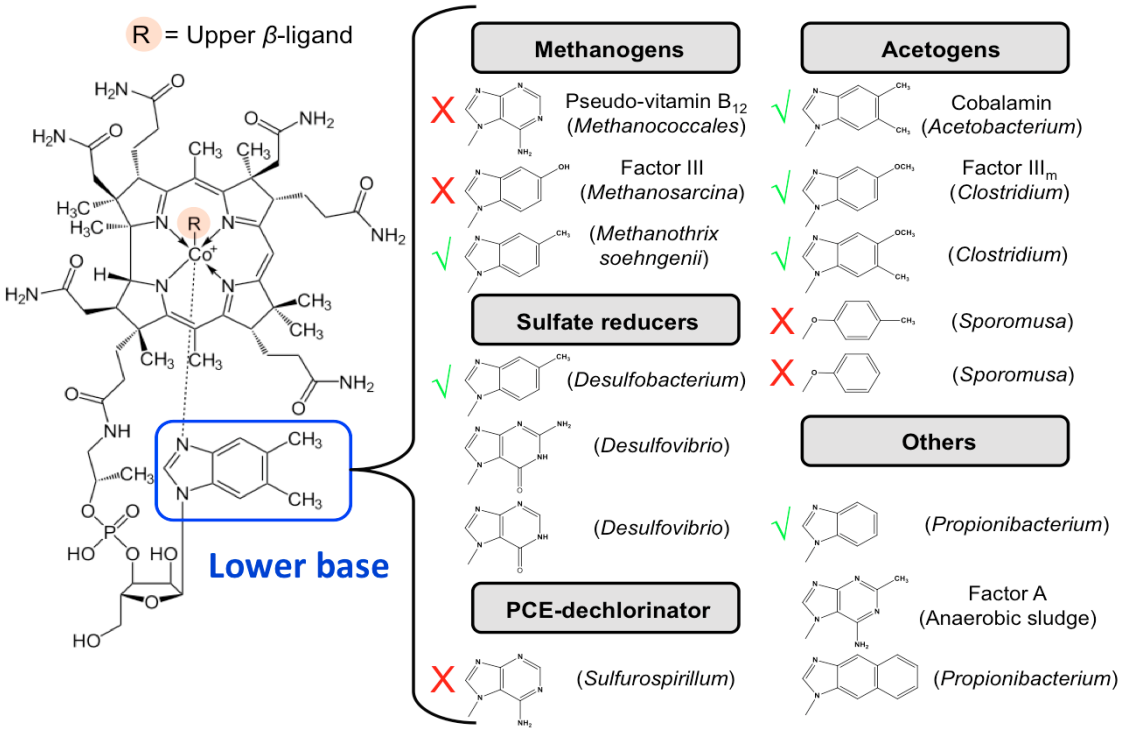

Figure 1.2. Structures of cobalamin and lower bases of several characterized cobamides

Nomenclature: Corrinoids contain the corrin ring system. Corrinoids with a central cobalt atom and both the upper β-base (i.e., ligand) and lower α-base are cobamides. Cobamides with 5,6-dimethyl-benzimidazole (DMB) as the lower α-base are cobalamins; the addition of a CN-group to the upper β-ligand position generates cyanocobalamin (vitamin B12). Cobamides lacking a lower base are cobinamides. The lower ligands marked with "√" re-present cobamides that support *Dhc* growth and "X" depicts cobamides not utilized by *Dhc* (Figure 1.2. courtesy of Dr. Jun Yan).

Unexpectedly, recent findings demonstrated that *Dhc* require specific types of corrinoids, and the methanogens and acetogens tested could not produce corrinoids that support *Dhc* activity.[63, 68, 69] On the other hand, *Geobacter* spp. possess complete pathways for *de novo* corrinoid biosynthesis. In *Dhc*-*Geobacter* co-cultures, both *Geobacter lovleyi* strain SZ and *Geobacter sulfurreducens* produced extracellular corrinoids; however only the corrinoid produced by *Geobacter lovelyi* strain SZ supported *Dhc* growth.[68] Dechlorination in inactive co-cultures reconstructed following the addition of 5',6'-dimethylbenzimidazole (DMB), as the lower base of cobalamin (i.e., vitamin B_{12}). This demonstrates that cobamide with DMB as lower base supports *Dhc* reductive dechlorination activity. These and recent findings show that a range of cobamides support *Dhc* activity; however reductive dechlorination rates and extents show differences in response to different types of comabides.[70] Depending on the site biogeochemical conditions and the type of substrate(s) (i.e., electron donors) used for biostimulation (i.e., electron donors), microorganisms that do not produce corrinoids that support *Dhc* growth, may dominate. In such scenarios, continuous biostimulation and bioaugmentation will not lead to the most efficient contaminant detoxification. Hence, knowledge of the community members producing lower bases and/or corrinoid(s) that support *Dhc* activity, as well as the biogeochemical conditions that favor the synthesis of required growth factors by community members, will enable strategies to overcome bioremediation limitations and promote efficient chlorinated compound detoxification.

1.2.5 *Dehalococcoides* strain diversity and adaptive evolution

While PCE and TCE are reductively dechlorinated to DCE by phylogenetically diverse bacteria, only certain strains of *Dhc* have been shown to completely dechlorinate PCE beyond DCE to benign ethane.[29] *Dhc* have streamlined, small genomes that reflect their highly specialized metabolic capabilities[51]. Each *Dhc* strain contains a unique complement of up to 36 RDase genes, whereas their 16S rRNA gene sequences show 98-100% sequence identity.[60, 71] The majority of RDases are encoded within two variable regions on the chromosome, termed high plasticity regions (HPRs) neighboring the origin of replication.[60 51]. The remainder of the genomes (~90%) is highly conserved between

strains. Within the genus, *Dhc* phylogeny is structured into 3 subgroups (i.e., Pinellas, Victoria and Cornell). Members of these subgroups have been obtained from geographically dispersed contaminated sites and pristine environments.[21, 29, 43, 51, 72] Unexpectedly, members from different geographic sources with distinct dechlorination capabilities often cohabit the same contaminating sediments,[64] raising the questions about *Dhc* substrate specificity and integrated reductive dechlorination by multiple strains. To date, all *Dhc* isolates or chlorinated ethene-dechlorinating consortia were derived with amendment of PCE or TCE at the beginning, before sub-culturing with *c*DCE and VC to enrich *c*DCE and VC dechlorinating strains (i.e., BAV1, VS and GT). The presence of such a large variety (up to 36) of RDase genes in *Dhc* genomes suggests that each *Dhc* strain may derive energy from wider yet undiscovered-variety of chlorinated compounds in the environment; however, the relationship between the complement of RDase genes (i.e., different strain) and *Dhc* substrate specificity is still unclear. Knowledge of the strains (and RDase genes) that are selected under different geochemical conditions (e.g., different chlorinated ethene concentrations), may enable strategies to overcome VC stalls and promote complete detoxification.

Despite the fact that *Dhc* reductive dechlorination has been studied for many years, important knowledge gaps remain. Many argue that within the last 100 years, anthropogenic dissemination of chlorinated solvents resulted in the evolution of reductive dechlorination, and todays *Dhc* is a product of the recent selection pressure. On the other hand, recent studies revealed the widespread presence of phylogenetically diverse RDase-homologous genes and frequent detection of *Dhc*-related 16S rRNA genes in pristine subsurface marine sediments.[73, 74] The metabolic properties of these diverse RDases are not clear yet[74] but dehalogenation of chlorinated compounds was detected in marine sediments,[75] indicating the evolutionary history of reductive dechlorination is much older than can be explained by the relatively recent anthropogenic release of chlorinated compounds. The complement of RDase genes is dynamic and appears to undergo continuous exchange, thus allowing evolution of new dehalogenation phenotypes.[60, 76] Recent comparative genomic analysis revealed that both VC RDase genes, *bvcA* and

vcrA, appear to be horizontally acquired, and specialization of *Dhc* for organohalide respiration is not strictly the result of the anthropogenic release of chlorinated compounds.[60, 77] Based on these observations and findings, the factors that control *Dhc* strain diversity and evolution remain unknown. This question will be addressed in this research by exploring the effect of available electron acceptors and corrinoids on *Dhc* strain selection.

1.3 Thesis Rationale and Research Objectives

The application of microbial reductive dechlorination process and especially of organohalide-respiring *Dhc* in bioremediation have been widely accepted as a cost-effective and efficient option for remediation of chlorinated solvent-contaminated sites. For successful implementation, the microbial interactions and environmental parameters, which control *Dhc* reductive dechlorination must be elucidated. Understanding *Dhc* interactions with other microorganisms and environmental factors that control *Dhc* reductive dechlorination activity can advance development of better and site-specific bioremediation and bioaugmentation strategies. Also of interest is elucidating environmental factors that control *Dhc* strain selection in the environment, and contribute to its evolutionary development. The overall objective of this research, therefore, was to address the key gaps in the scientific understanding of the controls over *Dhc* reductive dechlorination and strains selection, and *Dhc* interactions with other microorganisms in the environment. The specific objectives of this research, the results of which are detailed in **Chapter 2-5** are outlined below. A review of relevant literature was presented in **Chapter 1**, and overall summary and conclusions of this research are presented in **Chapter 6**.

<u>The current research is based on the following hypotheses:</u>

1. The Third Creek fracture-sediment interface is a natural barrier preventing contaminant discharge into surface water.

2. Community members control *Dhc* reductive dechlorination activity by supporting *Dhc* growth requirements (this research focuses especially on corrinoid cofactor).
3. Chlorinated solvents affect composition of dechlorinating microbial populations, and *Dhc* strain selection in the environment.
4. Corrinoid and lower base bioavailability is one of the major controls over *Dhc* reductive dechlorination activity, and strain selection.
5. Site specific geochemical conditions affect available corrinoid pools, hence *Dhc* activity.

Research Objective 1: Evaluate the role of anaerobic microbial activities for contaminant attenuation at a fracture-sediment interface at the Third Creek site.
Research Objective 2: Identify the key community members, and their contribution to *Dhc* reductive dechlorination activity.
Research Objective 3: Demonstrate the effects of available electron acceptors on composition of microbial populations, and *Dhc* strain selection
Research Objective 4: Identify the key community members that sustain *Dhc* corrinoid requirements and demonstrate that the community and site-specific geochemical conditions affect the available corrinoid pool, and hence *Dhc* activity.

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2 Natural Attenuation in Streambed Sediment Receiving Chlorinated Solvents from Underlying Fracture Networks

2.1 Abstract

Contaminant discharge from fractured bedrock formations remains a remediation challenge, mainly due to difficulties in characterizing complicated fracture networks and implementing corrective actions. We applied an integrated approach to assess the natural attenuation potential of sediment that forms the transition zone between upwelling groundwater from a chlorinated solvent-contaminated fractured bedrock aquifer and the receiving surface water. *In situ* measurements demonstrated that reductive dechlorination in the sediment prevented chlorinated compounds from reaching the water column. Microcosms established with creek sediment or *in situ* incubated Bio-Sep® beads dechlorinated chlorinated ethenes to ethene, 1,1,1-trichloroethane to chloroethane, 1,2-dichloropropane to propene, carbon tetrachloride to chloroform, and chloroform to dichloromethane, which was further degraded. Quantitative PCR (qPCR) and 16S rRNA gene amplicon pyrosequencing revealed the abundance and spatial distribution of known dechlorinator biomarker genes within the creek sediment. Phylogenetic classification of bacterial and archaeal sequences revealed a relatively uniform distribution of microorganisms at a spatial horizontal scale of 300 meters, but both *Dehalococcoides* and *Dehalobacter* were more abundant in deeper sediment, where $5.7 \pm 0.4 \times 10^5$ and $5.4 \pm 0.9 \times 10^6$ 16S rRNA gene copies per gram of sediment were measured, respectively. The characterization demonstrates that (i) conditions conducive for chlorinated solvent degradation exist near the fractured bedrock-sediment interface, (ii) multiple dechlorinator populations degrading chlorinated C_1-C_3 alkanes and alkenes coinhabit the sediment, and (iii) multiple sampling strategies are required to fully assess a site's natural attenuation potential. Microbial processes at the fractured bedrock-sediment interface were crucial for preventing contaminant mobilization to the water column, emphasizing the relevance of this sediment environment (critical zone) for contaminant attenuation.

2.2 Introduction

Chlorinated solvents have been widely used in a variety of industrial, military, and household applications since the 1940s.[1-4] Extensive use, improper handling and disposal resulted in widespread subsurface and groundwater contamination.[2, 5] Common groundwater contaminants include toxic tetrachloroethene (PCE) and trichloroethene (TCE), which pose a significant risk to human health.[6, 7] Chlorinated solvents tend to form dense non-aqueous phase liquids (DNAPL), which move gravitationally along interconnected fractures and form pools in low points in fractured bedrock formations. In addition to DNAPL pools, a significant mass of chlorinated solvents may penetrate low-permeability zones[8-12] where back diffusion from the rock matrix into water-bearing fractures can cause long-term groundwater contamination.[10, 13-16]

In the last two decades, various *in situ* remediation technologies, including bioremediation,[13, 17] thermal[15, 18] and chemical[3] treatments have been successfully applied to treat chlorinated solvent contamination in porous medium aquifers; however, the remediation of fractured bedrock formations remains challenging due to high flow rates in main fractures, back diffusion of contaminant from low-permeability zones, difficulties in characterizing complicated fracture networks, and the challenge of targeted delivery of remedial fluids.[1, 3, 4, 8, 13, 15, 19] An alternate remedial approach focuses on treatment at the fractured bedrock-sediment interface, where contaminated groundwater discharges to surface waters. Recent studies have shown that "hotspots" of anaerobic microbial activities play relevant roles for contaminant attenuation, including in hyporheic zones (i.e., aquifer/fractured bedrock-to-sediment transition zones).[20-27]

Organohalide-respiring bacteria use chlorinated hydrocarbons as terminal electron acceptors, and a number of species belonging to different genera (e.g., *Geobacter, Dehalobacter* (*Dhb*), *Desulfitobacterium*, and *Sulfurosprillum*) have been demonstrated to couple PCE reductive dechlorination to TCE or *cis*-1,2-dichloroethene (*c*DCE) for energy conservation.[17] To date, only strains of the species *Dehalococcoides mccartyi*

(*Dhc*) have been implicated in complete reductive dechlorination to environmentally benign ethene.[17] *Dhb* belong to the phylum *Firmicutes* and are involved in dechlorination of a range of chlorinated compounds including PCE, TCE, chlorinated ethanes[28] and chlorinated methanes.[29-31] *Dehalogenimonas* (*Dhgm*) spp. have been implicated in dehalogenation of chlorinated alkanes[32] but recently also in *trans*-1,2-dichloroethene (*t*DCE) reductive dechlorination to VC[33]. 16S rRNA genes from known dechlorinating bacteria and reductive dehalogenase (RDase) genes, which encode the key enzymes responsible for catalyzing reductive dechlorination reactions,[34] serve as biomarkers to assess natural attenuation potential and monitor bioremediation performance.

At a former metal manufacturing facility located adjacent to Third Creek, a Tennessee River tributary in Knoxville, TN (Figure 2.1A), chlorinated solvents, primarily PCE, TCE, 1,1,1-trichloroethane (TCA), and carbon tetrachloride (CT) were released and penetrated the underlying fractured bedrock formation. Observations made during various groundwater investigations suggested that contaminants migrated through the regolith and entered karst voids and fractures. Figure S2.1 presents the conceptual migration pathways for cVOCs based on depictions of potential source areas at the site and the current understanding of bedrock structure. The bedrock structural features influence the movement of cVOCs in the aquifer. High groundwater cVOC concentrations, exceeding 10 mg/L, were measured in bedrock groundwater samples, with the highest groundwater cVOC concentrations were detected at MW-100 and MW-102 (Figure S2.2). The persistence of solvents at such high concentrations indicates the accumulation of immobile separate-phase cVOCs in poorly interconnected fractures characteristic of the bedrock and the slow dissolution of cVOCs from these fractures. PCE and TCE comprised most of the cVOCs in bedrock groundwater samples (i.e., >95 mg/L, 96-100% of total cVOC concentrations), whereas concentrations of TCA and CT did not exceed 0.1 mg/L (data is not shown). In a few locations, cDCE and VC were detected in bedrock groundwater samples but they did not comprise more than 35% and 4% (mg/mg) of total cVOC concentrations, respectively (data is not shown). Ethene was not detected in bedrock groundwater samples.

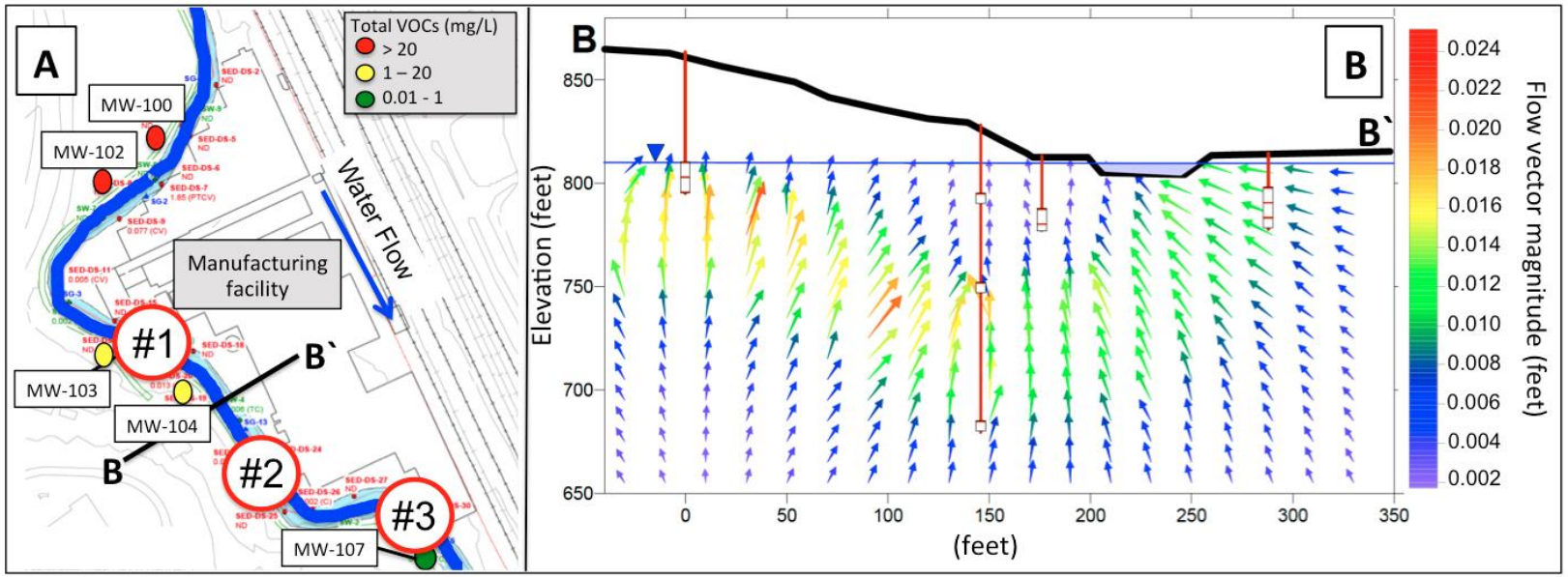

Figure 2.1. The Third Creek site and vertical groundwater gradients at the Third Creek site
A) The Third Creek site. The colored circles show the bedrock groundwater cVOC concentrations. The red circles (with #1, #2, #3) indicate the locations of seepage measurements and sediment collection for microbial analyses. **B)** Vertical groundwater gradients at the Third Creek site. The arrows indicate the direction and the magnitude of the groundwater flow direction [1 foot = 0.3 meter].

DCA was sporadically measured but CA was not detected in bedrock groundwater samples. These findings indicate limited natural attenuation of contaminants in the bedrock groundwater. Limited microbial transformation of contaminants in the fractured bedrock indicated the necessity of alternative remedies at the Third Creek site.

This study evaluated the role of the creek sediment, which separates groundwater in the fractured bedrock from surface water, as a natural barrier preventing contaminant discharge into Third Creek surface water. Integrated efforts characterizing the hydrogeological (e.g., flow paths) and microbiological site conditions at the Third Creek site demonstrated efficient natural attenuation in the sediment.

2.3 Material and Methods

2.3.1 Chemicals

Chlorinated compounds were of >99% purity. PCE and CT were purchased from ACROS Organics (Morris Plains, NJ), TCE was obtained from Fisher Scientific (Pittsburgh, PA), and *c*DCE, vinyl chloride (VC), ethene, TCA, dichloromethane (DCM), chloroform (CF), chloromethane (CM), 1,2-dichloropropane (1,2-D), 1,1-dichloroethane (DCA), and chloroethane (CA) were obtained from Sigma-Aldrich-Fluka (St. Louis, MO).

2.3.2 Site characterization

The manufacturing site is bounded by Third Creek on its west side (Figure 2.1A). The facility used chlorinated solvents as degreasers from the mid-1930s ending in the late 1990s. During the course of manufacturing activities, chlorinated solvents, primarily PCE, TCE, and, to a smaller extent TCA and CT, were released in different locations, resulting in contamination of the underlying groundwater-bearing fractured bedrock (Figure S2.1). Direct observation of soil and bedrock cores failed to locate DNAPL although contaminant concentrations as high as 120 mg/L were measured in non-flowing fractures (Figure S2.2). The presence of cDCE, VC, DCA and CF in several bedrock

monitoring wells indicated that some contaminant transformation occurred; however, high concentrations of parent compounds and no ethene and ethane formation indicated limited dechlorination capacity within the fracture network. Groundwater seepage rates were measured with leak-tested seepage meters.[35] Horizontal and vertical bedrock groundwater flow direction was evaluated using seasonal groundwater elevations from monitoring wells screened at multiple depths and staff gauges in the creek. Gravity-influenced cVOC movement likely contributed to the distribution of cVOCs within the aquifer, but the absence of any evidence for mobile DNAPL indicates that advective transport of cVOCs controlled by groundwater flow is the primary means of ongoing cVOC migration. Aquifer hydraulics and creek elevation appear to be the factors controlling migration and extent of cVOCs in groundwater. Horizontal gradients within the valley and upward vertical gradients in bedrocks demonstrated that contaminated groundwater discharges to the creek. Downstream of MW-103 towards to MW-107 (Figure S2.2, between locations #1 and #3 in Figure 2.1), the creek changes from a shallow pool and riffle stream to a deeper, slow moving stream. The bottom of the creek also transitions from sand, gravel and rock to silt, detritus and mud in this area.

2.3.3 Sediment pore water diffusion sampling for geochemical and contaminant measurements.

To measure concentrations of cVOCs, methane, ethene, and ethane, as well as geochemical parameters, sediment pore water diffusion samples were collected with depth-discrete diffusion samplers loaded with 40-mL glass vials.[36] Before placement of the samplers into the sediment, the 40-mL glass vials were filled with deionized water and covered with either polyethylene film for cVOC sampling, or a porous, nonwoven fabric for measurement of geochemical parameters. The samplers were installed in the same locations as the seepage meters were installed, 0 – 0.55 m (0-1.8 ft) below the sediment surface, and left in the creek for 2 weeks to achieve equilibrium with the pore water. After recovery of the samplers, the sample vials were immediately sealed and shipped to analytical laboratories (SiREM, Guelph, Canada and Microbac, Maryville, TN).

2.3.4 Sediment collection

Grab samples and sediment cores were collected from locations #1, #2 and #3 (Figure 2.1A). These locations were chosen based on the observed sediment cVOC concentrations and the site's hydrogeological characteristics. Top layer sediment samples were collected using autoclaved, sealable glass containers (Mason jars). Deeper sediment layers were obtained using direct push tools (AMS, Inc., American Falls, ID). The plastic liners and caps were wiped with 70% ethanol before use. All other materials (spatulas, containers, etc.) were autoclaved and aseptic techniques were applied to the extent feasible. All core samples were immediately transferred to sterile Mason jars, filled completely with creek water to exclude air, capped and placed in a cooler with ice packs.

2.3.5 Depth-resolved sediment collection

Depth-discrete diffusion samplers were employed simultaneously to collect sediment at multiple depths. Location #3 with measurable sediment pore water cVOC concentrations was selected to explore the depth distribution of dechlorinating populations. The diffusion sampler was loaded with eight 40-mL glass vials evenly spaced over a length of 99 cm (3.2 ft). The vial openings were covered with plastic mosquito netting (1-mm mesh size) held in place with rubber bands. The loaded sampler was pushed into the sediment to a depth of about 55 cm (1.8 ft). A second sampler loaded with customized Bio-Trap samplers (about 200 Bio-Sep beads per sampler, Microbial Insights, www.microbe.com) was placed in the sediment in the same location. After a 1-month incubation period, the samplers were removed to collect the glass vials with sediment material and the Bio-Sep bead cartridges. The vials were immediately closed with sterile Teflon-lined rubber septa and placed individually in Ziploc bags. The samples were immediately transferred to a cooler with ice and transported to the laboratory for microcosm setup and DNA extraction.

2.3.6 Sample handling

Depth-discrete diffusion samplers were employed simultaneously to collect sediment at multiple depths. Location #3 with measurable sediment pore water cVOC concentrations was selected to explore the depth distribution of dechlorinating populations. The diffusion sampler was loaded with eight 40-mL glass vials evenly spaced over a length of 99 cm (3.2 ft). The vial openings were covered with plastic mosquito netting (1-mm mesh size) held in place with rubber bands. The loaded sampler was pushed into the sediment to a depth of about 55 cm (1.8 ft). A second sampler loaded with customized Bio-Trap samplers (about 200 Bio-Sep beads per sampler, Microbial Insights, www.microbe.com) was placed in the sediment in the same location. After a 1-month incubation period, the samplers were removed to collect the glass vials with sediment material and the Bio-Sep bead cartridges. The vials were immediately closed with sterile Teflon-lined rubber septa and placed individually in Ziploc bags. The samples were immediately transferred to a cooler with ice and transported to the laboratory for microcosm setup and DNA extraction.

2.3.7 Microcosm setup

For sediment microcosm setup inside the anoxic glove box, approximately 4 g (wet weight) of the sediment from locations #1, #2, or #3 were transferred to sterile 60-mL glass serum bottles. Twenty-six mL of anoxic, bicarbonate-buffered mineral salts medium[37] amended with 5 mM lactate was added to each bottle before the vessels were sealed with autoclaved butyl rubber stoppers (Geo-Microbial Technologies, Inc., Ochelata, OK, USA). Neat PCE, TCE, *c*DCE, VC, TCA, DCA, 1,2-D, CT, CF, DCM, and CM were added into triplicate microcosms with 5 or 10 µL Hamilton glass syringes (Hamilton 85925 and 80370) to achieve initial aqueous phase concentrations of approximately 0.2 mM (12.5-33.1 mg/L). One microcosm for each treatment was autoclaved for 60 min at 121°C. An additional set of live control microcosms received all amendments except the cVOC additions.

To obtain depth-resolved information about microbial reductive dechlorination activity, additional microcosms were established with the *in situ* incubated Bio-Sep beads collected from the different depth intervals at Location #3. Inside the glove box, five beads were transferred to sterile 60-mL glass serum bottles before 30 mL of reduced mineral salts medium containing 5 mM lactate was added. The bottles were closed with butyl rubber stoppers and PCE, TCE, *c*DCE, VC, and TCA were added with a 5 or 10 uL Hamilton syringe to reach aqueous phase concentrations of approximately 0.2 mM. After a 2-month incubation period, 3% (vol/vol) culture suspension without beads was transferred to fresh medium amended with 5 mM lactate and 0.2 mM of the respective cVOC. Enrichment cultures that showed no reductive dechlorination received 6 mL of H_2 to ensure that electron donor availability was not a limitation. All microcosms and enrichment cultures were incubated statically at room temperature in the dark and monitored over a 20-month incubation period.

2.3.8 DNA isolation, qPCR, and 16S rRNA gene amplicon sequencing

To assess the presence of known dechlorinators in Third Creek sediment and to evaluate their spatial distribution in relation to the three different sampling locations and sediment depth at Location #3, qualitative PCR, quantitative PCR (qPCR), and 16S rRNA gene fragment amplicon sequencing were performed. DNA was extracted from 0.25 g of sediment samples using the MO BIO PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, CA). DNA extraction from Bio-Sep beads (~160 beads/depth) was performed by Microbial Insights using established procedures (www.microbe.com). Published primer sets and PCR conditions were used to amplify total bacterial,[38] *Dhc,*[39] *Dhb,*[40] *Dhgm*[32] and *Geobacter lovleyi* strain SZ (*GeoSZ*)[41] 16S rRNA genes and the 1,2-D to propene RDase gene *dcpA*.[42] For increased sensitivity of detection, a nested PCR approach was applied.[43] Initially, bacterial 16S rRNA genes were amplified using general primers,[44] followed by a second round of PCR using specific primer sets (Table S2.1) targeting the 16S rRNA genes of the dechlorinator of interest. A nested PCR approach was also performed to detect 1,2-D to propene RDase gene. Briefly, the degenerate primers B1R and RR2F[45]

and 10 to 20 ng of DNA extracted from sediment samples or from Bio-Sep beads were used in the initial PCR. The second round PCR used 1:10 dilutions of the amplicons from the first round PCR as template. The limits of detection for both direct and nested PCR assays were determined by amplification of the target genes using 10-fold serial dilutions of plasmids (~10^8 to 10^0 gene copies per µL template DNA) with the respective genes of interest, followed by visualization of the amplicons in 1 % agarose gels stained with SYBR Safe dye (Thermo Fisher Scientific, Waltham, Massachusetts, USA). About 10^7 and 10^4 gene copies per µL of template DNA were required for target gene detection in direct and nested PCR assays, respectively. qPCR to enumerate total bacterial, *Dhc* and *Dhb* 16S rRNA genes, as well as the *bvcA*, *vcrA*, *tceA,* and *cfrA* RDase genes used published primers and probes and followed established protocols (Table S2.1).

To compare the microbial communities at locations #1, #2 and #3, amplification of the hypervariable V1-V3 and V3-V5 regions of the 16S rRNA gene and subsequent pyrosequencing of the PCR amplicons was performed with barcoded-primers[46-49] (Table S2.1). Library preparation followed an established protocol[49] and pyrosequencing was performed on a 454 FLX Life Sciences Genome Sequencer (Roche Diagnostics) according to manufacturer's instructions. Sequences were processed and analyzed using the QIIME software package (v.1.04).[50] Briefly, sequences were first assigned to samples based on barcode matches, then denoised using the Denoise pipeline included in QIIME with default parameters to remove sequencing errors. Sequences were grouped into OTUs and clustered at a threshold of 97% sequence similarity using UCLUST.[51] The sequences were checked for chimeras using ChimeraSlayer with QIIME default parameters and aligned with the PyNAST algorithm.[52] The Greengenes database (http://Greengenes.secondgenome.com) was used for taxonomic assignments of OTUs. Alpha diversity values (i.e., within-sample diversity) were calculated using both Chao1 and OTU richness diversity metrics within the QIIME pipeline. Due to the variation in total sequence reads produced for each sample, the sequence data were normalized for the number of sequences in each sample by randomly sampling the minimum number of sequences per sample that was observed in each data set.[50]

2.3.9 Analytical methods

cVOCs, ethene, ethane, propene and methane were monitored using an Agilent 7890 gas chromatograph (GC) equipped with a flame ionization detector and a DB-624 capillary column (60 m by 0.32 mm with a film thickness of 1.18 µm) as described[53]. Standards were prepared by adding known amounts of individual analytes to 160-mL glass serum bottles containing 100 mL of water. The method provided linear detector responses for quantification of each analyte to 0.7 mM aqueous phase concentrations with a detection limit of about 2.5 µM.

2.4 Results

2.4.1 Third Creek site hydrogeological features

The groundwater seepages meters placed in creek sediment revealed maximum, mean and median seepage rates of 314, 67 and 19 g/ft^2 day, respectively, at Location #3. The broad range of measured seepage rates appeared to be influenced by the creek stage, location in the creek, and groundwater elevation. The lowest mean and median seepage rates of 4.4 and 0.5 g/ft^{2}d, respectively, were measured near Location #1, and occasionally negative values (i.e., loosing water) were observed. The seepage measurements and vertical and horizontal groundwater gradients demonstrated that Third Creek received groundwater from underlying fractures downstream of Location #1 (Figure 2.1B). Based on elevation data, the creek appears to transition from a losing to a gaining creek along the perimeter of the contaminated area near Location #1. Based on the average creek widths of 7.3 m (24 ft) between locations #1 and #2 and 10.4 m (34 ft) between locations #2 and #3, segment lengths of 226.5 m (743 ft) between locations #1 and #2 and 77.1 m (253 ft) between locations #2 and #3, the yearly volume of groundwater seeping into Third Creek between locations #1 and #3 was estimated at 11,000 to 45,000 gal/year using median and mean seepage rates, respectively. At Location #3, sediment pore water measurements detected *c*DCE, VC, ethene, ethane and methane in the deep sediment, whereas significantly lower cVOC concentrations were

measured near the sediment-surface water interface. Concentrations varied with depth (Figure 2.2) and maximum ethene, ethane and methane concentrations of 0.25, 0.08 and 5.2 mg/L, respectively, were measured near the sediment-surface water interface. cVOC concentrations were lower near the sediment-surface water interface and the *c*DCE and VC concentrations were less than the method detection limit of < 0.01 mg/L. Maximum *c*DCE and VC concentrations of 0.78 mg/L and 0.33 mg/L, respectively, were observed in the deeper sediment at Location #3. With the exception of *c*DCE (0.03 mg/L) in the deep sediment at Location #2, no cVOCs were detected in sediment pore waters collected at locations #1 and #2. TCE concentrations were generally below 0.01 mg/L in the deep sediment, but TCE was occasionally detected near the sediment-surface water interface (Figure 2.2, Table S2.2). In addition, concentration gradients of volatile fatty acids (VFAs) and sulfate were observed in the sediment at Location #3. VFA concentrations of 374 mg/L in the deep sediment pore water decreased to 4 mg/L in pore water collected near the sediment-surface water interface, and sulfate concentrations increased from <0.8 mg/L at deep sediment to 12 mg/L in the upper sediment layers at Location #3. Maximum sulfate concentrations of 130 mg/L were detected in the sediment layers at Location #2. Tables S2.2 and S2.3 summarize sediment pore water cVOC, ethene, ethane and methane concentrations along with geochemical parameters observed at the three sampling locations.

2.4.2 Reductive dechlorination in microcosms

The dechlorination patterns and dechlorination end products observed in the sediment microcosms are summarized in Table 2.1. In microcosms established with sediments collected at locations #1, #2, and #3, reductive dechlorination of chlorinated ethenes started within 1 week and stoichiometric conversion to environmentally benign ethene occurred within 4-5 weeks. In microcosms amended with TCA, sequential reductive dechlorination to DCA and CA occurred; however, ethane was not detected (Table 1). In both live and killed CT-amended microcosms, CT disappeared and no more than 40% of the initial amount of CT was recovered as CF, which was not further degraded. No further degradation of CF was observed.

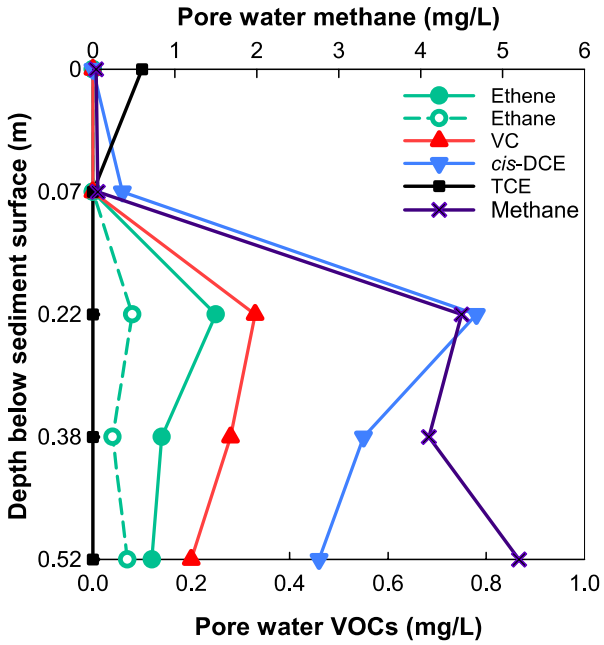

Figure 2.2. Sediment depth profile concentrations of cVOCs, ethene, ethane and methane at Location #3

In CF-amended microcosms (in the absence of CT), DCM formed transiently; however, CM, the reductive dechlorination daughter product of DCM, was not observed. In CM-amended microcosms, CM was not degraded. Microcosms amended with 1,2-D produced propene without intermediate formation of monochlorinated propanes suggesting a dichloroelimination reaction. Methane was produced in all live microcosms except in those amended with CT. No dechlorination daughter product formation and no methane formation occurred in control microcosms (except for the limited, abiotic CT-to-CF transformation observed in the CT-amended microcosms).

Table 2.1 summarizes the major dechlorination end products in microcosms established with *in situ* incubated Bio-Sep beads collected from eight discrete depth intervals. Initially, cVOCs could not be measured due to sorption to the Bio-Sep beads but quantitative analysis was possible in transfer cultures.

Table 2.1. Dechlorination products in sediment microcosms and Bio-Sep bead enrichment cultures

	cVOC substrate - Major degradation product (s)								
Sediment microcosms	**PCE**	**TCE**	***cis*-DCE**	**VC**	**TCA**	**DCA**	**1,2-D**	**CT**	**CF**
Location #1	Ethene[5]	Ethene[5]	Ethene[5]	Ethene[5]	CA[5]	ND	Propene[5]	CF[3]	DCM[#]
Location #2	Ethene[5]	Ethene[5]	Ethene[5]	Ethene[5]	CA[5]	ND	Propene[5]	CF[3]	DCM[#]
Location #3	Ethene[5]	Ethene[5]	Ethene[5]	Ethene[5]	CA[5]	CA[5]	Propene[5]	CF[3]	DCM[#]
Bio-Sep bead-derived enrichment cultures depth (m)	**PCE**		**TCE**			***cis*-DCE**		**VC**	**TCA**
Location #3 - 0	*cis*-DCE[3], VC[3], ethene[3]		TCE[1], *cis*DCE[1],VC[2], ethene[4]			Ethene[5]		VC[1], ethene[5]	TCA[5]
Location #3 - 0.07	VC[2], ethene[5]		Ethene[5]			VC[3], ethene[4]		Ethene[5]	TCA[5]
Location #3 - 0.17	Ethene[5]		*cis*-DCE[1], VC[2], ethene[5]			*cis*-DCE[3], VC[3], ethene[3]		VC[1], ethene[5]	TCA[5]
Location #3 – 0.22	*cis*-DCE[3], VC[3], ethene[3]		*cis*-DCE[2], VC[2], ethene[4]			Ethene[5]		Ethene[5]	TCA[5]
Location #3 – 0.30	*cis*-DCE[3], VC[3], ethene[3]		*cis*-DCE[1], VC[3], ethene[4]			Ethene[5]		Ethene[5]	TCA[5]
Location #3 – 0.38	Ethene[5]		Ethene[5]			Ethene[5]		Ethene[5]	TCA[5]
Location #3 – 0.45	*cis*-DCE[2], VC[2], ethene[4]		*cis*-DCE[2], VC[2], ethene[4]			*cis*-DCE[2], VC[2], ethene[5]		Ethene[5]	TCA[5]
Location #3 – 0.52	*cis*-DCE[3], VC[3], ethene[3]		Ethene[5]			VC[2], ethene[5]		Ethene[5]	TCA[5]

[1] < 10%; [2] 10-25 %; [3] 25-50 %; [4] 50-75 %; [5] 75-100 % (of total mass/bottle), ND: not determined

[#] DCM was further degraded with no evidence for reductive dechlorination because CM was not detected and microcosms failed to degrade CM.

PCE to TCE dechlorination occurred in cultures derived from all depth horizons; however, complete reductive dechlorination of polychlorinated ethenes to ethene was observed in cultures derived from only two of the eight depth horizons. Interestingly, VC-to-ethene reductive dechlorination occurred in cultures derived from all discrete depth horizons. In contrast to the sediment microcosms, TCA reductive dechlorination did not occur in any of the Bio-Sep bead-derived enrichment cultures. The addition of H_2 to transfer cultures that showed no or limited activity did not stimulate reductive dechlorination indicating that electron donor availability was not a limiting factor. Methane production occurred in all Bio-Sep bead microcosms and transfer cultures.

2.4.3 Detection and quantification of known dechlorinators and RDase genes

Direct PCR using DNA extracted from sediments as well as Bio-Sep beads did not yield target gene-specific amplicons. Nested PCR detected *Dhc* and *Dhb* 16S rRNA genes in all sediment samples from the three locations, including the depth-discrete sediment samples and the Bio-Sep beads collected at Location #3. Similarly, *Dhgm* 16S rRNA genes were detected with nested PCR in the majority of samples tested. *GeoSZ* 16S rRNA and *dcpA* genes were present in most of the sediment samples tested, but found in only one of the Bio-Sep bead samples (Table S2.4). About 10^5 *Dhc* 16S rRNA gene copies per gram of wet sediment were measured at sampling locations #1, #2 and #3. Somewhat greater variability was observed with *Dhb* 16S rRNA genes, and $2.1 \pm 0.5 \times 10^5$, $1.7 \pm 0.1 \times 10^6$, and $6.4 \pm 0.7 \times 10^4$ copies were measured per gram of wet sediment at locations #1, #2 and #3, respectively. The qPCR approach also revealed the spatial distribution of *Dhc* and *Dhb* 16S rRNA genes as well as RDase genes in the sediment column at Location #3 (Table 2.2). *Dhc* 16S rRNA genes were distributed throughout the sediment column and generally more abundant in deeper sediments layers with cell numbers exceeding 8.0×10^4 per gram of wet sediment or 4.0×10^4 per gram of wet Bio-Sep beads. The *bvcA* gene was present in numbers exceeding 7.0×10^5 per gram of wet sediment or 4.0×10^5 per gram of Bio-Sep beads (wet weight), and could be quantified in all sediment depth horizons. The *vcrA* gene was distributed throughout the sediment but was present in lower abundances, and could be quantified in only two sediment depths.

Table 2.2. Quantification of dechlorinator 16S rRNA genes and RDase genes via qPCR

Depth (m) Sediment samples	Gene copies per gram of wet sediment or gram of wet Bio-Sep bead samples					
	Bac 16S rRNA	***Dhc* 16S rRNA**	***Dhb* 16S rRNA**	***vcrA***	***bvcA***	***tceA***
0*	$3.7 \pm 0.1 \times 10^9$	$1.1 \pm 0.2 \times 10^5$	DNQ	DNQ[#]	$1.4 \pm 0.1 \times 10^6$	$3.5 \pm 0.2 \times 10^5$
0.07	$2.5 \pm 0.4 \times 10^9$	$8.9 \pm 0.8 \times 10^4$	$1.1 \pm 0.1 \times 10^5$	DNQ	$1.5 \pm 0.1 \times 10^6$	$3.2 \pm 0.2 \times 10^5$
0.17	$1.1 \pm 0.1 \times 10^9$	$9.9 \pm 1.5 \times 10^4$	DNQ	DNQ	$2.7 \pm 0.9 \times 10^6$	$1.9 \pm 0.3 \times 10^5$
0.22	$3.4 \pm 0.1 \times 10^9$	$8.7 \pm 1.2 \times 10^4$	$4.5 \pm 0.6 \times 10^5$	DNQ	$7.7 \pm 2.7 \times 10^5$	$1.8 \pm 0.1 \times 10^5$
0.30	$4.7 \pm 0.3 \times 10^8$	$9.2 \pm 0.8 \times 10^4$	DNQ	$1.3 \pm 0.2 \times 10^5$	$8.5 \pm 2.3 \times 10^5$	$2.8 \pm 0.6 \times 10^5$
0.38	$9.6 \pm 0.4 \times 10^8$	$1.4 \pm 1.5 \times 10^5$	$1.5 \pm 0.2 \times 10^6$	DNQ	$1.3 \pm 0.2 \times 10^6$	$1.9 \pm 0.4 \times 10^5$
0.45	$6.7 \pm 0.4 \times 10^8$	$1.3 \pm 0.1 \times 10^5$	$9.5 \pm 0.2 \times 10^5$	DNQ	$1.5 \pm 0.4 \times 10^6$	$1.6 \pm 0.9 \times 10^5$
0.52	$1.8 \pm 0.1 \times 10^8$	$5.7 \pm 0.4 \times 10^5$	$5.4 \pm 0.9 \times 10^6$	$7.1 \pm 1.9 \times 10^4$	$2.1 \pm 0.6 \times 10^6$	$2.2 \pm 0.6 \times 10^5$
Bio-Sep bead samples	**Bac 16S rRNA**	***Dhc* 16S rRNA**	***Dhb* 16S rRNA**	***vcrA***	***bvcA***	***tceA***
0*	$3.8 \pm 0.4 \times 10^7$	$6.9 \pm 1.6 \times 10^4$	$3.3 \pm 0.2 \times 10^5$	DNQ	$5.3 \pm 0.3 \times 10^5$	$2.6 \pm 0.3 \times 10^5$
0.07	$6.4 \pm 0.1 \times 10^7$	$4.3 \pm 0.7 \times 10^4$	$7.5 \pm 1.0 \times 10^4$	DNQ	$4.4 \pm 0.4 \times 10^5$	$9.9 \pm 1.1 \times 10^4$
0.17	$1.0 \pm 0.0 \times 10^8$	$7.4 \pm 0.9 \times 10^4$	DNQ	DNQ	$4.9 \pm 0.8 \times 10^5$	$2.9 \pm 0.4 \times 10^5$
0.22	$3.8 \pm 0.3 \times 10^8$	$7.5 \pm 0.8 \times 10^4$	DNQ	DNQ	$5.6 \pm 0.6 \times 10^5$	$2.1 \pm 0.1 \times 10^5$
0.30	$9.5 \pm 0.3 \times 10^7$	$1.2 \pm 0.1 \times 10^5$	$4.5 \pm 0.9 \times 10^4$	DNQ	$5.0 \pm 0.2 \times 10^5$	$2.4 \pm 0.2 \times 10^5$
0.38	$2.5 \pm 0.2 \times 10^8$	$1.1 \pm 0.4 \times 10^5$	$1.0 \pm 0.2 \times 10^5$	DNQ	$5.7 \pm 0.3 \times 10^5$	$2.1 \pm 0.8 \times 10^6$
0.45	$3.1 \pm 0.0 \times 10^8$	$9.2 \pm 2.2 \times 10^4$	$1.1 \pm 0.1 \times 10^5$	DNQ	$6.4 \pm 0.3 \times 10^5$	$4.7 \pm 0.2 \times 10^5$
0.52	$3.3 \pm 0.2 \times 10^8$	$3.8 \pm 0.2 \times 10^5$	$5.9 \pm 1.2 \times 10^4$	DNQ	$8.7 \pm 2.7 \times 10^5$	$7.6 \pm 1.4 \times 10^5$

*: Surface water – groundwater transition depth, [#]DNQ: Detected but not quantifiable.
All listed samples were from Location #3.

The *tceA* gene was present in higher numbers exceeding 1.0×10^5 per gram of wet sediment or 9.0×10^4 per gram of Bio-Sep beads throughout the sediment column. *Dhb* 16S rRNA genes were also detected throughout the sediment column but in greater abundance in deeper sediment horizons. The CF and TCA reductase gene, *cfrA*, was not detected in any sediment samples, and only one Bio-Sep bead sample (0.45 m depth, $1.7 \pm 0.2 \times 10^3$ gene copies per gram of wet Bio-Sep beads) tested positive.

2.4.4 Community structure of the Third Creek sediment

Barcoded pyrosequencing of bacterial and archaeal 16S rRNA gene amplicons from Third Creek sediment samples collected at locations #1, # 2 and # 3 resulted in a total 53,815 high-quality sequences after data processing. Comparison of the V1-V3 and V3-V5 regions sequencing results are summarized in Table S2.5 and Figure S2.3. Notably, the Location #3 sediment sample had the lowest bacterial operational taxonomic unit (OTU) richness among the sediment samples based on both Chao1 and OTU richness estimates (i.e., averages of 4,507 and 836, respectively). Location #1 had the highest Chao1 and OTU richness estimates (i.e., averages of 9,359 and 1,630, respectively) (Figure S2.4).

Based on the analysis of bacterial V3-V5 sequences, a total of 47 bacterial phyla could be assigned across all samples. Taxonomic classification of the bacterial OTUs at the class level is shown in Figure 2.3. The majority (up to 65%) of the OTUs belonged to the *Proteobacteria*, and *Bacteroidetes* and *Chloroflexi,* which contributed, on average, 15% and 6% of all V3-V5 bacterial sequences, respectively. *Nitrospirae* comprised less than 0.2% of the total 16S rRNA gene sequences in Location #3 sediment, whereas this group contributed more than 2% at locations #1 and #2. In sediment samples from locations #1 and #2, methanotrophs including members of family *Methylococcaceae* contributed up to 0.2% of total bacterial OTUs, but this group was not detected in sediment samples from Location #3. Sulfate reducers including members of the orders *Desulfobacterales*, *Desulfovibrionales*, and *Syntrohobacterales* contributed up to 4% of total bacterial sequences.

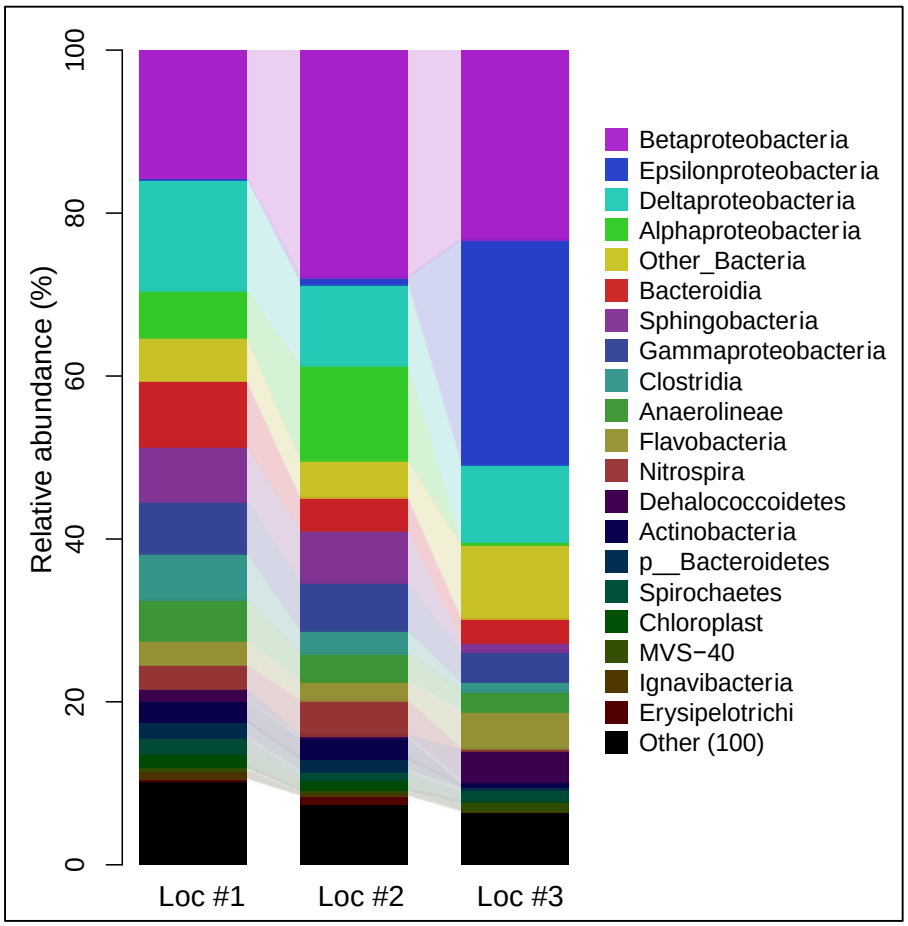

Figure 2.3. Bacterial community structure in Third Creek sediment at locations #1, #2 and #3

Classification was made at the class level based on 16S rRNA gene amplicon sequence analysis (V3-V5 region). "Other" refers to lower-abundance OTUs. The category 'Other_Bacteria' includes sequences that could not be classified at any taxonomic level. Sequences, which were classified at class level, are represented with their phylum level classification (i.e., p__). Loc #1, Loc#2 and Loc#3 represent the locations 1, 2,3 shown in Figure 2.

Based on genus level taxonomic classification, *Sulfuricurvum* (27% of total V3-V5 bacterial 16S rRNA gene sequences) was the most abundant genus in the sediment sample from Location #3, whereas this group contributed to less than 1% of the total OTUs observed in the sediments from other two locations. The application of a second primer (V1-V3) set corroborated the dominance of *Sulfuricurvum* 16S rRNA gene amplicons at Location #3.

Based on the class level taxonomic classification, OTUs belonging to the class *Dehalococcoidia* comprised up to 4% of the total bacterial sequences; however, sequences of the species *Dhc* were detected in lower abundances, comprising less than 0.1% of the total bacterial OTUs in the sediment samples collected from locations #1, #2 and #3. *Dhb* OTUs were only detected in the V3-V5 region sequence data of Location #1 in low abundances (< 0.1% of the total bacterial sequences). *Geobacter* OTU abundance exceeded 3% of the V3-V5 bacterial amplicon sequences obtained from locations #1 and #2, whereas less than 0.1% of the total bacterial sequences of the Location #3 sediment sample could be assigned to *Geobacter*. None of the amplicon sequences affiliated with the genus *Dhgm*. The analysis of bacterial V1-V3 region sequences contributed two additional phyla (*ABY1_OD1* and *Lentisphaerae)* both with average abundances of less than 0.1% of total bacterial sequences. Across all samples analyzed, the V3-V5 amplicon analysis contributed 11 phyla with total sequence abundances not exceeding 0.5%, which were not detected among the V1-V3 region amplicons. Despite these differences, taxonomic classification of bacterial amplicons based on V1-V3 and V3-V5 sequence analysis revealed similar results when OTUs that contributed at least 1% of the total sequences were included.

Archaeal V1-V3 sequences grouped into a total of 1,462, 1,296 and 1,779 OTUs for locations #1, #2 and #3, respectively. Based on the Chao1 and OTU richness estimates, the sediment sample from Location #2 had the lowest archaeal species richness, followed by the sediment sample from Location #3 (Figure S2.5). Taxonomic classification of the archaeal OTUs at the family level is shown in Figure 2.4.

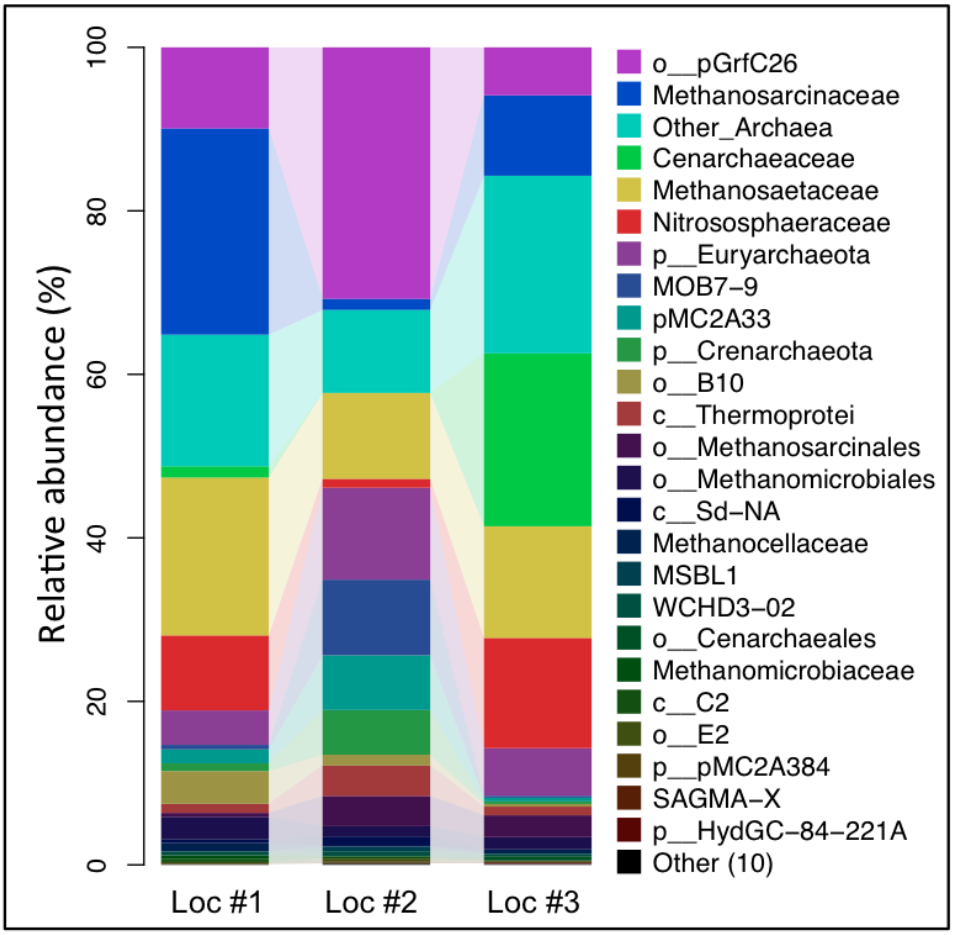

Figure 2.4. Comparison of archaeal communities in Third /creek sediment locations #1, #2 and #3

Classification was represented at the family level. "Other" refers to lower-abundance OTUs (see Table 7c). Sequences that could not be classified at any level of taxonomy are represented in the 'Other_Archaea' category. Sequences not classified at the family level are represented with their order (o), class (c) or phylum (p) classifications. Loc #1, Loc#2 and Loc#3 represent locations 1, 2, and 3 shown in Figure 2.1A

The majority (up to 60%) of archaeal OTUs belonged to the *Euryarchaeota*, followed by *Crenarchaeota* varying between 27 and 44% of the archaeal sequences). In the sediment sample from Location #2, order *pGrfC26* within the phylum *Crenarchaeota* was the most abundant group (30% of the total archaeal sequences). Sequences representing this group comprised less than 10% in sediment samples from locations #1 and #3. Sequences that could not be assigned to a genus within the family *Cenarchaeaceae* constituted the second most abundant group of sequences (15% of the total archaeal sequences) in the sediment sample from Location #3. Interestingly, this sequence cluster represented less than 1% of the archaeal sequences in the sediment samples from other locations. Sequences representing *Methanosarcina, Merhanosaeta*, and *Candidatus Nitrososphaera* were also abundant in the Third Creek sediments. It should be noted that the number of unique 16S rRNA gene amplicon sequences from all sediment samples did not reach saturation (Figures S2.4, S2.5), suggesting that the amplicon data underestimate the true species diversity in Third Creek sediment samples.

2.5 Discussion

2.5.1 The importance of critical zone interfaces for contaminant attenuation

Although microbial activity within fractures has been demonstrated,[4, 11, 54] poor matrix continuity, high flow rates, absence of dechlorinators, and unfavorable geochemical conditions often limit natural attenuation capacity.[11] At the Third Creek site, *in situ* dechlorination within the fracture network is insufficient to prevent cVOC from discharging into Third Creek sediment. Remarkably, contaminant release to surface waters is negligible, indicating that fractured bedrock-sediment interface mediates contaminant attenuation. Favorable biogeochemical conditions for organohalide-respiring bacteria exist within the Third Creek streambed sediment. The sustained presence and activity of organohalide-respiring bacteria allows the sediment to serve as an effective, natural reactive barrier that dechlorinates cVOCs before they are expressed into surface water.

2.5.2 Distribution of dechlorinators in Third Creek sediment

Enhanced biotransformation activity of chlorinated solvents has been observed in sediments receiving chlorinated solvent-polluted groundwater.[22-24, 26, 55] Although spatial variations in degradation activity have been reported within a 45-m long test area and apparently limited to certain activity hot spots in some sites,[22, 24, 26] the three tested Third Creek locations spanning approximately 300 m showed very consistent genetic markers for dechlorinating bacteria and dechlorinating activity in microcosm tests. Both *Dhc* and *Dhb* 16S rRNA genes were more abundant in deeper, and corresponding higher concentrations of dechlorination daughter products and VFAs were measured. Similarly, correlations between the detection of *Dhc*, organic carbon and contaminant concentration and composition in sediments have been observed at other sites.[23, 25, 56]

The microcosm experiments and molecular analyses demonstrated the presence of multiple *Dhc* strains with different dechlorination capabilities (i.e., *tceA*, *bvcA*, *vcrA,* and *dcpA* genes) in Third Creek sediment. Similarly, the distribution of multiple *Dhc* strains (with *bvcA*, *vcrA* and *tceA*) was observed in contaminated Zenne River (Belgium) sediment.[57] However, it is unclear whether different *Dhc* strains (i.e., *Dhc* populations with different RDase genes) have distinct properties that affect their dispersal and distribution throughout the sediment. For example, *dcpA* gene was detected and 1,2-D was dechlorinated to propene in the microcosms although 1,2-D was not a known contaminant used at the former manufacturing facility. Why the *dcpA* gene was present and able to be expressed in the absence of 1,2-D is unclear. While an upstream source of 1,2-D is possible, the chemical would be carried to the test area in surface water and the gaining nature of the stream should prevent penetration into the sediment. Therefore, the *dcpA* gene appears to be sustained in the absence of its primary known substrate.

In some samples *bvcA* and *tceA* gene copies were five- to ten-fold higher than the *Dhc* 16S rRNA gene copies (Table 2.2). This appears to be a common phenomenon that has been observed by others.[23, 58] It is curious that elevated RDase gene copies are only observed in certain “hot spot” locations. *Dhc* genomics analyses demonstrated that

individual RDase genes occur as single copy genes and there is currently no evidence for gene duplications as a possible explanation. Recent comparative genomic analyses suggested that *tceA*[59, 60] and VC RDases (i.e., *bvcA* and *vcrA*)[61, 62] can be horizontally acquired. These results could be evidence that non-*Dhc* hosts carry these functional genes in Third Creek sediment.

2.5.3 Co-occurrence of multiple contaminants at the Third Creek site

CT, CF, and TCA are potent inhibitors of reductive dechlorination of chlorinated ethenes;[29, 63] therefore, successful remediation requires a sequential treatment to alleviate the inhibition.[28] At the Third Creek Site, CF and TCA reductase *cfrA* was detected only in deep sediment layers, indicating that chlorinated methanes were degraded in the deeper sediment, allowing the degradation of chlorinated ethenes by *Dhc* strains in upper sediment layers. Such a stratification of organohalide-respiring organisms with distinct dechlorination capabilities can effectively overcome inhibition in mixed contaminant plumes.

2.5.4 Sediment versus Bio-Sep bead analysis

PCR analysis detected *Dhc*, *Dhb*, and *Dhgm* 16S rRNA genes in both sediment- and bead-derived DNA. In contrast, *GeoSZ* 16S rRNA genes were detected in most sediment samples but in only one Bio-Trap. These observations indicate that *in situ* enrichment can bias the results interpretation. Possible reasons why *GeoSZ* biomarkers were not detected with Bio-Sep beads include (1) the limited 30-day incubation period, which may have been insufficient for all sediment populations to colonize the beads, or (2) organism-specific differences in attachment behavior. The distribution of *Dhc* in porous matrices is obviously relevant for bioaugmentation applications, and studies have demonstrated that *Dhc* cells disperse following injection.[64, 65] What has not been explored is if individual *Dhc* strains with unique reductive dechlorination capabilities exhibit distinct propensity for attachment and biofilm formation. The lack of TCA dechlorinating activity in cultures initiated with beads suggests that the *Dhb* populations did not colonize the beads,

or were so strongly attached to the beads that the organism could not be transferred with culture supernatant.

2.5.5 Microbial community and heterogeneity in Third Creek sediment

The pyrosequencing and PCR data led to comparable conclusions regarding the presence of known organohalide-respiring populations, although *Dhb* and *Dhgm* 16S rRNA genes were only detected with PCR in several samples. Rarefaction analysis showed that saturation had not been reached (Figure S2.4) and insufficient sequence depth is a possible explanation for why *Dhb* and *Dhgm* OTUs were not identified using the sequencing approach.

Recovery of sequences representing members of the *Methylococcaceae* indicates the potential for oxidative cometabolism to contribute to contaminant transformation near the anoxic-oxic transition zone. Methanotrophs have been observed in oxic-anoxic transition zones in chlorinated solvent-contaminated sediments and groundwater plumes[66, 67], and implicated in contaminant cometabolism[66-68].

Despite of low nitrite concentrations in sediment pore water samples from location #3 (Table S2.3), a high abundance of sequences belonging to ammonia-oxidizers of the family *Cenarchaeaceae*[69, 70] was observed. To date, these organisms are not known to use other metabolic pathways for energy conservation and their abundance may present yet-to-be discovered metabolic versatility. Indeed, the genome sequence of the ammonia-oxidizing *Candidatus Nitrososphaera gargensis* showed the potential for utilization of a greater substrate spectrum.[71] Additionally, Third Creek is considered impaired due to urban run-off with inorganic nutrients, including ammonium, being recognized water contaminants.

An interesting observation was the high abundance of sequences (i.e., 27 % of total bacterial sequences) belonging to *Sulfuricurvum* sp. in the sediment sample collected at Location #3. Sequencing of the two regions of the 16S rRNA gene (i.e., V1-V3 and V3-

V5) yielded similar results demonstrating that this observation is not an artifact. The characterized *Sulfuricurvum* spp. are facultative anaerobes capable of oxidizing inorganic sulfur compounds (i.e., S^0, $S_2O_3^{-2}$, H_2S) common in anoxic sediments with NO_3^- or O_2 as electron acceptors.[72, 73, 74, 75] Sulfate concentrations increased towards the oxic-anoxic transition zone (Table S2.3), showing that the oxidation of sulfur compounds mediated by *Sulfuricurvum* spp. is a relevant process in Third Creek sediment near Location #3. Additional DNA sequencing from the same location one year later revealed much lower *Sulfuricurvum* abundances (i.e., <0.02% of total sequences, data not shown) indicating that the population size is undergoing dynamic changes. The first sampling event may have captured a *Sulfuricurvum* bloom, indicating that a single sampling event may not enough for robust site characterization.

2.5.6 Potential electron donors supporting reductive dechlorination

A major challenge for sustained reductive dechlorination is the supply of fermentable substrates to maintain hydrogen concentrations that do not constrain the activity of organohalide-respiring bacteria. Bioavailable organic carbon (BOC) has been reported as an important parameter affecting natural attenuation at chlorinated solvent-contaminated sites.[76] The quality and quantity of BOC are affected by hydrogeological, physicochemical and biological processes in the subsurface and the BOC pool has been demonstrated to undergo dynamic spatial and temporal variations.[77, 78] High concentrations of VFAs and methane, as well as depletion of sulfate in deeper sediment horizons of Third Creek suggested infiltration of organic material from underlying fractures. Quantifying the relationship between BOC, such as petroleum hydrocarbons, and cVOC dechlorination is of interest for estimating the persistence of reductive dechlorination during the natural attenuation of the cVOC contaminated groundwater. Operating data were too vague quantify the amount of cVOC and dissolved BOC released at the site. Without reliable data on the mass of contaminants released to the subsurface, accurate relationships between BOC and reductive dechlorination potential cannot be calculated.

Hypothetically, open-top cleaning technologies in metal manufacturing industries generated petroleum hydrocarbon-laden waste streams, and this process required approximately 750 kg of chlorinated solvents per 100 kg of petroleum hydrocarbons removed.[79] Petroleum compounds are often a major component of chlorinated solvent DNAPLs, and their fermentation can be a major source of VFAs and hydrogen.[76, 80] Assuming that about two-thirds of the chlorinated solvent volatilized,[79] the spent metal-degreasing fluid that penetrated the subsurface contained substantial amounts of petroleum hydrocarbons. Using a measured groundwater discharge of 45,000 gal/year and the highest measured cVOC concentration of 95 mg/L, the total annual cVOC mass discharged into Third Creek sediment would be about 16.1 kg. Based on a presumed chlorinated solvent to petroleum hydrocarbon ratio of 2.5:1 in the NAPL phase, an electron donor demand of 1 mol of H_2 to release 1 mol of chloride in reductive dechlorination, and an average of 51 mol of H_2 released during the fermentation of 1 kg of hydrocarbons, the theoretical H_2 release approximately meets 85% of the theoretical electron donor demand (see Appendix 2 for calculations). Due to hydrocarbons dissolution and fermentation, the chlorinated solvent:petroleum hydrocarbon ratio will likely decrease over time. In addition, hydrogen is a preferred electron donor for other respiratory processes, including ferric iron reduction, sulfidogenesis and methanogenesis, and not all hydrogen produced in petroleum hydrocarbon fermentation will be available for reductive dechlorination. Based on these assumptions and calculations, the amount of fermentable petroleum hydrocarbons will probably not be sufficient to maintain a long-term hydrogen flux to sustain reductive dechlorination of the chlorinated solvent mass residing in the fractures. Thus, lasting natural attenuation must rely on organic materials (e.g., plant debris) settling through the water column and fermentation processes occurring in the upper layers of the creek sediment. In contrast to the current situation, fermentation reactions will increase hydrogen flux in the upper sediment horizons (i.e., the hydrogen concentrations gradients will invert), suggesting that the zone of active reductive dechlorination may transition to shallower sediment zones. The flow of energy within the creek sediments and the microbial community that it sustains is complex and not fully characterized; however, the sediment ecosystem has been shown to have rich

genetic and metabolic diversity that seems to be persistent and self-sustaining. Although the current situation is stable and the infiltration of chlorinated solvents into the Third Creek water column is not a concern, careful monitoring is warranted to ensure microbial activity is sustained and chlorinated solvent concentrations remain below regulatory limits in the water column.

The detailed hydrological, geochemical and microbial characterization of the Third Creek site attributed a critical role to the streambed sediment for contaminant attenuation. Phylogenetically distinct groups of organohalide-respiring bacteria co-exist in the sediment and apparently effectively avoid cVOC toxicity and inhibition resulting in effective contaminant degradation. A major goal should be to define the environmental conditions that allow a perturbed (i.e., chlorinated solvent spills) system to adapt and sustain a microbial community that effectively detoxifies the contaminants. If the boundary conditions required for a natural attenuation system to emerge can be defined, and become analytically tractable, monitored natural attenuation can possibly be successfully implemented at many more sites.

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2.8 Appendix 2

Petroleum Hydrocarbon-electron donor calculations

Assumptions:

1. *Oil content:*
   - 70% of total hydrocarbons are paraffinic hydrocarbons containing an average of 30 carbon atoms (30C).
   - 30% is total hydrocarbons are aromatic hydrocarbons containing average of 6 carbons (6C).
   - 50% of paraffinic hydrocarbons are saturated; the other 50% is unsaturated.
   - Unsaturated paraffinic hydrocarbons contain 15 double bonds; and the structure of unsaturated paraffinic hydrocarbons does not contain any triple bonds.
2. Total mass of released hydrocarbons is fermented completely (100% fermentation) in the Third Creek sediment, and H_2 is one of the fermentation products.
3. All calculations were made based on total mass of 100 kg of hydrocarbon released.

Step 1: Total H_2 (mol) released from degradation of saturated paraffinic hydrocarbons

Molar mass of saturated paraffinic (MW_{sp}) 30C-hydrocarbon:

$MW_{sp} = C_nH_{2n+2}$

MW_{sp} (n=30) = (12 g/mol × 30) + [1g/mol × (2 × 30 + 2)],

MW_{sp} ($C_{30}H_{62}$) = 422 g/mol

Total mass of released oil (kg) = 100 kg

Paraffinic ratio (%) = 70

Saturated paraffinic ratio (%) = 50

Total mass of saturated paraffinic hydrocarbons (M_{sp}, kg):

M_{sp} = 100 kg × 0.7 × 0.5, **M_{sp} = 35 kg**

Total mole of saturated paraffinic hydrocarbons (M_{sp}, mol):

M_{sp} = 35 kg ÷ 422 g/mol × (1000 g ÷ 1 kg), M_{sp} = **82.94 mol**

Total mole of H_2 released from degradation of 82.94 mol saturated paraffinic 30C-hydrocarbons ($M_{H2,\ sp}$, mol)

$M_{H2,\ sp}$ = 82.94 mol of $C_{30}H_{62}$ × (31 mol of H_2 ÷ 1 mol of $C_{30}H_{62}$)

$M_{H2,\ sp}$ = 2571. 1 mol

Step 2: Total H_2 (mol) released from degradation of unsaturated paraffinic hydrocarbons

Molar mass of unsaturated paraffinic (MW_{usp}) 30C-hydrocarbon:

Number of double bounds (DB)= 15

$MW_{usp} = C_nH_{(2n+2\ -\ DB\ \times\ 2)}$

MW_{usp} (n=30) = (12 g/mol × 30) + [1g/mol × (2 × 30 + 2 -15 × 2)]

MW_{usp} ($C_{30}H_{32}$) = 392 g/mol

Unsaturated paraffinic ratio (%) = 50

Total mass of unsaturated paraffinic hydrocarbons (M_{usp}, kg):

M_{usp} = 100 kg × 0.7 × 0.5, **M_{usp} = 35 kg**

Total mole of saturated paraffinic hydrocarbons (M_{usp}, mol):

M_{usp} = 35 kg ÷ 392 g/mol × (1000 g ÷ 1 kg), **M_{usp} = 89.3 mol**

Total mole of H_2 released from degradation of 89.3 mol unsaturated paraffinic 30C-hydrocarbons ($M_{H2,\ usp}$, mol):

$M_{H2,\ usp}$= 89.3 mol of $C_{30}H_{32}$ × (16 mol of H_2 ÷ 1 mol of $C_{30}H_{32}$),

$M_{H2,\ usp}$ = 1428.8 mol

Step 3: Total H_2 (mol) released from degradation of aromatic hydrocarbons

Molar mass of aromatic (MW_a) 6C-hydrocarbon:

$MW_a = C_nH_n$

MW_a (n=6) = (12 g/mol × 6) + (1g/mol × 6)

MW_a (C_6H_6) = 78 g/mol

Aromatic ratio (%) = 30

Total mass of unsaturated paraffinic hydrocarbons (M_a, kg):

M_a = 100 kg × 0.3, **M_a = 30 kg**

Total mole of saturated paraffinic hydrocarbons (M_a, mol):

M_a = 30 kg ÷ 78 g/mol × (1000 g ÷ 1 kg), **M_a = 384.6 mol**

Total mole of H_2 released from degradation of 384.6 mol aromatic 6C-hydrocarbons ($M_{H2, a}$, mol):

$M_{H2, a}$= 384.6 mol of C_6H_6 × (3 mol of H_2 ÷ 1 mol of C_6H_6),

$M_{H2, a}$ = 1153.8 mol

Step 4: Total moles of H_2 released from complete fermentation of 100 kg of hydrocarbons ($M_{H2, 100\ kg\ hydrocarbon}$, mol)

$M_{H2, 100\ kg\ hydrocarbon} = M_{H2, sp} + M_{H2, usp} + M_{H2, a}$

$M_{H2, 100\ kg\ hydrocarbon}$ = 2571. 1 mol + 1428.8 mol + 1153.8 mol

$M_{H2, 100\ kg\ hydrocarbon}$ = 5153.7 mol

Total of 5153.7 mol of H_2 is produced by degradation of 100 kg of hydrocarbons.

Electron donor demand (H_2) for contaminant attenuation at Third Creek sediment:

Step 5: Total moles of H_2 required to dechlorinate 1 mole of PCE to ethene

Assumption: Although PCE is not the only contaminant released into Third Creek sediment; in next steps we use only PCE (C_2Cl_4) to ethene (C_2H_4) reductive dechlorination as a model reaction to simplify calculations. Many organohalide-respiring bacteria use H_2 as electron donor for reductive dechlorination and we assume that H_2 is used for the stepwise reductive dechlorination of PCE to ethene dechlorination:

Redox reactions:

$4H_2 \rightarrow 8H^+ + 8e^-$

$C_2Cl_4 + 8H^+ + 8e^- \rightarrow C_2H_4 + 4H^+ + 4Cl^-$

$C_2Cl_4 + 4H_2 \rightarrow C_2H_4 + 4H^+ + 4Cl^-$

4 moles of H_2 are required to dechlorinate **1 mole of PCE to 1 mole of ethene.**

Step 6: Total mass of cVOCs discharged into Third Creek sediment

Volume of groundwater discharging into Third Creek sediment (V_{gw}, gal/yr)

V_{gw} = 45000 gal/yr

Concentration of total cVOCs (C_{cVOCs}, mg/L):

C_{cVOCs} = 95 mg/L – latest highest concentration detected in bedrock groundwater measurements.

Total mass of cVOCs discharge into Third Creek sediment ($M_{cVOCs,}$ kg):

M_{cVOCs} = **V_{gw}** × **C_{cVOC}**

M_{cVOCs} = 45000 gal/yr × 95 mg/L x (3.76 L ÷ 1 gal) x (1 kg ÷ 10^6 mg)

M_{cVOCs} = 16.1 kg/yr

Assumptions:

- Open-top cleaning technologies were used in manufacturing site at Third Creek area. The assumption is that this technology cleaned 100 kg of oil using 750 kg of chlorinated solvent. Evaporative chlorinated solvent loss is 500 kg, and 250 kg was spilled into subsurface[1].Thus, 100 kg of hydrocarbons were released together with 250 kg of solvent (solvent:hydrocarbon ratio is 2.5:1).
- PCE was the main contaminant and PCE was used for electron donor demand calculations.

Step 7: Total mole of PCE ($M_{PCE, 250kg}$, mol)

MW_{PCE} = 165,83 g/mol

$M_{PCE, 250kg}$ = 250 kg ÷ 165.83 g/mol × (1000g ÷ 1kg)

$M_{PCE, 250kg}$ = 1507.5 mol (PCE)

Step 8: Total moles of H_2 required for the reductive dechlorination of 250 kg PCE ($MH_{2, demand}$)

If H_2 is the only electron donor used for complete dechlorination of PCE to ethene:

$M_{H2, demand of 250 kg PCE}$ = 1507.5 mol of PCE × (4 mol of H_2 ÷ 1 mol of PCE)

$M_{H2, demand of 250 kg PCE}$ = 6030 mol

Step 9: What percentage of the H_2 requirement for dechlorination of 250 kg PCE can be sustained from petroleum hydrocarbon fermentation (%)?

In Step 4, we found that total of 5153.7 mol of H_2 is produced by complete fermentation of 100 kg of hydrocarbons (based on described assumptions).

$M_{H2a,\ demand\ of\ 250\ kg\ PCE}$ = 6030 mol (Step 8, for PCE to ethene dechlorination)

$M_{H2,\ 100\ kg\ hydrocarbon}$ = 5153.7 mol (Step 4)

% H_2 required for dechlorination, which can be supplied from hydrocarbons fermentation

a) *for PCE to ethene dechlorination*

% H_2 demand = $M_{H2,\ 100\ kg\ hydrocarbon} \div M_{H2a,\ demand\ of\ 250\ kg\ PCE} \times 100$

% H_2 demand = 5153.7 mol ÷ 6030 mol × 100

% H_2 requirement = 85.5 %

85.5 % of H_2 required for dechlorination of PCE can be supplied by hydrocarbon fermentation.

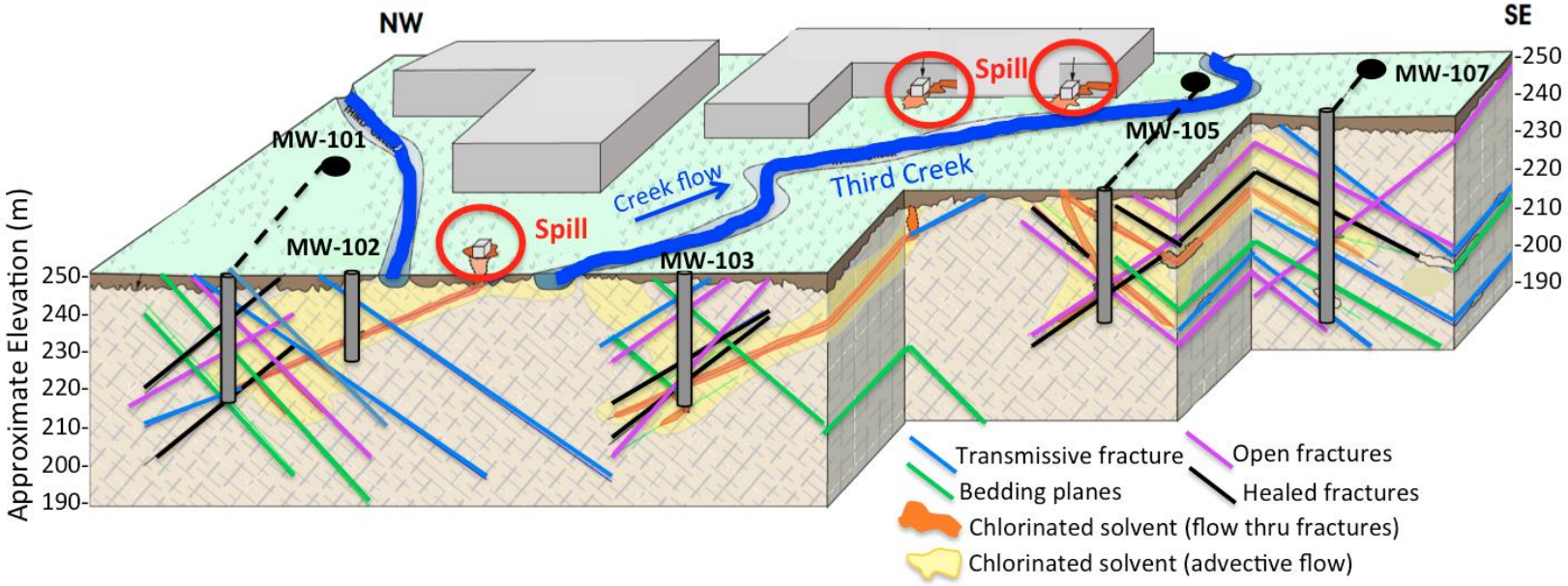

Figure S2.1. Conceptual site model for contaminants (cVOCs) migration pathways at Third Creek site

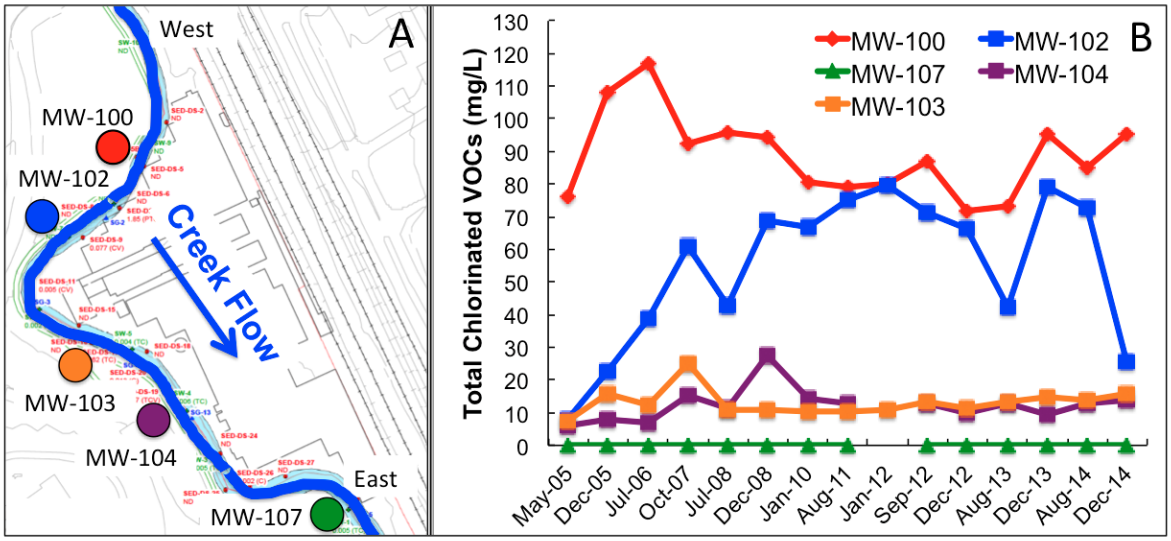

Figure S2.2. Bedrock groundwater cVOC concentrations over time
Locations of bedrock wells are shown with colored-circles (panel A). The same colors were used for corresponding well's groundwater cVOC concentrations measured at 20 m below ground surface over time (panel B).

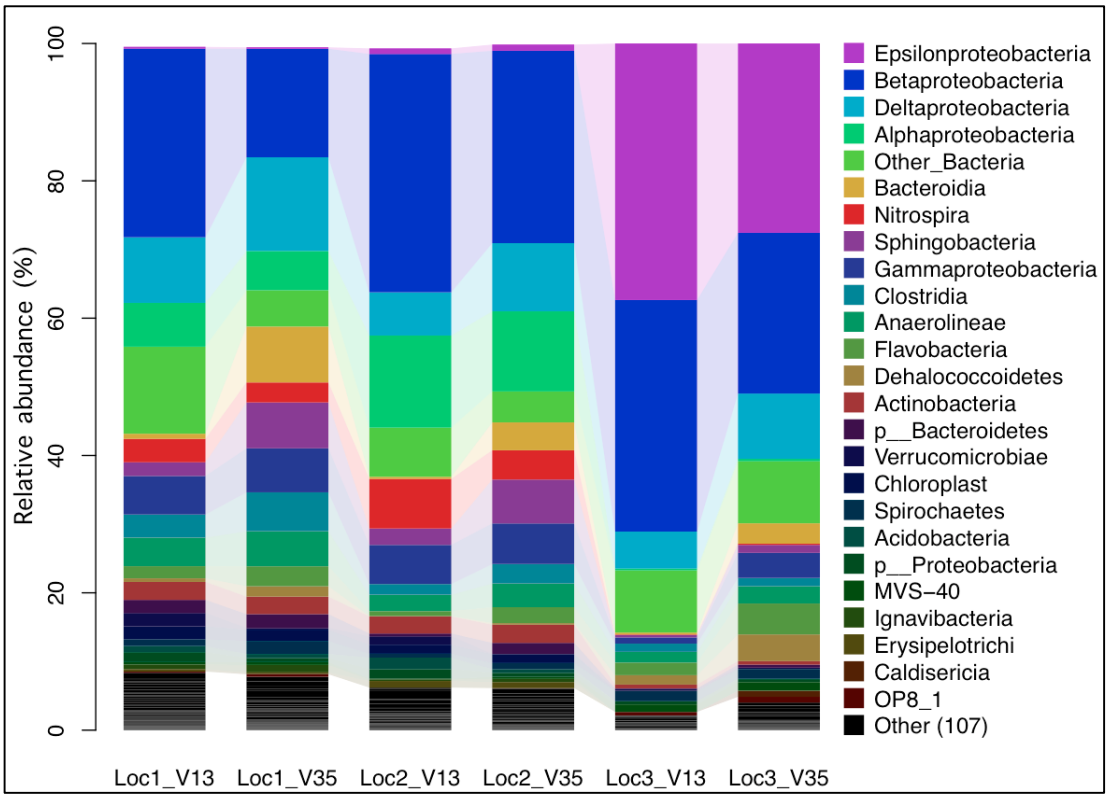

Figure S2.3. Comparison of bacterial communities in Third Creek using V1-V3 and V3-V5 region sequences

Comparison of bacterial communities in Third Creek sediment locations #1, #2 and #3 at the class level using V1-V3 and V3-V5 region sequences. "Other" refers to low abundance OTUs. Sequences that could not be classified at any level of taxonomy are represented in the 'Other_Bacteria' category. Sequences not classified at the class level are represented with their phylum (p) classification. Loc #1, Loc#2 and Loc#3 represent the locations 1, 2, and 3 illustrated in Figure 2.1A.

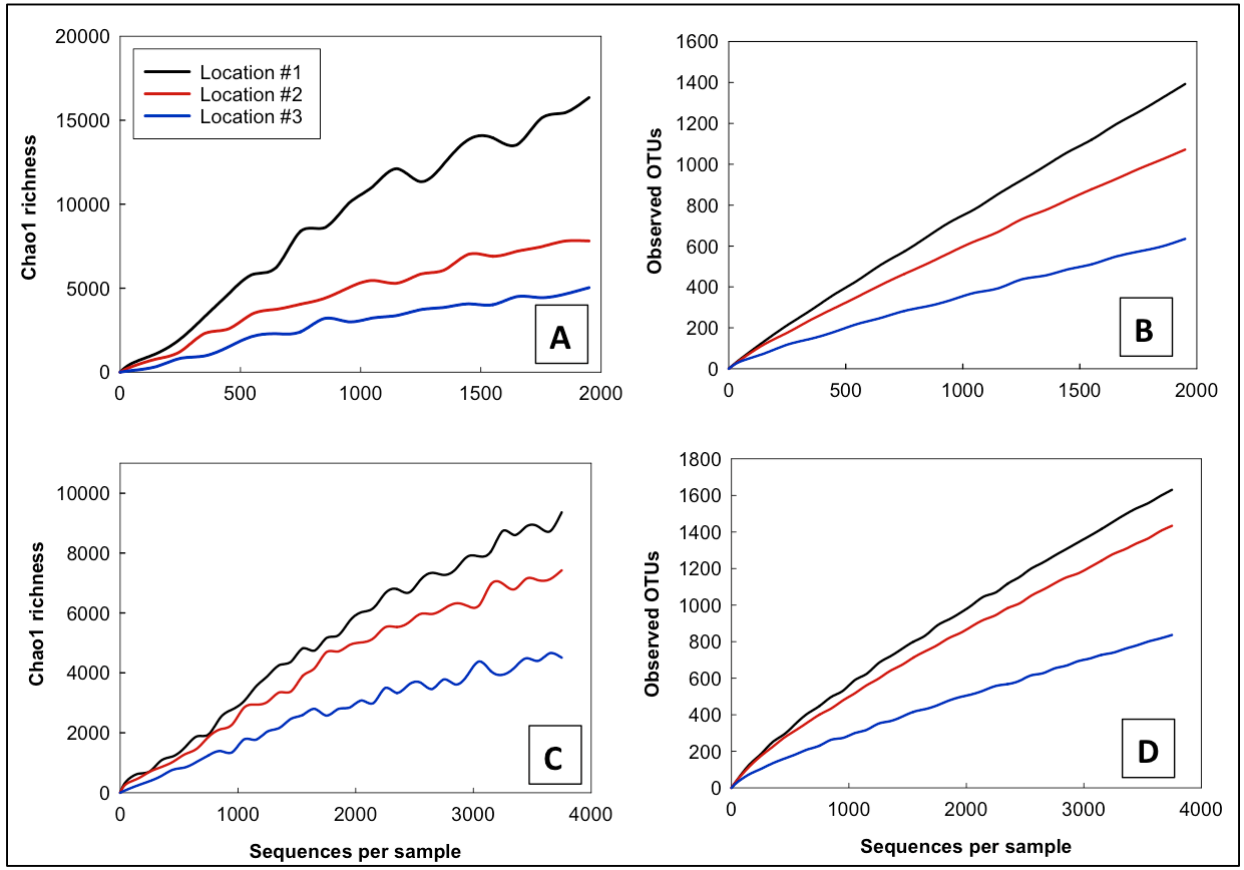

Figure S2.4. Rarefaction curves of bacterial 16S rRNA gene amplicon sequences
Sequences were recovered from Third Creek sediment samples. The y-axes represent Chao1 [panel A, V1-V3 region; C, V3-V5 region]) or OTU richness estimates [panel B, V1-V3 region; D, V3-V5 region) for the number of sequences analyzed (x-axes).

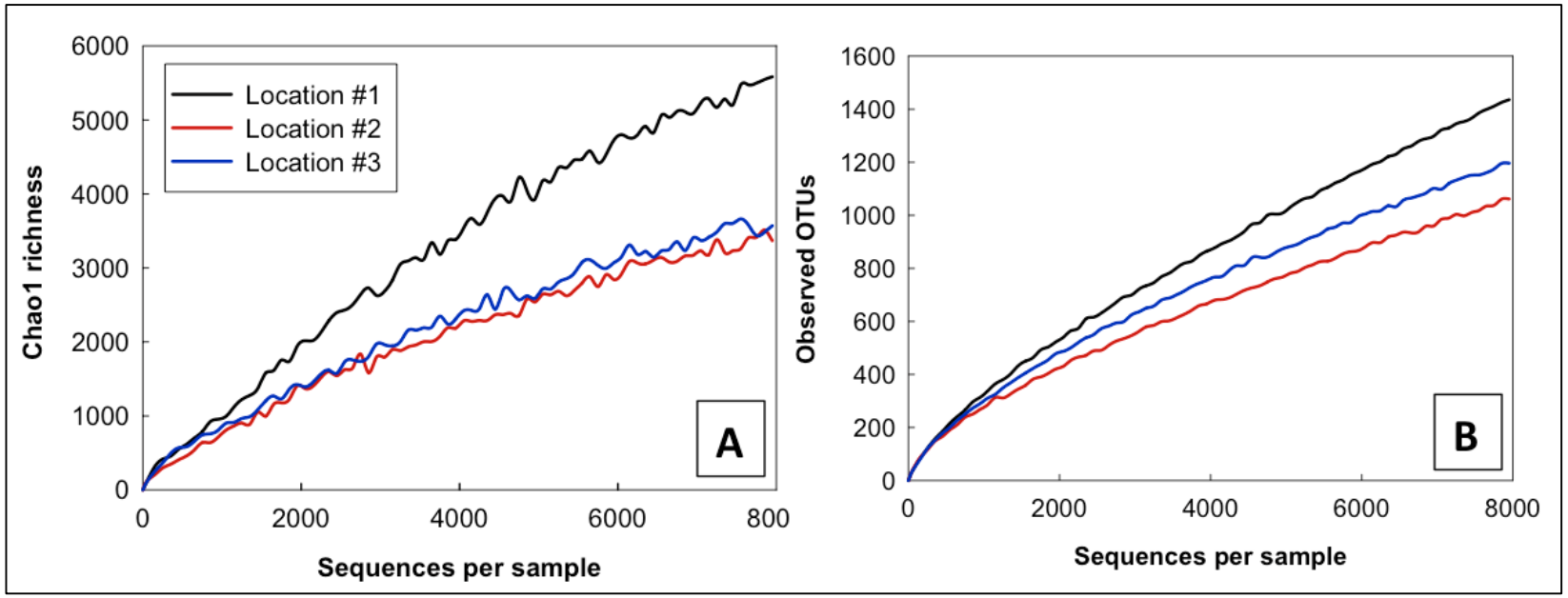

Figure S2.5. Rarefaction curves of archaeal 16S rRNA gene sequences
Sequences were recovered from Third Creek sediment samples. The y-axes represents Chao1 (panel A) or OTU (panel B) richness estimates for the number of sequences analyzed (x-axes).

Table S2.1. Primers and probes used in this study

Primer or probe	Target	Primer/probe sequence (5'-3')
Bac-8F Bac-1541R	Total bacterial 16S rRNA gene	AGAGTTTGATCCTGGCTCAG AAGGAGGTGATCCAGCCGCA
Dhc-730F *Dhc*-1350R	*Dhc* 16S rRNA gene	GCGGTTTTCTAGGTTGTC CAC CTT GCT GAT ATG CGG
Dhb-179F *Dhb*-1007R	*Dhb* 16S rRNA gene	TGTATTGTCCGAGAGGCA ACTCCCATATCTCTACGG
Dhgm-631F *Dhgm*-796R	*Dhgm* 16S rRNA gene	GGTCATCTGATACTGTTGGACTTGAGTATG ACCCAGTGTTTAGGGCGTGGACTACCAGG
Geo-196F *Geo*-999R	*GeoSZ* 16S rRNA gene	GAATATGCTCCTGATTC ACCCTCTACTTTCAT AG
RRF2 B1R	RDase A RRDFMK motif RDase B WYEW motif	SHMGBMGWGATTTYATGAARR CHADHAGCCAYTCRTACCA
dcpA-360F *dcpA*-1449R	*dcpA* gene	TTGCGTGATCAAATTGGAGCCTGG TTTAAACAGCGGGCAGGTACTGGT
Bac-1055F *Bac-1492R* *Bac-1115probe*	Total bacterial 16S rRNA gene	ATGGYTGTCGTCAGCT ACGGGCGGTGTGTAC FAM-CAACGAGCGCAACCC-TAMRA
Dhc-1200F *Dhc*-1271R *Dhc*-1240Probe	*Dhc* 16S rRNA gene	CTGGAGCTAATCCCCAAAGCT CAACTTCATGCAGGCGGG FAM-TCCTCAGTTCGGATTGCAGGCTGAA-TAMRA
Dhb-F *Dhb*-R *Dhb-probe*	*Dhb* 16S rRNA gene	GCCGCGAGGTGAAGCA CAGCCTGCAATCCGAACTG 6FAM- ATCCGAGAAAGCCGTTC (MGB)
cfrA- F *cfrA*-R *cfrA*-722probe	*cfrA* gene	CCCAAGCATGTAATCTCGATGA GAATGCAGCATATCCAATCTGG 6FAM-CAGAAGGAGTAAAGTGTGAC-MGB
bvcA-925R *bvcA*-1017F *bvcA*-977probe	*bvcA* gene	AAAAGCACTTGGCTATCAAGGAC CCAAAAGCACCACCAGGTC FAM-TGGTGGCGACGTGGCTATGTGG-BHQ1
vcrA-1022F *vcrA*-1093F *vcrA*-1042probe	*vcrA* gene	CGGGCGGATGCACTATTTT GAATAGTCCGTGCCCTTCCTC FAM-CGCAGTAACTCAACCATTTCCTGGTAGTGG-BHQ1
tceA-1270F *tceA-1336R* *tceA-1294probe*	*tceA* gene	ATCCAGATTATGACCCTGGTGAA GCGGCATATATTAGGGCATCTT FAM-TGGGCTATGGCGACCGCAGG-BHQ1
27YMF (bac) *534R (bac)*	V13-bacterial 16S rRNA gene	AGAGTTTGATYMTGGCTCAG TYACCGCGGCTGCTGG
341F(bac) *926R (bac)*	V35-bacterial 16S rRNA gene	CCTACGGGAGGCAGCAG CCGTCAATTCMTTTRAGT (80) CCGYCAATTYYTTTRWGT (20)
A2FA 571R	V13-archaeal 16S rRNA gene	TCYSGTTGATCCYGCSRG GCTACRGVYSCTTTARRC

Abbreviations for degenerate nucleotides positions are as follows: R =A or G; K = G or T; M =A or C; S =C or G; W=A or T; Y =C or T; B =C, G, or T; D = A, G, or T; V =A, C, or G; H =A, C, or T
References of each primers probes are cited in original text

Table S2.2. cVOC, ethene , ethane and methane concentrations measured in Creek sediment pore water

Location-depth (m)	Methane (mg/L)	Ethene (mg/L)	Ethane (mg/L)	VC (mg/L)	*cis*-DCE (mg/L)	TCE (mg/L)
#1 - 0.05	0.13	<0.10	<0.10	<0.10	<0.10	<0.10
#1 - 0.20	0.56	<0.10	<0.10	<0.10	0.03	<0.10
#1 – 0.35	0.98	<0.10	<0.10	<0.10	<0.10	<0.10
#2 - 0.00	0.04	<0.10	<0.10	<0.10	<0.10	<0.10
#2 - 0.10	1.70	<0.10	<0.10	<0.10	<0.10	<0.10
#2 – 0.25	0.85	<0.10	<0.10	<0.10	<0.10	<0.10
#2 – 0.40	0.43	<0.10	<0.10	<0.10	<0.10	<0.10
#2 – 0.55	0.17	<0.10	<0.10	<0.10	<0.10	0.82
#3 – 0.00	0.04	<0.10	<0.10	<0.10	<0.10	0.10
#3 – 0.07	0.06	<0.10	<0.10	<0.10	0.06	<0.10
#3 – 0.22	4.50	0.25	0.08	0.33	0.78	<0.10
#3 – 0.37	4.10	0.14	0.04	0.28	0.55	<0.10
#3 – 0.52	5.20	0.12	0.07	0.20	0.46	<0.10

Table S2.3. Third Creek sediment pore water geochemical measurements

Location-depth (m)	Total VFA (mg/L)	Sulfate (mg/L)	Chloride (mg/L)	Nitrite-N (mg/L)	Nitrate-N (mg/L)
#1 – 0.00	4.6	14	44	<0.10	1.0
#1 - 0.12	4.0	17	41	<0.10	<0.10
#1 - 0.27	2.8	7.2	13	<0.10	<0.10
#2 - 0.02	2.8	72	31	<0.10	0.18
#2 - 0.18	4.8	130	20	<0.10	<0.10
#2 – 0.33	2.4	92	14	<0.10	<0.10
#2 – 0.48	3.5	25	5.3	<0.10	<0.10
#3 – 0.00	4.0	12	24	<0.10	0.82
#3 - 0.14	2.6	4.5	28	<0.10	<0.10
#3 - 0.30	374	<0.73	23	<0.10	<0.10
#3- 0.45	5.3	<0.73	14	<0.10	0.11

Table S2.4. Detection of dechlorinator 16S rRNA genes and *dcpA* reductive dehalogenase gene via nested PCR

	Biomarker								RDase gene	
	16S rRNA genes									
Sediment samples	***Dhc***		***Dhb***		***Geo***		***Dhgm***		***dcpA***	
Location #1	+		+		+		+		+	
Location #2	+		+		+		+		+	
Location #3	+		+		-		+		+	
Depth-resolved samples Location #3	**Sed**	**Bio**	**Sed**	**Bio**	**Sed**	**Bio**	**Sed**	**Bio**	**Sed**	**Bio**
Depth*(m) 0	+	+	+	+	+	-	+	-	+	+
0.07	+	+	+	+	+	+	+	+	+	-
0.17	+	+	+	+	-	-	+	+	+	-
0.22	+	+	+	+	+	-	+	-	+	-
0.30	+	+	+	+	+	-	+	+	+	-
0.38	+	+	+	+	+	-	+	+	+	-
0.45	+	+	+	+	+	-	+	+	+	-
0.52	+	+	+	+	-	-	-	-	-	-

Sed: sediment sample; Bio: Bio-Sep bead sample. *Depth below surface water-sediment interface

Table S2.5. Comparison of results of V1-V3 and V3-V5 region sequencing results

	16S rRNA gene region	
	V1 – V3	**V3 – V5**
Total number of sequences obtained	6967	14,365
Total number of high-quality sequence after data processing	6791	13848
Minimum number of seqs/sample	1968	3869
Maximum number of seqs/sample	2562	5431
Total number of OTUs (locations #1/#2/#3 in order)	1593, 1080, 798	1740, 1174, 792
Total number of phyla assigned	38	47
Phyla only identified in each region sequences	*ABY1_OD1, Lentisphaerae*	*49S1_2B, BRC1, Caldiserica, Caldithrix, GN06, HDBW-WB69, LCP-89, MVP-15, OP9, SC3, SM2F11*

3 Third Creek Reductively Dechlorinating Consortia: Effects of Chlorinated Solvents on Community Structure and Strain Selection of Dechlorinators

3.1 Abstract

Chlorinated solvents including tetrachloroethene (PCE), trichloroethene (TCE) and 1,1,1-trichloroethane are commonly encountered toxic groundwater contaminants. Bacterial organohalide respiration of toxic contaminants has emerged as a cost-effective and productive bioremediation approach at contaminated sites. Among organohalide respiring bacteria *Dehalococcoides mccartyi* (*Dhc*) and *Dehalobacter* strains are key players in bioremediation. Non-dechlorinating community members sustain *Dhc* and *Dhb* reductive dechlorination by providing essential growth nutrients including hydrogen, acetate and corrinoid cofactor, and maintaining anoxic conditions. In this study, various chlorinated-ethenes, -ethanes, -propanes or -methanes dechlorinating enrichment cultures were derived from a site contaminated with mixture of chlorinated solvents. A combination of 16S rRNA gene sequencing and qPCR analysis was applied to explore the effect of available electron acceptors on development of dechlorinating communities and dechlorinator strain selection. Different strains of *Dhc* were enriched in chlorinated ethenes and 1,2-Dichloropropane (1,2-D) -amended cultures, while *Dhb* strains highly enriched in chlorinated ethanes- or chloroform (CF) -amended cultures. Sequential transfers yielded stable enrichment cultures with significantly reduced microbial diversity. Fermenting microbial populations including *Clostridium* spp., *Acetobacterium* spp., *Sedimentbacter* spp., were enriched alongside with *Dhc* or *Dhb* strains. 16S rRNA gene sequencing revealed possible syntrophic interactions between fermenting communities such as *Desulfovibrio,* and methanogenic or dechlorinating communities in enrichment cultures. Significant variation in methanogenic populations with respect to amendment of different chlorinated ethenes, chlorinated ethanes and chlorinated methanes, and chlorinated-propane, indicated the selective effect of electron acceptors on community composition. Knowledge of the specific dechlorinators and dechlorinating

communities enriched with respect to different chlorinated-solvent amendment provide fundamental information for development of specific bioaugmentation consortia, which can be used at specific chlorinated-solvent contaminate sites to achieve successful contaminated-site cleanup.

3.2 Introduction

Polluting organohalides including tetracloroethene (PCE), trichloroethene (TCE) and 1,1,1-trichloroethane (TCA) have been heavily used in various industrial processes, and improper handling of these compounds has caused widespread groundwater contamination[1-3]. The accumulation of these pollutants in the environment is problematic and a major concern because of their probable human carcinogenic and toxic nature.[3, 4] Microbial organohalide respiration is an energy-conserving respiratory process, and can transfer or reduce the halogenated-pollutants into less toxic forms.[5] [6] Organohalide-respiring bacteria (ORHB), which are bacteria capable of deriving energy for growth from organohalide respiration of halogenated compounds, are of environmental importance in detoxification of toxic chlorinated compounds in the environment.[4]

OHRB have been identified from distinctly related bacterial genera belonging to phyla including *Chloroflexi*, *Firmicutes* and *Proteobacteria*.[7] Diverse group of bacteria can contribute to reductive dechlorination of PCE to TCE or *cis*-1,2-dichloroethene (*c*DCE) including proteobacteria OHRB *Geobacter*, *Desulfuromonas*, *Sulfurisprillum* and fermicutes OHRB including *Desulfitobacterium* and *Dehalobacter* (*Dhb*).[7, 8] In addition to PCE reductive dechlorination, some strains of *Dhb* are capable of dechlorination of other chlorinated compounds, including chlorinated ethanes[9] and chlorinated methanes.[10-12] To date, only *Dehalococcoides* (*Dhc*) strains within the *Chloroflexi* have been implicated in complete reductive dechlorination to benign ethene.[13-15] *Dhc* are key players in organohalide respiration and bioremediation. Their obligate use of chlorinated solvents as energy source, has allowed successful enrichment of *Dhc*-containing microbial mixed cultures for bioaugmentation of chlorinated ethene-contaminated sites.[16-

[18] *Dhc* has a highly restricted lifestyle and require specific chlorinated compounds as electron acceptors, acetate as carbon source and hydrogen as electron donor to fuel their energy metabolism.[15] *Dhc* grow best in dechlorinating-mixed cultures in terms of *Dhc* growth yields and dechlorination rates, and they depend on other microbial populations to provide essential nutrients (e.g., H_2, corrinoid cofactor).[19-22] The detection of *Dhc* 16S rRNA genes and reductive dehalogenase (RDase) genes, which encode the key enzymes responsible for catalyzing reductive dechlorination reactions[15] in contaminated sites does not always represent a scenario of complete dechlorination of the contaminants, which can be attributed to the composition of supportive (e.g., corrinoid-producing) community members[39,40]. Relevant members in *Dhc*-containing mixed cultures are typically fermentative, acetogenic bacteria and methanogens.[23-26]

Dhc strains contain unique complement of up to 36 RDase genes, whereas their 16S rRNA genes sequences are highly conserved between the strains.[15] *Dhc* strains or *Dhc*-containing consortia have been obtained from geographically dispersed contaminated sites or pristine environments,[13, 15, 20, 27, 28] and unexpectedly, members from geographically different sources with distinct dechlorination capabilities often found to cohabit the same contaminated environments.[29] In addition, recent study such that comparative metagenomics of three *Dhc*-containing consortia, which were derived from geographically distant locations and containing different strains of *Dhc*, revealed functional similarities (i.e., complete reductive dechlorination of PCE or TCE to ethene) although taxonomic variations were apparent both in dechlorinator and non-dechlorinating community.[28, 30-32] The presence of such a large variety of RDase genes in *Dhc* genomes and geographical-origin based taxonomic variations between the strains suggest that *Dhc* may use wider yet undiscovered-variety of chlorinated compounds in the environment; however, the relationship between the complement of RDase genes (i.e., different strain) and *Dhc* substrate specificity is still unclear. To date, all *Dhc* isolates or *Dhc*-containing dechlorinating consortia were derived with amendment of PCE or TCE at the beginning and using environmental materials (e.g., groundwater, sediment) from geographically distinct locations, before sub-culturing with *cis*-DCE and

VC to enrich *cis*-DCE and VC dechlorinating strains (i.e, BAV1, VS and GT), thus isolation of the *Dhc* stains mostly depended on parent compounds PCE or TCE amended-conditions. Based on listed observations and studies, it is still not clear if available chlorinated-electron acceptors affect *Dhc* strain selection, and also structure of dechlorinating microbial communities.

Third Creek site (details in Chapter 2), which is co-contaminated with PCE, TCE, TCA, and small amount of chloroform (CF) is a good source to explore the role of electron acceptor in dechlorinator strain selection, and dechlorinating-community development. In previous study (Chapter 2), we have demonstrated that multiple dechlorinators including *Dhc* strains, PCE, chlorinated-ethanes, and -methanes dechlorinator *Dhb*[9] [10-12, 33] and chlorinated-alkanes or trans-dichloroethene dechlorinator *Dehalogenimonas* (*Dhgm*) [34 35] co-existed in Third Creek sediments, and were key players in detoxification of chlorinated-contaminants reaching into the sediment. In this study, we used various chlorinated-ethenes, -ethanes, -propanes or -methanes dechlorinating enrichment cultures, which were derived from a same source (that is Third Creek Location #3 sediment, Chapter 2) and investigated the effect of initial-exposed electron acceptor on microbial community development and dechlorinator strain selection especially *Dhc* and *Dhb*. Knowledge of the strains and dechlorinating-communities that are selected under different geochemical conditions (e.g., available chlorinated solvents), may enable development of better bioremediation strategies to achieve successful contaminated-site cleanup.

3.3 Material and Methods

3.3.1 Chemicals

Chlorinated compounds were of >99% purity. PCE was purchased from ACROS Organics (Morris Plains, NJ), TCE was obtained from Fisher Scientific (Pittsburgh, PA), and *c*DCE, vinyl chloride (VC), ethene, TCA, dichloromethane (DCM), chloroform (CF),

chloromethane (CM), 1,2-dichloropropane (1,2-D), 1,1-dichloroethane (DCA), and chloroethane (CA) were obtained from Sigma-Aldrich-Fluka (St. Louis, MO).

3.3.2 Third Creek (TC) dechlorinating enrichment cultures

As outlined in Chapter 2, microcosms were established using sediment samples collected from the chlorinated solvent-contaminated Third Creek sediment in Knoxville, TN (from Location #3, see Chapter 2). Separate microcosms received PCE, TCE, *c*DCE, VC, 1,2-D, TCA, DCA, or CF as electron acceptor and lactate as electron donor. PCE, TCE, *c*DCE, VC, 1,2-D, TCA, DCA and CF-dechlorinating enrichment cultures were derived from Third Creek microcosms by transferring 3% (v/v) microcosms in to fresh medium after initial electron acceptors were degraded. TC dechlorinating enrichment cultures were maintained in batch cultures in anoxic bicarbonate-buffered, defined mineral salts medium with a N_2/CO_2 (80/20, v/v) headspace[36] amended with 0.4-0.5 mM corresponding chlorinated compounds as electron acceptor, and 5 mM lactate as electron donor. The culture was routinely allowed to dechlorinate amended electron acceptors, followed by transferring 3% (v/v) cultures in to fresh medium. All culture bottles were incubated stationary at 30°C in the dark.

3.3.3 16S rRNA gene sequencing

16S rRNA gene sequencing was performed using DNA samples from TC enrichment cultures using different platforms due to technological development over years. *454-sequencing.* Cells were harvested on to 0.22 μm membrane filters (Merc Milipore Ltd, Darmstadt) in triplicates from 20 mL of transfer 3 (T3) and transfer 8 (T8) of PCE, TCE, cDCE, VC and TCA enrichment cultures, and DNA was extracted using MO BIO Soil Kit following the manufacturer's protocols. Amplification of the hypervariable V3-V5 region of the bacterial and V1-V3 region of the archaeal 16S rRNA genes and subsequent pyrosequencing of the PCR amplicons was performed with barcoded-primers.[37-40] Library preparation, sequencing and data analyses were performed as described in details in

Chapter 2. Sequences derived from Third Creek Location #3 sediment samples, which described in details in Chapter 2, were used for community comparison analysis.

Illumina MiSeq sequencing. Cells were harvested on to 0.22 μm membrane filters in triplicates from 5 mL of PCE, TCE, cDCE, VC, 12-D, TCA, DCA and CF-dechlorinating enrichment cultures (maintained under same conditions for 3+ years), after a cycle of reductive dechlorination of corresponding electron acceptor to expected final product. DNA was extracted from collected cells using MO BIO Soil Kit following the manufacturer's protocols. Following extraction, DNA samples were purified using the Genomic DNA Clean and Concentrator Kit (Zymo Research, Irvine, CA). Purified DNA samples were pooled and then samples were PCR amplified by targeting the V4 region of the 16S rRNA gene using primers F515/R806. [41, 42] Amplification was performed in 50 μL assays consisting of 10 uL of sample DNA, 1 μL of barcoded-primer (10 μM) and 39 μL of a mixture of 22 μL de-ionized water (5 PRIME, Gaithersburg, MD), 5 μL Invitrogen *Pfx50*™ buffer (Invitrogen, Carlsbad, CA), 1 μL CAP 515F primer (10 μM), 5 μL dNTP (Invitrogen, Carlsbad, CA), 1 μL Invitrogen *Pfx50*™ Polymerase (Invitrogen, Carlsbad, CA) and 5 μL of $MgCl_2$ (25mM). Thermo cycling program included denaturation at 94°C for 3 min followed by 35 cycles at 94°C for 45 sec, annealing at 55°C for 60 sec, and extension at 72°C for 90 sec, and final extension at 72°C for 10 min. Quality (size) of produced amplicons was checked using High Sensitive DNA Kit on a model 2100 Bioanalyzer (Agilent, Santa Clara, CA). Relative concentrations of the individual samples were estimated based on the peak height at the appropriate size, and pooled to equal amounts. Pooled samples were purified with SPRI magnetic beads (Beckman Coulter, Inc., Indianapolis, IN). The products from purification step were analyzed again with High Sensitive DNA kit for quality assurance and verification of the removal of primer dimers. Before sequencing, concentrations of pooled amplicons were determined using Illumina Library Quantification kit (KAPA Biosystems, Boston. MA) following the manufacturer's protocol. Quantification of each sample was determined based on amplicon adaptors. The amplicon library was diluted to a starting concentration of 10 nM, followed by paired end sequencing on the Illumina

MiSeq sequencer (Illumina, Inc., San Diego, CA). After sequencing was performed, base calling, demultiplexing and adopter trimming were performed using MiSeq reporter using the default parameters. Raw 16S rRNA gene amplicon sequences were then analyzed using CLC Genomic workbench (v9.0) with CLC Microbial Genomics Module (v1.3.1) from Qiagen. Briefly, all reads were initially subjected to quality control: reads were paired, merged, quality trimmed and filtered based on the presence of the ambiguous nucleotides using the CLC Microbial Genomics default parameters. Following the quality control, sequences were clustered into operational taxonomic units (OTUs) at a threshold of 97% sequence similarity, checked for chimeras with CLC Microbial genomics default parameters and aligned with the MUSCLE algorithm[43]. The Greengenes database (http://Greengenes.secondgenome.com) was used for taxonomic assignments of OTUs.

3.3.4 Quantification of dechlorinator 16S rRNA genes and RDase genes

Cells were harvested on to 0.22 μm membrane filters in triplicates from 5 mL of PCE, TCE, cDCE, VC, 12-D, TCA, DCA and CF-dechlorinating enrichment cultures just after a complete cycle of reductive dechlorination of corresponding electron acceptor to expected final product. DNA was extracted from the filters using MO BIO Soil Kit following the manufacturer's protocols. Following extraction, *Dhc*, *Dhb* and *Dhgm* 16S rRNA gene, RDases *tceA*, *vcrA*, *bvcA*, *Dhc* strain 195 *pceA, dcpA* and *cfrA* were quantified using primers and probe sets of Dhc1200F/ Dhc1271R/ Dhc1240Probe[44]; DhbF (GCCGCGAGGTGAAGCA)/ DhbR (CAGCCTGCAATCCGAACTG)/ DhbProbe(6FAM- ATCCGAGAAAGCCGTTC-MGB)[www.microbe.com]; Dhgm478F (AGCAGCCGCGGTAATACG)/ Dhgm536R (CCACTTTACGCCCAATAAATCC)/ Dhgm500Probe (6FAM-AGGCGAGCGTTATC-MGB); TceA1270F/ TceA1336R/ TceA1294Probe[44]; VcrA401F[44]/ VcrA471R[44]/ VcrA1042Probe (VcrA1042Probe 6FAM-TACCAGGAAATGGTTGAGTTAC-MGB); BvcA58F (GCGGGAGCAGGGATAGGT)/ BvcA116R (TCATCCAAGTCGTGAAAATTCG)/ BvcA77P (6FAM-CCGCGACTTCAGTTAT-MGB); Dhc195-PceA1111F (CCACCCCGGTTCGTTCA)/ Dhc195-PceA1171F (CGGCGTGGTGGAAACAG)/ Dhc195-PceA1134Probe (6FAM-ACATGACGTAGTCAAGGG-MGB);

DcpA491F/DcpA549R/DcpA511Probe[45]; CfrAF (CCCAAGCATGTAATCTCGATGA)/ CfrAR (GAATGCAGCATATCCAATCTGG)/ CfrA722Probe (6FAM-CAGAAGGAGTAAAGTGTGAC-MGB) [www.microbe.com], respectively following the established qPCR protocols.[44] Standard curves for each qPCR assays were generated with 10-fold serial dilutions (10^8 to 10^0 gene copies/µL template) of a cloned-target gene fragments. All assays exhibited amplification efficiencies ranging from 90-100% (i.e., slope of the regression lines were between -3.6 and -3.1) and the linear standard curves had R^2 values > 0.98. Nuclease free water replaced template DNA in control assays and verified template DNA-specific fluorescence increase.

3.3.5 Analytical methods

Chlorinated-ethenes, -ethanes, -methanes 1,2-D, ethene, ethane, propene and methane were monitored using an Agilent 7890 gas chromatograph (GC) equipped with a flame ionization detector and a DB-624 capillary column (60 m by 0.32 mm with a film thickness of 1.18 µm) as described[46]. The method provided linear detector responses for quantification of each analyte to 0.7 mM aqueous phase concentrations with a detection limit of about 2.5 µM. Standards curves were prepared by adding known amounts of individual analytes to 160-mL glass serum bottles containing 100 mL of water, and analysing via GC. The concentrations of the compounds were calculated by normalizing the peak area measurements to standard curves.

3.4 Results

3.4.1 Reductive dechlorination in Third Creek (TC) enrichment cultures

Stable PCE-, TCE-, cDCE-, VC-, TCA-, DCA-, 1,2-D and CF-dechlorinating enrichment cultures were enriched from initial TC microcosms. Enrichment cultures were maintained for over 3 years with corresponding electron acceptor and lactate amendments. The dechlorination patterns and dechlorination end products observed in the TC dechlorinating enrichment cultures are summarized in Table 3.1. PCE-, TCE-, cDCE- and

VC-amended enrichment cultures dechlorinated corresponding chlorinated-ethenes to environmentally benign ethene in 1-2 months. In enrichment cultures-amended with 1,2-D, 1,2-D was reduced to propene without observing intermediate monochlorinated-propanes. In enrichment cultures amended with TCA, sequential reductive dechlorination to DCA and CA occurred; however, ethane was not produced. DCA-amended enrichment cultures reductively dechlorinated DCA to CA, but not further degradation was measured. In CF-amended enrichment cultures, DCM was formed transiently, which was further degraded; however, CM, the expected reductive dechlorination daughter product of DCM, was not detected. Methane was also produced in all enrichment cultures.

Table 3.1. Degradation products in TC-dechlorinating enrichment cultures

Enrichment culture	Electron Acceptor	Major degradation product
TC-PCE	PCE	Ethene
TC-TCE	TCE	Ethene
TC-*c*DCE	*c*DCE	Ethene
TC-VC	VC	Ethene
TC-TCA	TCA	CA
TC-DCA	DCA	CA
TC-CF	CF	DCM[#]
TC-1,2-D	1,2-D	Propene

[#] DCM further degraded with no evidence for reductive dechlorination because CM was not detected and microcosms failed to degrade CM.

3.4.2 Microbial community structures of TC dechlorinating enrichment cultures

Results of bacterial and archaeal 16S rRNA genes sequencing using different platforms and different barcoded primers are discussed in separate sections due to variations caused by different sequencing platforms and library preparation. Bacterial 16S rRNA gene amplicon (V3-V5 region) sequencing of TC sediment samples and samples from third and eight transfer (T3 and T8) of PCE, TCE, cDCE, VC and TCA cultures were generated from 454 Titanium libraries, and resulted in total of 97,310 high sequences

after raw data were denoised and processed for other quality filtering (e.g., excluding chimeric reads). The number of sequences originated from eight of the samples varied from 4,545 to 25,095 (Table S3.1). Three of the data sets (PCE_T8, VC_T8, TCA_T8) were excluded from further analyses due to recovery of low number of sequences (i.e., <500 sequences). Number of unique 16S rRNA gene amplicon sequences from the samples reached saturation except for sediment sample, showing that the sequences derived from cultures were estimated true species diversities (Figure S3.1). Maximum number of OTUs (972) was assigned at 97% nucleotide sequence identity using Greengenes database to sequences derived from sediment samples. Number of the observed OTUs obtained from the other samples varied from 111 to 323 per sample (Table 3.1). Taxonomic classification of observed OTUs at genus level is represented in Figure 3.1. Community compositions of cultures amended with chlorinated-ethenes and chlorinated-ethanes showed significant differences in terms of dechlorinator populations. Cultures amended with chlorinated-ethenes were highly enriched with *Dhc* OTUs (~40 – 75 % of total sequences), whereas *Dhb* OTUs were most abundant in TCA-amended cultures (~67 % of sequences). Following dechlorinators *Dhc* or *Dhb*, fermenting and acetogenic populations were the most abundant groups across all samples, and did not show significant differences between chlorinated-ethenes or –ethanes amended cultures. OTUs assigned to acetogens and fermenters *Acetobacterium*, *Clostridium*, *Sedimentibacter* and OTUs assigned to order of *Bacteroidales* contributed to great portion of the total sequences (Figure 3.1). Another fermenter *Citrobacter* also contributed to more than 1 % of the total bacterial OTUs in PCE-, TCE- and TCA-amended cultures, and were detected at much less abundances in other cultures.

In PCE_T3, PCE dechlorinating *Sulfurospirrillum*, *Desulfitobacterium* and *Geobacter* contributed up to 2. 5, 2.2 and <1% of the total sequences, respectively. *Sulfurospirrillum* OTUs were also detected in other samples at less than 1 % of sequence abundances whereas contributed to more than 2% in cDCE_T8 sample. Other than detection in PCE_T3 sample, *Desulfitobacterium* were detected at abundances less than 0.5 % of the total sequences in TCE, TCE and cDCE cultures, but not detected in TCA or VC cultures.

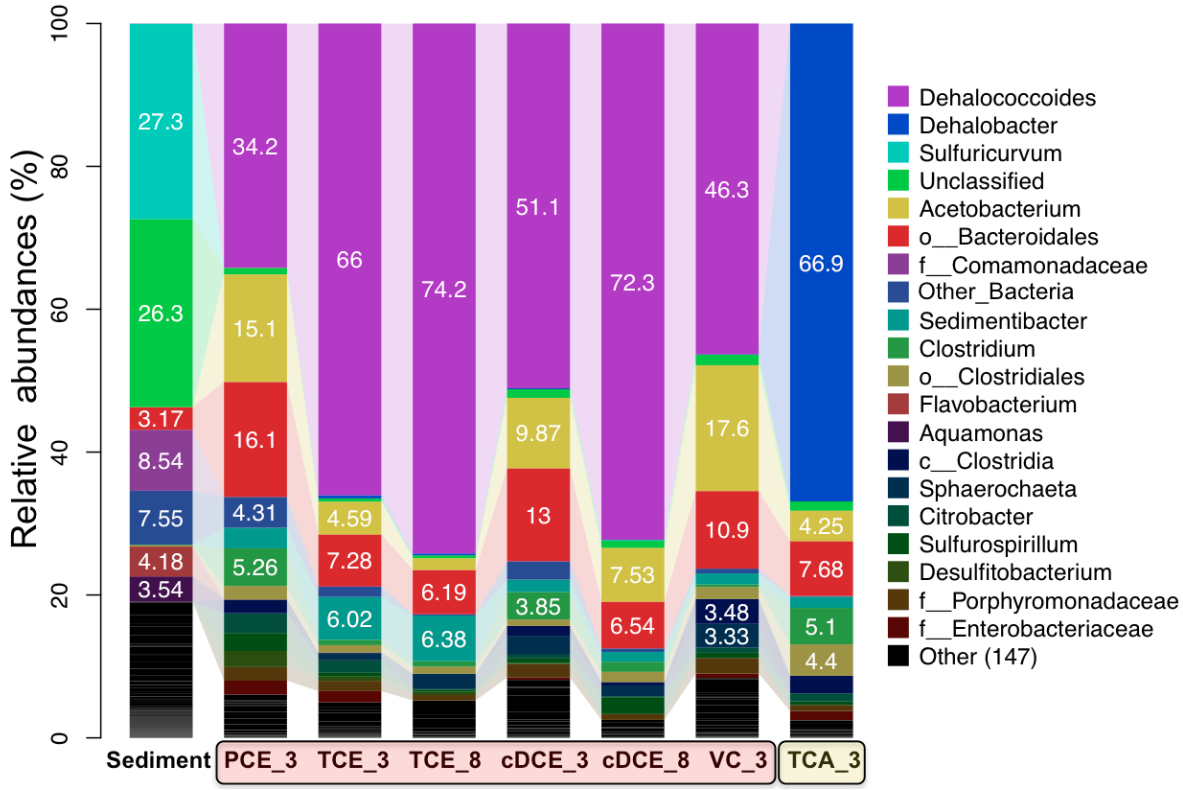

Figure 3.1. Microbial community structures of TC-dechlorinating enrichment cultures
Phylogenetic classification is presented at the genus level based on bacterial 16S rRNA gene (V3-V5 region) amplicon sequence analysis. Numbers in culture names represent the generation of the culture (i.e., 3rd and 8th generations). Top 20 genus were listed, other low-abundance OTUs (147) are grouped in "Others". Sequences that could not be classified at any level of taxonomy are represented in the 'Other_Bacteria' category. Low-abundance OTUs (<0.01 %) that could not be assigned to any genus are grouped in "Unclassified". Numbers on bar graphs represent the relative abundances of each taxonomic OTUs (only >3 % were represented). OTUs, which could not be assigned to any genus, represented with its family, order or class level classification [i.e., order (o), class (c) or family (f)]

OTUs assigned to sugar fermenters *Sphaerochaeta*[47] contributed to more than 1 % of total sequences in TCE, cDCE and VC-amended cultures, but not detected in cultures received PCE or TCA as electron acceptors. Community compositions of third- and eight-transfer cDCE or TCE cultures showed very similar profile for top 20 OTUs, while number of the total OTUs reduced significantly (Table S3.1), demonstrating that sequential transfers yielded stable enrichment cultures with significantly reduced microbial diversity (Figure 3.1).

Illumina MiSeq 16S rRNA gene amplicon sequencing of Third Creek TCA (18^{th} generation), 1,2-D, DCA, and CF cultures, which were enriched for 3+ years, produced total of 283,269 high-quality 16S rRNA gene amplicon sequences, after quality filtering (i.e., trimming, excluding chimeric reads). The number of the sequences and OTUs obtained for each samples varied from 55,032 to 94,172 sequences, and 126 to 181 OTUs, respectively (Table S3.1). Total of 11 to 16 bacterial phyla were assigned to OTUs recovered from all samples. Majority (ranged between 55 to 88 % of the total sequences) of the bacterial OTUs belonged to *Firmicutes*, followed by *Proteobacteria* (ranged between 1 to 13 % of total sequences). Taxonomic classification of OTUs assigned to sequences from 12-D, TCA, DCA and CF cultures is presented in Figure 3.2. OTUs belonging to fermenters *Clostridium* contributed to 25% of the total sequences recovered from 12-D amended cultures, representing the most abundant genera in the culture. Although *Clostridium* also contributed to significant portion of community OTUs (7 % of total sequences) in TCA-amended cultures, this group was detected at much lower abundances (less than 0.5 % of the total sequences in DCA and CF amended cultures. Genera *Dhc*, *Sedimentibacter*, *Syntrophomonas* contributed to 10 % (each) of the total sequences in 12-D amended cultures. Sequences, which could not be assigned to any genus within the family *Desulfovibrioceae* via Greengenes database but showed 100 % nucleotide sequence similarity to a uncultured *Desulfovibrio* sp. in NCBI database contributed to 6 % of the sequences from 12-D cultures, and followed by genus *Syntrophus* (6% of the sequences).

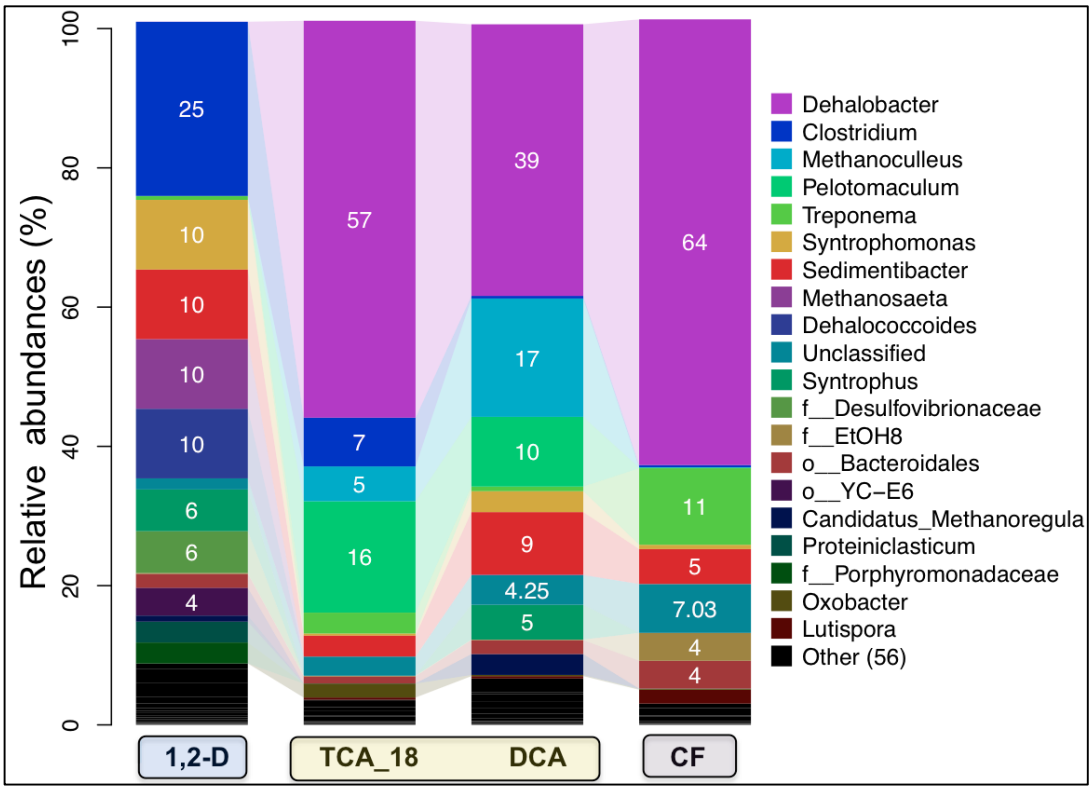

Figure 3.2. Microbial community structure in TC 1,2-D, TCA, DCA and CF-dechlorinating enrichment cultures

Phylogenetic classification is presented at the genus level based on 16S rRNA gene amplicon sequence analysis. Top 20 genus were listed, other low-abundance OTUs (56) are grouped in "Others". Low-abundance OTUs that could not be assigned to any genus are grouped in "Unclassified". Numbers on bar graphs represent the relative abundances of each taxonomic OTUs (only >3 % were represented). OTUs, which could not be assigned to any genus, represented with its family or order level classification [i.e., order (o) or family (f).

Dhb OTUs, as being the most abundant OTUs, contributed to 57, 39 and 64 % of total sequences recovered from TCA-, DCA- and CF-amended cultures, respectively. Propionate-oxidizing *Pelotomaculum* was the second most abundant (16% of total sequences) group in TCA-amended cultures, and contributed to 10 % of the total sequences of DCA amended cultures; however was at much less abundances (less than 0.01 %) in CF and 12-D-amended cultures.

Fermenter *Sedimentibacter* comprised 3, 9 and 5 % of sequences from TCA-, DCA, and CF-amended cultures. OTUs, which could not be assigned to any genus or family within the order of *Bacteroidales* were detected in high abundance in TCA, DCA and CF amended cultures (1, 2 and 3 % of the total sequences, respectively). Genus *Treponema* was the second most abundant group in CF-amended cultures (11 % of the total sequences), and also detected at 3% abundance in TCA-amended cultures; but at much lower (less than 0.6 %) abundances in DCA and 12-D amended cultures. OTUs assigned to genus *Geobacter* contributed to 1 % of total sequences recovered from DCA cultures; but at much lower abundances in other cultures. Interestingly, *Dhc* OTUs were also detected in TCA-, DCA-, and CF-amended cultures, but at abundances lower than 0.01 % of the total sequences.

454 pyrosequencing of archaeal 16S rRNA gene produced very low number of sequences, which were not used for further analysis. Accordingly, all TC dechlorinating cultures (using DNA from > 3+ year enriched cultures), were re-sequenced using Illumina MiSeq platform, and sequences were analyzed using CLC Microbial Genomics Module as described in method section. Archaeal 16S rRNA amplicon sequences contributed to 5, 9, 10, 30, 16, 5, 20% of the total sequences (bacterial and archaeal) in PCE, TCE, cDCE, VC, 12-D, TCA and DCA amended cultures, respectively; but less than 1 % in CF-amended cultures. Taxonomic classification of archaeal OTUs recovered from TC cultures are represented in Figure 3.3. Surprisingly, there is a distinct differentiation of archaeal populations between chlorinated-ethenes, -ethanes, -propane and –methane amended cultures.

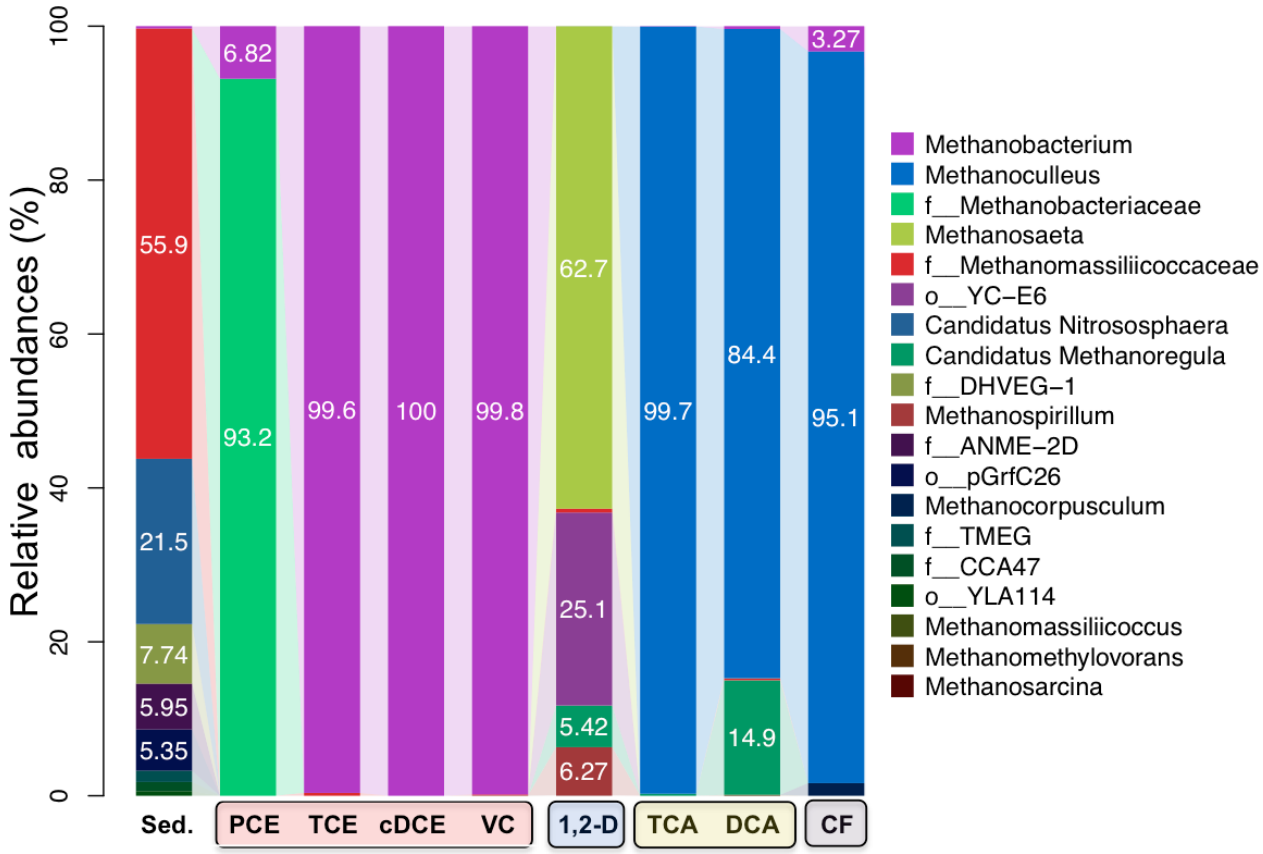

Figure 3.3. Archaeal community structure in TC- dechlorinating enrichment cultures
Phylogenetic classification is presented at the genus level based on archaeal 16S rRNA gene amplicon sequence analysis. Numbers on bar graphs represent the relative abundances of each taxonomic OTUs. OTUs, which could not be assigned to any genus, represented with its family or order level classification [i.e., order (o) or family (f)].

OTUs that could not be assigned to any genus within the family *Methanobacteriaceae* comprised 93 % of the total archaeal sequences recovered from PCE-amended cultures; however this group was not detected in any other TC cultures. Although methanogen *Methanobacterium* OTUs were enriched (more than 99% of the archaeal 16S rRNA gene amplicon sequences) in chlorinated-ethenes TCE, cDCE and VC-amended cultures, other group of methanogen *Methanoculleus* were enriched in chlorinated-ethanes (TCA and DCA) and chlorinated-methane (CF) amended cultures.

In 12-D cultures methanogens *Methanosaeta* was the most abundant group, contributing 62 % of the total archaeal sequences, followed by OTUs (25% of the total archaeal sequences), which could not be assigned to any genus in Greengenes database within the family *Methanobacteriaceae,* but share 100 % sequence similarity with an uncultured *Methanosaeta* in NCBI database). OTUs assigned to another methanogens *Methanoregula* was other most abundant group observed in DCA-amended culture.

3.4.3 Effect of electron acceptors on dechlorinating community

To explore if different dechlorinating strains (with different RDase genes) were selected in TC dechlorinating enrichment cultures amended with different electron acceptors, we quantified the known dechlorinators *Dhc*, *Dhb*, and *Dhgm* 16S rRNA genes, *Dhc* RDase genes *tceA*, *vcrA*, *bvcA*, strain 195 *pceA*, *dcpA* (1,2-D reductase) and TCA and CF reductive dehalogenase gene *cfrA* (assuming that different RDases represent different dechlorinator strains) in enrichment cultures. Figure 3.4A shows qPCR quantification of RDase genes *vcrA*, *bvcA*, *tceA*, and *dcpA* along with *Dhc* 16S rRNA gene in all TC dechlorinating enrichment cultures. *Dhc* 16S rRNA gene were abundant in chlorinated–ethenes and 1,2-D-amended cultures at numbers exceeding 6.0×10^7 gene copies/mL culture. *Dhc* 16S rRNA gene were also at quantifiable numbers in DCA and CF-amended cultures, whereas detected and not quantifiable in TCA-cultures (lower than 10^3 gene copies/mL).

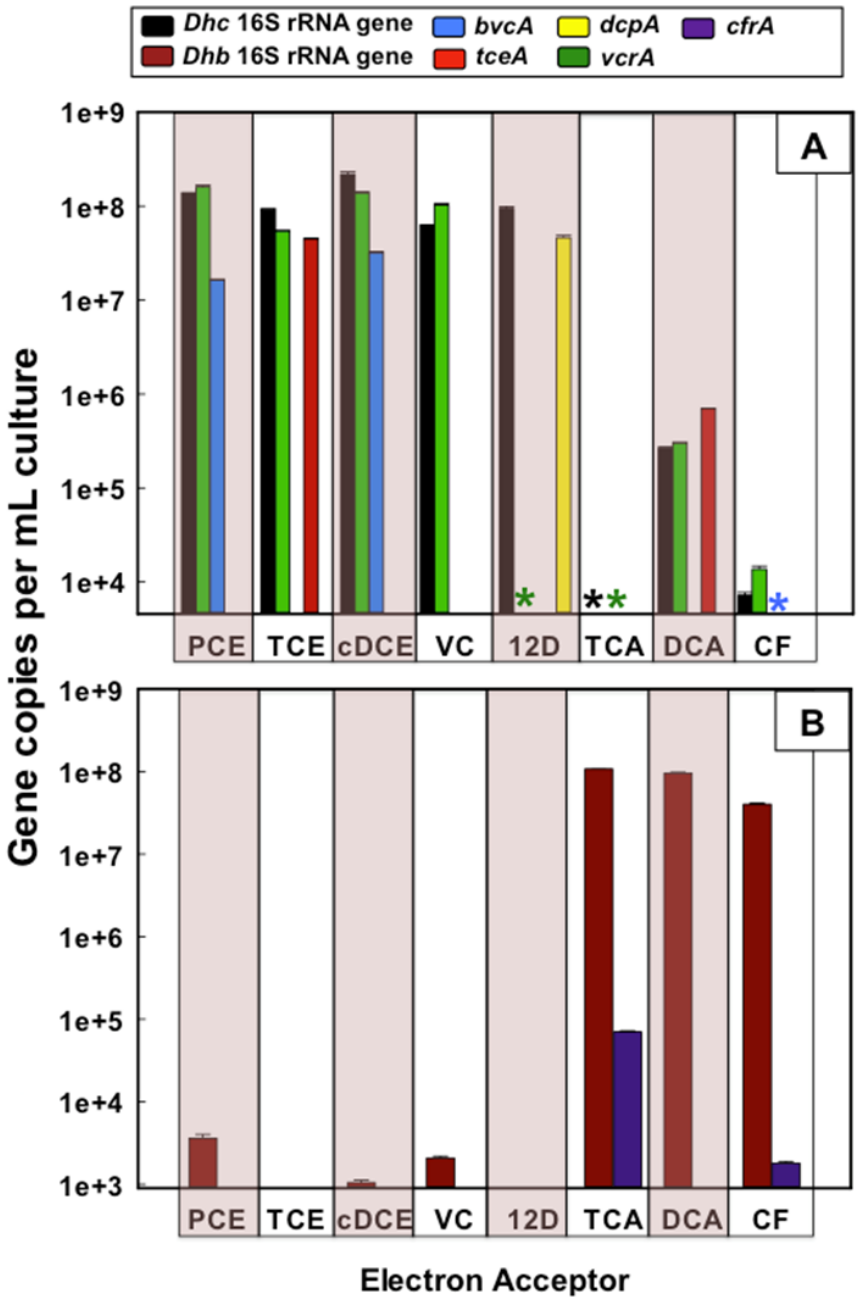

Figure 3.4. Quantification of *Dhc*, *Dhb* 16S rRNA genes, and RDase genes in TC enrichment cultures
A) *Dhc* 16S rRNA gene (black bars), *vcrA* (green bars), *bvcA* (blue bars) and *tceA* (red bars), *dcpA* (yellow bars) averaged gene copies in samples harvested from TC enrichment cultures. **B)** *Dhb* 16S rRNA gene (claret red) and *cfrA* (dark purple) averaged gene copies in samples harvested from TC enrichment. Error bars represent the standard error of triplicate samples. Stars (*) represent if gene detected in the sample, but now quantifiable, color of the stars matches with color of corresponding gene.

Although low *Dhc* cells density of $7.2 \pm 0.9 \times 10^3$ gene copies/mL were measured in CF-amended cultures, surprisingly higher cell density of $2.7 \pm 0.2 \times 10^5$ gene copies/mL were quantified in DCA-amended cultures. *Dhc* strains carrying cDCE to ethene reductive dehalogenase gene *vcrA* was the most abundant *Dhc* populations in all chlorinated-ethenes amended cultures at numbers exceeding 5.0×10^7 gene copies/mL, however detected but quantifiable in TCA and 1,2-D amended cultures. Similar to detection of *Dhc* 16S rRNA gene, *vcrA* gene was detected at numbers of $3.0 \pm 0.1 \times 10^5$ and $1.4 \pm 0.2 \times 10^4$ gene copies/mL in DCA and CF-amended cultures, respectively. cDCE-ethene reductive dehalogenase gene *bvcA* was only detected in PCE- and cDCE cultures among all chlorinated-ethenes-amended cultures at high numbers of $1.6 \pm 0.1 \times 10^7$ and $3.2 \pm 0.2 \times 10^7$ gene copies/mL, respectively. *bvcA* gene was also detected in CF-amended cultures, but at numbers below quantification limit. TCE reductive dehalogenase gene *tceA* was abundant only in TCE-amended cultures, and was also detected in DCA-amended cultures at numbers of $7.0 \pm 0.1 \times 10^5$ gene copies/mL, but not detected in other cultures. 1,2-D reductive dehalogenase *dcpA*[45] gene was only detected in 1,2-D-amended cultures at densities similar to *Dhc* 16S rRNA gene (Figure 3.4A). *Dhc* strain 195 *pceA* gene was not detected in any TC cultures, indicating that PCE reductive dehalogenation was carried out by other PCE dechlorinators such as *Geobacter* and *Desulfitobacterium* in PCE-amended cultures.

Figure 3.4B shows enumeration of *Dhb* 16S rRNA gene and TCA and CF reductive dehalogenase *cfrA* gene in all TC dechlorinating enrichment cultures. *Dhb* 16S rRNA genes were abundant in chlorinated-ethanes and CF-amended cultures at numbers exceeding 4.0×10^7 gene copies/mL, and also detected in PCE-and cDCE- and VC-amended cultures at much lower densities (lower than 5.0×10^3 gene copies/mL). *Dhb cfrA* gene, which catalyzes TCA to DCA and CF to DCM reductive dechlorination reactions[48] was detected only in TCA-amended and CF-amended cultures, but at low numbers of $7.0 \pm 0.4 \times 10^4$ and $1.8 \pm 0.2 \times 10^3$ gene copies/mL, respectively. Although detected in Third Creek sediment sample (Chapter 2), chlorinated-alkanes or trans-

dichloroethene dechlorinator *Dhgm* 16S rRNA was not enriched in any TC cultures including 1,2-D-amended cultures.

3.5 Discussion

Dhc are mediators in bioremediation and detoxification of toxic chlorinated aliphatic compounds. However detection of *Dhc* 16S rRNA or RDase genes at contaminated sites does not always indicative for the complete detoxification of contaminants, and stalls of more toxic degradation products cDCE or VC present worse scenarios. The reason for stalled or incomplete dechlorination in the contaminated sites may be lack of the right supportive microbial populations providing constant flux of required nutrients (i.e., corrinoid cofactors, H_2). To date, *Dhc* containing enrichment cultures or *Dhc* isolates have been derived from geographically distant contaminated sites or pristine environments, and subcultures growing on dechlorination products were only derived after a long period of growth of parent cultures on PCE or TCE.[13, 15, 20, 27, 28, 30] Therefore, development of dechlorinating communities and dechlorinator diversities could only be attributed to effect of different electron donor amendments, the geographical differences, or the original microcosms conditions (i.e., PCE, TCE amendment for a long time,).[24, 30, 32] In this study, the role of available electron acceptors on dechlorinating community composition, and dechlorinator strain selection were explored. For this purpose, TC chlorinated-ethene, -ethane, 1,2-D, and CF dechlorinating enrichment cultures, which were derived from a same source (that is TC contaminated sediment) and amended only with the corresponding electron acceptors over years, were investigated.

Both 16S rRNA gene sequencing and qPCR analysis revealed a distinct selection of dechlorinators *Dhc* and *Dhb* with respect to amendment with chlorinated-ethenes, -propanes, -ethanes or -methanes (Figures 3.1 and 3.2). Results demonstrated that *Dhc* populations had strong substrate specificity to chlorinated-ethenes and 1,2-D; whereas *Dhb* populations to chlorinated-ethanes or CF. *Dhc* strains carrying *vcrA* genes were enriched in all of the chlorinated-ethenes amended cultures (Figure 3.4A), indicating that

these strains have broad substrate ranges. These results are consistent with previous observation of that VcrA can catalyze dechlorination of broad range of substrates.[49] In contrast, *Dhc* strains carrying *tceA* gene or *bvcA* genes showed more specific enrichment under different chlorinated-ethenes amendments. *Dhc* strains carrying *bvcA* genes were only enriched in PCE and cDCE amended cultures and diluted out from TCE- and VC-amended cultures during over 3+ years enrichment. Similarly, *Dhc* strains carrying *tceA* genes were only enriched in TCE-amended cultures, but diluted out from other chlorinated-ethene amended cultures. These results indicated that *Dhc* strains were either in a competition with each other for the chlorinated ethenes in cultures, therefore some were diluted out, or each *Dhc* strains has high specificity to certain chlorinated-ethenes. Enrichment of *Dhc* strains carrying *dcpA* gene in 1,2-D-amended cultures, but not in any other tested electron acceptor conditions indicated that *Dhc* strains with *dcpA* gene have a high substrate specify to 1,2-D. Detection of known PCE-to-cDCE dechlorinator *Dhb*[50] in PCE-amended cultures indicated their possible contribution to PCE or TCE dechlorination. Conversely, detection of *Dhb* in cDCE- and VC-amended cultures at quantifiable copies after 3+ year-enrichment was somewhat unexpected and could be an indication for a role of *Dhb* in dechlorination of these compounds, which needs to be further investigated. RDase *cfrA* from *Dehalobacter* strain CF was reported to catalyze TCA-to-DCA or CF-to-DCM reductive dechlorination reaction.[10, 48] Detection of *cfrA* genes at high numbers in TCA cultures showed that *Dhb* strains carrying *cfrA* gene were selected under TCA-amendment (Figure 3.4B). In contrast, detection of *cfrA* at very low numbers (~10^3 gene copies/mL) despite of detection of *Dhb* 16S rRNA gene at high numbers (>10^7 gene copies/mL) in CF-amended cultures was unexpected. Although bacterial transformations of CF have been also observed in methanogenic and sulfate reducing environments[51, 52], measurement of high *Dhb* 16S rRNA gene numbers indicated that growth of different *Dhb* strains (with different RDase) are more likely to be occurring via dechlorination of CF-to-DCM in CF-amended cultures.

Another interesting outcome of this study was detection of high number of *Dhc* 16S rRNA genes along with high numbers of RDase genes *vcrA* and *tceA* in DCA-amended

cultures (Figure 3.4A). These high numbers of *Dhc* 16S rRNA and RDase genes presented possible growth of *Dhc* strains carrying *vcrA* or *tceA* RDase genes under DCA-amended conditions. Previously, *Dhc* strain 195 was reported to dechlorinate 1,2-dichloroethane (1,2-DCA),[53] in addition, enzymes *BvcA*, *VcrA* were also reported to catalyze dehaloelimination of 1,2-DCA to ethane reaction.[49, 54] Further experiments to explore whether *vcrA*, *tceA* or other *Dhc* RDase genes involved in the dechlorination of DCA can provide more insights into *Dhc* substrate range. The knowledge of specific strains selection with certain electron acceptor amendments from the same source has implications for bioremediation. Different strains of dechlorinators especially *Dhc* have specific requirements (e.g., corrinoid cofactors[55], which is further discussed Chapter 4), and knowledge of the conditions favoring the specific dechlorinators may enable the development of strategies with strain specific-amendments. In addition, bioremediation strategies where bioaugmentation with specific *Dhc* populations or *Dhc*-containing enrichment cultures (e.g., enriched with VC amendment only) could be used under specific electron acceptor contamination to overcome stalls/slow dechlorination *in situ*.

Microbial community analyses based on 16S rRNA gene amplicon sequencing revealed effect of electron acceptors on non-dechlorinating community structure. The primary role of the non-dechlorinating microbial communities in dechlorinating cultures is to ferment complex organic compounds to H_2 and acetate required electron donor and growth substrate for *Dhc* reductive dechlorination activity, respectively. Acetogens, fermenters and methanogens frequently co-exist with *Dhc*- and *Dhb*-containing enrichment cultures.[30, 56] Fermenters or acetogens including Firmicutes *Clostridium*, *Sedimentibacter*, *Acetobacterium*, and members of order of *Bacteroideles* were the most abundant enriched members after *Dhc* in all chlorinated-ethenes amended cultures, indicating that there was no significant effect of electron acceptors on fermenting populations. In contrast, previous studies comparing three *Dhc*-contaning dechlorinating cultures (i.e., KB-1, ANAS, and Donnall amended with methanol, lactate and butyrate, respectively; and TCE or PCE as electron acceptor) showed taxonomic variations between the fermenting and acetogenic populations due to maintenance (i.e., electron donor) and geographical

differences.[24] Similar to TC-fermenting populations in chlorinated-ethene amendment, enrichment of Firmicute *Clostridium*, and *Bacteroides* were also reported for ANAS culture; but different populations other than Firmicute group were enriched KB-1 fed with methanol. Results of this research combination with previously reported literature, demonstrate that electron donor effect is more robust on development of fermenting and acetogenic populations.

In contrast to observation made with chlorinated-ethene dechlorinating cultures, we observed significant effect of chlorinated ethanes and CF on dechlorinating cultures development. Although acetogenic *Acetobacterium* OTUs, a well-known corrinoid producers[57] were recovered at high abundances (>4 % of the total bacterial OTUs) in TCA_T3 cultures, *Acetobacterium* OTUs were detected at abundance of less than 0.01 % of the community in TCA_T18 (enriched over 3+ years). Similar observation was made with DCA-, and CF-amended cultures. This could be reason of inhibition of TCA on non-dechlorinating populations like acetogenic *Acetobacterium*. This is relevant finding for bioremediation of sites co-contaminated with mixture of chlorinated solvents. TCA, DCA and CF inhibition on reductive decflorination of chlorinated ethenes especially beyond cDCE level were previously reported, and attributed as the reason of cDCE stalls in co-contaminated sites.[9, 30] Inhibition of chlorinated ethanes and CF on supportive communities (e.g., corrinoid producers) could also indirectly inhibit reductive dechlorination activity.

TC dechlorinating cultures were amended with lactate as electron donor, which can be metabolized by fermenting organism such as *Clostridium* and *Desulfovibrio* to products including acetate, propionate and H_2.[58] Methanogenesis or other hydrogen utilizing processes like reductive dechlorination make propionate-oxidation energetically feasible by keeping the H_2 concentrations at low levels.[59, 60] Enrichment of propionate-oxidizing bacteria *Pelomaculum* sp. at very high abundances (>10 % of total OTUs) along with methanogen *Methanoculleus*, and *Dehalobacter* indicated possible syntrophic interactions in TCA and DCA amended cultures. Similar syntrophic interactions with

different populations were observed in 1,2-D amended cultures, where mixed-fatty acid oxidizing syntrophic genera *Syntrophomonas*, and *Synropohus*[61] along with H_2 utilizing methanogens *Methanosaeta* and *Dhc* enriched at high abundances. The reason for differences in syntrophic communities could be different chlorinated compound-amendments (i.e., chlorinated ethane-amendment vs chlorinated-propane amendment). Although methanogens have been considered as competitors for H_2 in dechlorinating communities, recent studies emphasized their role in providing essential nutrients including corrinoid cofactor in dechlorinating communties.[24] Such development of syntrophic interactions in dechlorinating cultures may outweigh their role as H_2 competitors with dechlorinators, particularly when excess amount of electron donor is present. High sequence abundances of OTUs assigned to genus *Sytnrophomonas* (10 %) and members of *Desulfovibrionaceae* (6 %) in *Dhc* containing 1,2-D dechlorinating cultures indicated other possible syntrophic interactions sustaining *Dhc* growth in the community. In previous studies of *Dhc* strain 195 co-culture experiments with lactate fermenter *Desulfovibrio* and butyrate fermenter *Sytnrophomonas*, revealed that these fermenters could enhance *Dhc* growth and dechlorination rates by providing fermentation products H_2 as electron donor and acetate as growth substrate.[21, 62 63] In addition, *Desulfovibrio* and *Syntrophomonas* reduced toxic effect of carbon monoxide (CO), which accumulated as byproduct during the methionine biosynthesis via incomplete Wood-Ljungdahl pathway by *Dhc* strain 195 and showed to inhibit sustainable growth of *Dhc,*[63, 64] by using it as supplemental energy source. In contrast to observations with chlorinated-ethane dechlorinating cultures, syntrophic partners such as *Syntrophomonas*, *Syntrophus or Pelomaculum* did not enriched along with *Dhb* populations; instead another fermenting population *Treponema* within the phylum of *Spirochaetes*, were highly enriched in CF-amended cultures. Since lactate was the only electron donor source in all TC cultures, differences between supporting community members can be attributed to the effect of available electron acceptor on community development.

The most obvious effect of electron acceptors was observed on development of archaeal populations. Despite of their role as H_2 competitors, methanogenic archaea have been reported to be the one of the key supportive members in dechlorinating cultures,

sustaining reductive dechlorination activity by mitigating oxygen free radical damage on dechlorinators and also supporting required nutrients such as corrinoid cofactor.[24] Archaeal populations contributed to less than 0.1 % of community in CF-amended cultures, whereas contributed up to 30 % of the community in other TC-dechlorinating cultures. CF has been reported to inhibit methanogenic activities,[65, 66] which could explain the low abundances of archaeal populations in CF-amended cultures. Taxonomic differentiation on archaeal communities between cultures amended with chlorinated-ethenes, chlorinated-ethanes, 1,2-D and CF was significant (Figure 3.3). Although cultures were established from the same contaminated sediment, different genera of methanogens enriched in TC-cultures amended with different electron acceptors. *Methanobacterium* was highly enriched in chlorinated-ethenes amended cultures; *Methanoculleus* was enriched in chlorinated-ethane amended cultures, whereas *Methanosaeta* was the most abundant archaeal population in 1,2-D. Variation in archaeal groups was previously reported between the three dechlorinating enrichment cultures (ANAS, KB-1 and Donnall), derived from different sources, and amended with different electron donors (i.e., lactate, methanol, butyrate, respectively). The reason for this variation could be explained by geographical and maintenance differences (i.e., different electron donor- amendments) for these three cultures. However in TC cultures, the only difference between the cultures was amendment with different electron acceptors. Methane can be produced by methanogens through different pathways, each of which has different substrates. For example, hydrogenotrophic methanogens like *Methanobacterium* and *Methanoculleus* reduced CO_2 with H_2 as electron donor to methane; acetoclastic methanogens such as *Methanosaeta* and *Methanosarcina* convert methyl group of acetate to methane.[67, 68] The differentiation between methanogenic populations in TC cultures could be attributed to substrates they use for methanogenesis; however since H_2 and acetate were produced via lactate fermentation in all TC-cultures, the effect of different chlorinated solvent on different methanogenic populations become more likely.

To conclude, this study demonstrated the effect of electron acceptors on dechlorinator strain selection and dechlorinating community compositions. Variations in non-

dechlorinating members were detected between TC cultures, which were amended with different electron acceptors. Inhibition of compounds such as TCA or CF on non-dechlrinating members can affect *Dhc* reductive dechlorination due to lack of appropriate supportive members at contaminated sites. Therefore, observed variation between community-structure should be taken into consideration in developing bioremediation strategies for sites contaminated with mixtures of the compounds. Knowledge of the strains and dechlorinating-communities that are selected under different chlorinated electron acceptor will enable development of better bioremediation strategies to achieve successful contaminated-site cleanup.

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3.7 Appendix 3

Table S3.1. Summary of 16S rRNA gene sequencing results

Samples	Total number of high-quality sequences	Platform used for sequencing	Total OTUs/sample
Sediment (TC, Location #3)	4545	454	972
TC-PCE (3)	8527	454	323
TC-TCE (3)	8821	454	313
TCE-cDCE (3)	7601	454	245
TCE-VC (3)	8447	454	286
TC-TCA (3)	10346	454	231
TC-TCE (8)	22942	454	111
TC-cDCE (8)	25095	454	125
TC-1,2[#]	64147	Illumina MiSeq	126
TC-TCA (18)[#]	55032	Illumina MiSeq	138
TC-DCA[#]	69918	Illumina MiSeq	181
TC-CF[#]	94172	Illumina MiSeq	127

[#] DNA was obtained from cultures enriched for over 3+ years
Numbers in parentheses represent culture generation number

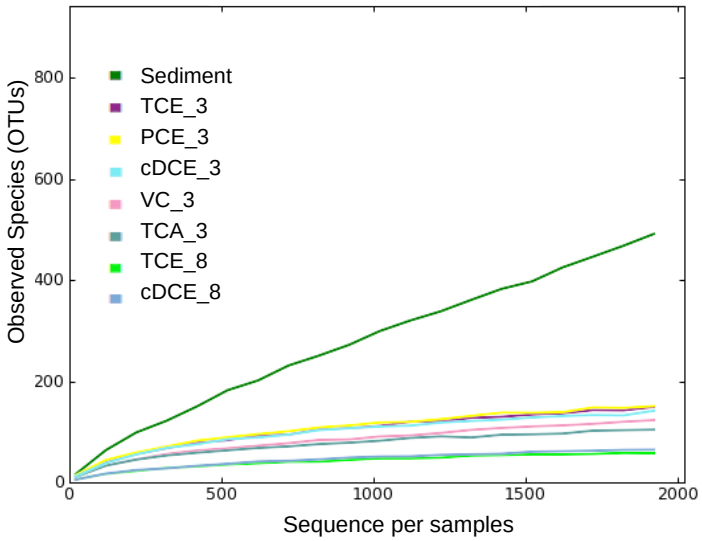

Figure S3.1. Rarefaction curves of bacterial 16S rRNA gene sequences recovered from TC enrichment cultures and sediment
Rarefaction curves of bacterial 16S rRNA gene amplicon sequences recovered from TC enrichment cultures. The y-axes represent OTU richness estimates for the number of sequences analyzed (x-axes)

4 Corrinoid and Lower Base Bioavailability Is a Major Control of *Dehalococcoides* Reductive Dechlorination Activity in Chlorinated Ethene-Dechlorinating Enrichment Cultures

4.1 Abstract

Organohalide-respiring *Dehalococcoides mccartyi* (*Dhc*) strains are keystone bacteria for detoxification of toxic and carcinogenic chlorinated contaminants. Corrinoid auxotroph *Dhc* has an obligate requirement for corrinoid cofactor for organohalide- respiration. In chlorinated-solvent contaminated environments, *Dhc* depend on corrinoid salvage to acquire essential corrinoid cofactor produced by other microorganisms. However, *Dhc* have highly specific corrinoid requirements that not all naturally occurring corrinoids support *Dhc* reductive dechlorination activity. In this study, we hypothesized that available lower bases controlled corrinoid pools in mixed microbial communities, and that the corresponding corrinoids effected *Dhc* reductive dechlorination activity and *Dhc* strain selection. Tetrachloroethene (PCE), *cis*-dichloroethene (*c*DCE) and vinyl chloride (VC) dechlorinating enrichment cultures (PCE-, cDCE-, and VC-noB_{12}) were derived from chlorinated solvent contaminated site sediment without vitamin B_{12} amendment. Relatively similar dechlorination rates of PCE and VC dechlorination were measured in PCE- and VC-dechlorinating cultures, amended with or without vitamin B_{12}, indicating that non-*Dhc* community members sustained *Dhc* reductive dechlorination by providing functional corrinoids. Corrinoid analysis revealed that 5,6-dimethylbenzimidazole-cobamide (DMB-Cba) was the most abundant corrinoid produced in cultures grown without B_{12} addition. In contrast to PCE and VC-noB_{12} cultures activity, incomplete cDCE dechlorination was demonstrated by very low dechlorination rates in cDCE-noB_{12} cultures. DMB amendment to cDCE-noB_{12} cultures did not enhance reductive dechlorination, indicating that cDCE was potent inhibitor of corrinoid producers. Furthermore, addition of lower bases 5,6-dibromobenzimidazole (5,6-DiBrBza) and benzimidazole (Bza) caused a shift from native DMB-Cba production to production of corrinoids 5,6-DiBrBza-Cba and Bza-Cba, and corresponding corrinoids affected *Dhc*

reductive dechlorination activity. 16S rRNA gene sequencing of PCE-, cDCE-, and VC-noB12 cultures revealed the high abundances of well-known corrinoid producers *Acetobacterium*, and *Clostridium*. In addition, corrinoid producing PCE-to-cDCE dechlorinator *Geobacter lovleyi* was the most abundant member in PCE-dechlorinating cultures, presenting its role in contribution to corrinoid pool. This study also demonstrated the possible effects of available corrinoids in determining *Dhc* strain selection, highlighting the role of corrinoids in *Dhc* diversity at contaminated sites. This study emphasizes that lower base- and corrinoid-producing community has a strong control over *Dhc* reductive dechlorination activity and its strain selection by controlling the corrinoid pool in the environments. Therefore, dechlorinating communities must be elucidated to predict *Dhc* activity and selection at chlorinated-solvent contaminated sites, and develop effective contaminated site cleanup strategies.

4.2 Introduction

Toxic chlorinated compounds, including tetrachloroethene (PCE) and trichloroethene (TCE) have been widely used in different areas of industry as solvents, biocides, plasticizers, dry cleaning, decreasing machinery components in the past century[1]. Improper management and accidental spills of chlorinated solvents have resulted in prevalent groundwater contamination in US.[1-3] Biodegradation shows great promise as an environment-friendly and cost-effective remediation strategy for a wide variety of toxic groundwater pollutants.[4-7] Organohalide-respiring *Dehalococcoides mccartyi (Dhc)* strains within the phylum *Chloroflexi*, couple growth with reductive dechlorination of a variety of priority chlorinated contaminants. Among *Dhc* strains are the only microorganisms so far capable of complete detoxification of toxic PCE, TCE and dechlorination daughter products dichloroethenes (DCEs) and vinyl chloride (VC) to non-toxic ethene, thus are of great interests to remedy aquifers contaminated with chlorinated ethenes[7-10]. *Dhc* have a unique and highly restricted lifestyle, and require specific chlorinated hydrocarbons as electron acceptors, acetate as carbon source and hydrogen as electron donor to fuel their energy metabolism.[8] The majority of *Dhc*-

containing dechlorinating cultures are maintained as mixed microbial consortia, because mixed cultures generally exceed pure cultures performances in terms of *Dhc* growth yields, dechlorination rates, and better stability.[8, 11-13] Community members sustain *Dhc* growth by maintaining a reducing environment and providing essential growth factors (e.g., H_2, corrinoid cofactor) and metabolites that *Dhc* requires.[8, 12, 14, 15]

The key catalysts for organohalide-respiration are reductive dehalogenase enzyme systems (RDases), which require a corrinoid cofactor for the activity.[16-19] Despite their dependence on corrinoid cofactor, *Dhc* strains lack the ability for *de novo* corrinoid biosynthesis[20-24] and the growth of *Dhc* pure cultures strictly depends on exogenous addition of vitamin B_{12} (cyanocobalamin).[19, 25-28] All sequenced *Dhc* strains possess the genes involved in corrinoid scavenging pathways, including corrinoid transport, cobinamide salvage/activation, and lower base activation, which may help to access growth supporting cobamides[14, 29, 30]. In various natural environments, methanogens, acetogens and other bacterial groups are corrinoid producers and are presumed to provide corrinoids to corrinoid auxotrophic *Dhc*.[12, 31-36] Naturally occurring corrinoids produced by these microorganisms have been found to be various types with different lower base structures, including benzimidazole, phenolic, and purine types[34, 36, 37]. Recent studies demonstrated that *Dhc* had highly specific corrinoid requirements that not all naturally occurring corrinoids fulfill *Dhc* reductive dechlorination activity in co-cultures [15, 29, 30, 38]. Recent studies revealed that different *Dhc* strains (different RDase genes) could have different preferences for different type of corrinoids. For example, growth of *Dhc* strain 195 harboring *tceA* RDase was sustained by corrinoids with DMB, 5-methylbenzimidazole (5-MeBza) and 5-methoxybenzimidazole (5-OMeBza) lower bases, not with benzimidazole (Bza), 5-hydroxybenzimidazole (5-OHBza) or phenolyl lower bases[14, 30]. VC dechlorinating *Dhc* strains BAV1 harboring *bvcA* RDase and GT harboring *vcrA* RDase also showed different preferences for the corrinoids.[29] While corrinoids with 5-OMeBza, Bza lower bases sustained strain BAV1 VC reductive dechlorination to non-toxic ethene, they failed to sustain growth of *Dhc* strain GT and toxic VC was not completely dechlorinated to ethene[29].

Availability of sufficient right type of corrinoids is also another important factor controlling *Dhc* reductive dechlorination. Growth of *Dhc* strains BAV1, GT and FL2 grown with limiting amounts of 1 µg/L exogenous vitamin B_{12} failed to completely detoxify chlorinated ethenes to ethene, which resulted in toxic vinyl chloride (VC) accumulation. In contrast, the same cultures amended with 25 µg/L vitamin B_{12} completely degraded chlorinated ethenes with much higher dechlorination rates.[38] These distinct responses of *Dhc* strains to different quantities and type of corrinoids with different lower bases indicate that availability of right type of the corrinoid and/or lower bases at enough quantities control *Dhc* activity and extents in contaminated environment. Remarkably, studies with pure *Dhc* strains and co-cultures with corrinoid producers demonstrated the essential role of corrinoids for *Dhc* reductive dechlorination. However, there is still a knowledge gap about the corrinoid-dependency of *Dhc* and corrinoid-related interactions between *Dhc* and other microorganisms in natural environments. In addition, key cofactor corrinoid's role in *Dhc* strain diversity (i.e., selection) has not been demonstrated yet. To fulfill the knowledge gaps and improve bioremediation strategies, in this study dechlorinating mixed cultures, which derived from a chlorinated-solvent contaminated site sediment, were used as representatives of microbial communities in contaminated-environments. We explored the importance of supporting microorganisms in terms of sustaining corrinoid cofactor to support *Dhc* reductive dechlorination, and the effects of available corrinoids on *Dhc* strain selection.

4.3 Material and Methods

4.3.1 Chemicals

Lower bases, DMB (≥ 99%), 5-MeBza (≥ 98%), 5-OMeBza (≥ 97%), Bza (≥ 98%), 5,6-DiBrBza, betaine (≥ 99%) were purchased from Sigma-Aldrich (St Louis, MO, USA). Yeast extract and casitone were purchased from Becton, Dickson and Company (Franklin Lakes, NJ, USA). Chlorinated compounds were of >99% purity. PCE was purchased from ACROS Organics (Morris Plains, NJ), TCE was obtained from Fisher Scientific

(Pittsburgh, PA), and *c*DCE, vinyl chloride (VC), ethene, were obtained from Sigma-Aldrich-Fluka (St. Louis, MO).

4.3.2 Environmental samples

Sediment samples were collected from the chlorinated solvent-contaminated Third Creek sediment in Knoxville. Sediment samples were obtained using direct push tools (AMS, Inc., American Falls, ID). The plastic liners and caps were wiped with 70% ethanol before use. All other materials (spatulas, containers, etc.) were autoclaved, and aseptic techniques were applied to the extent feasible. All core samples were immediately transferred to Mason jars, filled completely with creek water to exclude air, capped and placed in a cooler with ice packs. Sediment samples from the same locations were homogenized by mixing inside an anoxic glove box filled with H_2/N_2 (3/97%, vol/vol), and were kept at 4°C till microcosms were established (within 1 week of sample collection).

4.3.3 Microcosms and enrichment cultures with and without exogenous vitamin B_{12} amendment

Inside the anoxic glove box filled with H_2/N_2 (3/97%, v/v), two separate sets of duplicate microcosms were established using sediment samples (approximately 4 g, wet weight) in 60-mL glass serum bottles containing 26 mL defined, bicarbonate-buffered mineral salt medium with a N_2/CO_2 (80/20 [vol/vol]) headspace, and amended with 5 mM lactate as electron donor and 0.2 mM PCE as electron acceptor. One set was prepared with medium containing vitamin 25 µg/ L of vitamin B_{12} (withB_{12}), the other set was prepared with vitamin B_{12}-free medium (noB_{12}). After all PCE was dechlorinated to ethene, 3% (v/v) cultures were transferred into fresh 27-mL medium keeping the same amendments. After, all PCE was dechlorinated to ethene in the 1st transfer PCE-dechlorinating enrichment cultures, PCE, cDCE and VC-dechlorinating sub-cultures were derived by transferring 3% (v/v) PCE-dechlorinating parent cultures in to 160-mL glass serum bottles containing 100 mL vitamin B_{12}-free defined, bicarbonate-buffered mineral salt medium or medium

with 25 µg/ L of vitamin B_{12}. Sub-cultures received 5 mM lactate as electron donor and 0.5 mM PCE, cDCE or VC as electron acceptor. Sediment-free PCE, cDCE, and VC dechlorinating enrichment cultures with or without vitamin B_{12} amendment were derived by continuously transferring the cultures into fresh 100-mL medium keeping the same amendments over 3+ years (when electron acceptors were completely dechlorinated to ethene). PCE, cDCE and VC cultures derived with vitamin B_{12} amendment over +3 years were used in 16S rRNA gene community analysis. Cultures derived without vitamin B_{12} amendment (PCE–, cDCE–, and VC–noB_{12}) were used for further analyses to test corrinoid related questions in this research. For dechlorination rate comparison experiments, control group of PCE–, cDCE–, and VC–noB_{12} cultures did not receive vitamin B_{12}, another set of these cultures received 25 µg/ L of vitamin B_{12}. All microcosms and enrichment cultures were incubated statically at in dark at room temperature, and 30°C, respectively.

4.3.4 PCE enrichment cultures with different lower base amendments

To investigate if the available lower bases control the corrinoid production by the community members and corresponding corrinoids control *Dhc* reductive dechlorination activity in dechlorinating enrichment cultures, PCE-noB_{12} enrichment cultures were grown with different lower base amendments, with or without vitamin B_{12} amendment. Two percent (v/v) PCE-noB_{12} cultures were transferred into triplicate glass vials containing 200-mL vitamin B_{12}-free medium, amended with 5 mM lactate as electron donor and 0.5 mM PCE as electron acceptor, and received 25 µg/L vitamin B_{12} or 50 µM one of the following lower bases: DMB, Bza, 5,6-diromobenzimidazole (5,6-DiBrBza, an artificial lower base). Additional set of culture did not receive any lower base, served as a control group (i.e., no vitamin B_{12}, no lower base amendment). To obtain high biomass yield for corrinoid analysis, PCE-noB_{12} cultures were grown in larger volumes, in 2-L glass bottles containing 1.6-L vitamin B_{12}-free medium, amended with 5 mM lactate, and received 50 µM lower bases. One set of cultures did not receive any lower base or vitamin B_{12}, served as a native PCE-noB_{12}. To explore what type of corrinoid was produced by non-dechlorinating community members (e.g. fermenters, acetogens), and

available to *Dhc* under different lower base amendments, 1.6-L cultures did not receive PCE as electron acceptor. Cells from the cultures were harvested from 1.6-L cultures after 11 days, which was determined as the time when all PCE was reduced to cDCE in cultures amended with PCE, and lactate was completely fermented to acetate and propionate. All culture vessels were incubated statically in dark at 30°C.

4.3.5 cDCE-noB_{12} cultures with DMB amendment

To explore if DMB-addition to cDCE-noB_{12} cultures increase DMB-Cba production and recover *Dhc* reductive dechlorination activity, triplicate cDCE-noB_{12} cultures were grown in 100 mL vitamin-B_{12}-free medium amended with 0.5 mM cDCE as electron acceptor and 5 mM lactate as electron donor, and 50 μM DMB. Culture vessels were incubated statically in dark at 30°C.

4.3.6 Intracellular corrinoid extraction and purification

To investigate corrinoid production by the community members under different lower base amendment or no-amended conditions, cells from 1.6-L cultures were harvested by centrifugation in 500-mL plastic bottles at 15 000 × g for 20 min at room temperature. Intercellular total corrinoids were extracted in the cyano form and purified following the potassium cyanide (KCN) extraction protocol with following changes.[38] Briefly, following removal of supernatants, cell pellets were suspended in 5 mL of deionized water in sterile 50-mL plastic tubes, the pH was adjusted to 5−6 with 3 % (v/v) glacial acid, and KCN was added at 10 mM final concentration. The suspensions were vortexed, each transferred into sterile 50 mL-plastic tubes and incubated for 20 min in a boiling water bath. Following centrifugation 15 000 × g for 15 min, the supernatant collected, and to increase corrinoid recovery yield, corrinoids were extracted from the cell pellets second time by repeating the previous steps. The combined supernatants were loaded onto Sep-Pak C18 cartridge (Waters Corp, Milford, MA, USA), which was previously equilibrated with 2 mL of 100 % methanol and 40 mL of deionized water. Following sample loading, each cartridge was washed with 10 mL of deionized water and 7.5 mL 10

% methanol. Lastly, corrinoid on the cartridges was eluted with 4 mL of 100 % methanol. The final slightly pink-colored solutions were vacuum dried and residues were suspended in sterile tubes with 0.5 mL deionized water.

4.3.7 Identification and quantification of corrinoids produced in enrichment cultures

To use as standards for HPLC measurements, cobamides DMB-, 5-MeBza-, 5-OMeBza-, Bza-, 5-OHBza, 5,6-DiBrBza-Cba and phenolic type (Phe-Cba and *p*-Cre-Cba) were biosynthesized, extracted and purified following an established protocol.[29] Briefly, *Sporomusa* sp. strain KB-1 cultures growing in 1.2 L glass bottles using a defined medium (B_{12}-free) were amended yeast extract and casitone (2 g/L of each) and betaine (50 mM) and supplied with single lower base compounds. After static incubation at 30°, cobamides biosynthesized by *Sporomusa* sp. strain KB-1 were extracted and purified following KCN extraction protocol.[38] Absorbance of purified cobamides were measured with Lambda 35 UV/Vis spectrometer (PerkinElmer, Waltham, MA, USA) at 361 nm, and cobamide concentrations were estimated using a molar extinction coefficient of 28, 060 mol/cm.[39] 20 µL of corrinoid samples extracted from 1.6-L redox-enrichment cultures were analyzed with an Agilent (Santa Clara, CA, USA) 1200 high-performance liquid chromatography (HPLC) system with Eclipse XDB-C18 column (5 µm, 4.6 × 150 mm). Following injection onto column, samples were separated with a flow rate of 1mL/min at 30°C using 0.1 % (v/v) formic acid (≥ 88%, w/v) in water (eluent A) and 0.1% formic acid in methanol (eluent B), and a linear change to 25% A/ 75% over 3 min followed by a 5 min hold before the column was equilibrated to initial conditions. Cobamides were detected at 361 nm with an Agilent 1260 Infinity diode array detector and concentrations were quantified by comparing integrated peak areas with 4-point calibration curves, which were generated with purified cobamides.

4.3.8 Quantification of *Dhc* 16S rRNA, *bvcA*, *vcrA*, *tceA and pceA* genes

Cells were harvested on to 0.22 μm membrane filters (Merc Milipore Ltd, Darmstadt) in triplicates from 1 mL of PCE-noB_{12} cultures amended with different lower bases, or with/without vitamin B_{12} after a cycle of complete reductive dechlorination of PCE-to-ethene. DNA was extracted from the filters using MO BIO Soil Kit following the manufacturer's protocols. Following extraction, *Dhc* 16S rRNA gene, RDases *tceA*, *vcrA*, *bvcA*, and *Dhc* strain 195 *pceA* were quantified using primers and probe sets of Dhc1200F /Dhc1271R/ Dhc1240Probe[40], TceA1270F/ TceA1336R/ TceA1294Probe[40], VcrA401F[40]/ VcrA471R[40]/ VcrA1042Probe (VcrA1042Probe 6FAM-TACCAGGAAATGGTTGAGTTAC-MGB), BvcA58F (GCGGGAGCAGGGATAGGT)/ BvcA116R (TCATCCAAGTCGTGAAAATTCG)/ BvcA77P (6FAM-CCGCGACTTCAGTTAT-MGB), Dhc195-PceA1111F (CCACCCCGGTTCGTTCA)/ Dhc195-PceA1171F (CGGCGTGGTGGAAACAG)/ Dhc195-PceA1134Probe (6FAM-ACATGACGTAGTCAAGGG-MGB), respectively following the established qPCR protocols.[40] Standard curves for each qPCR assays were generated with 10-fold serial dilutions (10^8 to 10^0 gene copies/μL template) of a cloned-target gene fragments. All assays exhibited amplification efficiencies ranging from 90-100% (i.e., slope of the regression lines were between -3.6 and -3.1) and the linear standard curves had R^2 values > 0.98. Nuclease free water replaced template DNA in control assays and verified template DNA-specific fluorescence increase.

4.3.9 16S rRNA gene amplicon sequencing and sequence analysis

To perform 16S rRNA gene sequencing, cells were collected from 5 mL of triplicate PCE, cDCE and VC –noB_{12}, -withB_{12} enrichment cultures, after a cycle of complete reductive dechlorination of representative electron acceptor. DNA was extracted from collected cells using MO BIO Soil Kit following the manufacturer's protocols. Following extraction, DNA samples were purified using the Genomic DNA Clean and Concentrator Kit (Zymo Research, Irvine, CA). Purified DNA samples were pooled and then samples were PCR amplified by targeting the V4 region of the bacterial 16S rRNA gene using

primers F515/R806. [41, 42] Amplification was performed in 50 µL assays consisting of 10 uL of sample DNA, 1 µL of barcoded-primer (10 µM) and 39 µL of a mixture of 22 µL de-ionized water (5 PRIME, Gaithersburg, MD), 5 µL Invitrogen *Pfx50*™ buffer (Invitrogen, Carlsbad, CA), 1 µL CAP 515F primer (10 µM), 5 µL dNTP (Invitrogen, Carlsbad, CA), 1 µL Invitrogen *Pfx50*™ Polymerase (Invitrogen, Carlsbad, CA) and 5 µL of $MgCl_2$ (25mM). Thermo cycling program included denaturation at 94°C for 3 min followed by 35 cycles at 94°C for 45 sec, annealing at 55°C for 60 sec, and extension at 72°C for 90 sec, and final extension at 72°C for 10 min. Quality (size) of produced amplicons was checked using High Sensitive DNA Kit on a model 2100 Bioanalyzer (Agilent, Santa Clara, CA). Relative concentrations of the individual samples were estimated based on the peak height at the appropriate size, and pooled to equal amounts. Pooled samples were purified with SPRI magnetic beads (Beckman Coulter, Inc., Indianapolis, IN). The products from purification step were analyzed again with High Sensitive DNA kit for quality assurance and verification of the removal of primer dimers. Before sequencing, concentrations of pooled amplicons were determined using Illumina Library Quantification kit (KAPA Biosystems, Boston. MA) following the manufacturer's protocol. Quantification of each sample was determined based on amplicon adaptors. The amplicon library was diluted to a starting concentration of 10 nM, followed by paired end sequencing on the Illumina MiSeq sequencer (Illumina, Inc., San Diego, CA). After sequencing was performed, base calling, demultiplexing and adopter trimming were performed using MiSeq reporter using the default parameters. Raw 16S rRNA gene amplicon sequences were then analyzed using CLC Genomic workbench (v9.0) with CLC Microbial Genomics Module (v1.3.1) from Qiagen. Briefly, all reads were initially subjected to quality control: reads were paired, merged, quality trimmed and filtered based on the presence of the ambiguous nucleotides using the CLC Microbial Genomics default parameters. Following the quality control, sequences were clustered into operational taxonomic units (OTUs) at a threshold of 97% sequence similarity, checked for chimeras with CLC Microbial genomics default parameters and aligned with the MUSCLE algorithm[43]. The Greengenes database (http://Greengenes.secondgenome.com) was used for taxonomic assignments of OTUs.

4.3.10 Other analytical methods

Chlorinated ethenes and ethene were monitored using an Agilent 7890 gas chromatograph (GC) equipped with a flame ionization detector and a DB-624 capillary column (60 m by 0.32 mm with a film thickness of 1.18 μm) as described[44]. The method provided linear detector responses for quantification of each analyte to 0.7 mM aqueous phase concentrations with a detection limit of about 2.5 μM. Standards curves were prepared by adding known amounts of individual analytes to 160-mL glass serum bottles containing 100 mL of water, and analysing via GC. The concentrations of chlorinated ethenes were calculated by normalizing the peak area measurements to standard curves. Lactate and its fermentation products acetate and propionate were analyzed using an Agilent 1200 series HPLC system equipped with and Aminex HPX-97H column (Bio-Rad, Hercules, CA, USA). Samples were acidified with 1 M H_2S04 in a ratio of 19:1 (v/v), and separated with 4 mM aqueous H_2S04 as the mobile phase at a flow rate of 0.6 mL/min, and then quantified using a UV detector set to 210 nm.

4.4 Results

4.4.1 Dechlorination activity in PCE, cDCE and VC enrichment cultures with and without vitamin B_{12} amendment

In all PCE–, cDCE–, VC–noB_{12} enrichment cultures complete reductive dechlorination of corresponding chlorinated ethenes to non-toxic ethene occurred, demonstrating that non-*Dhc* microorganisms in the cultures (without exogenous vitamin B_{12} amendment) sustained *Dhc* reductive dechlorination activity by providing required corrinoid. Comparable PCE-to-ethene dechlorination rates of 41.1 ± 3.1 and 38.7 ± 3.6 μM Cl^- released per day were measured in PCE-noB_{12} cultures grown with and without vitamin B_{12}, respectively, and the initial amount of PCE was dechlorinated to ethene in 50 and 54 days, respectively (Figure 4.1A). Similarly, VC-noB_{12} grown with and without vitamin B_{12} amendment showed very similar dechlorination activity with measured rates of 14.21 ± 0.1 and 13.46 ± 0.5 μM Cl^- released per day, respectively (Figure 4.1B). In contrast,

although cDCE-noB_{12} cultures amended with B_{12} dechlorinated to initial amount of cDCE to ethene in 61 days at a dechlorination rate of 19.6 ± 1.4 μM Cl^- released per day, much lower dechlorination rate of 4.3 ± 0.6 μM Cl^- released per day was measured for cDCE-noB_{12} cultures grown without vitamin B_{12} amendment. In cDCE-noB_{12} cultures (no exogenous vitaminB_{12}), no more than 27 % (16 μmoles) of the total VC produced from cDCE dechlorination was further dechlorinated to ethene over 145-day activity monitoring period (Figure 4.1C). This discrete dechlorination activity in cDCE-noB_{12} cultures amended with and without vitamin B_{12} presented that availability of functional corrinoid (e.g., DMB-Cba) was the limiting factor, affecting the *Dhc* reductive dechlorination activity in cDCE-noB_{12} cultures. In previous cDCE-noB_{12} transfer more than 400-day incubation was needed for complete cDCE dechlorination to ethene without exogenous vitamin B_{12} amendment (Figure S4.1).

4.4.2 Community structure in PCE, cDCE and VC enrichment cultures with and without vitamin B_{12} amendment

Illumina MiSeq 16S rRNA gene amplicon sequencing produced a total of 524,005 16S rRNA gene V4 region sequences after raw pair-reads were merged and trimmed at fixed length of 252 bp. After further quality filtering (i.e., excluding chimeric reads), total of 440,843 high-quality 16S rRNA gene amplicon sequences were obtained. The number of sequences originated from different samples varied from 24,495 to 137,362 (Table S4.1). Total of 191 OTUs were assigned by clustering high-quality sequencing at 97% nucleotide sequence identity and taxonomic annotation using Greengenes database (Table S4.1). Total of 36 bacterial and 3 archaeal phyla were assigned across all samples. The majority of OTUs belonged to *Firmicutes* (31-79 % of total sequences) and *Chloroflexi* (9 to 15 % of total sequences), and *Bacteroidetes* (3 to 9 % of the total sequences), and *Proteobacteria* (2 to 42 % of the total sequences).

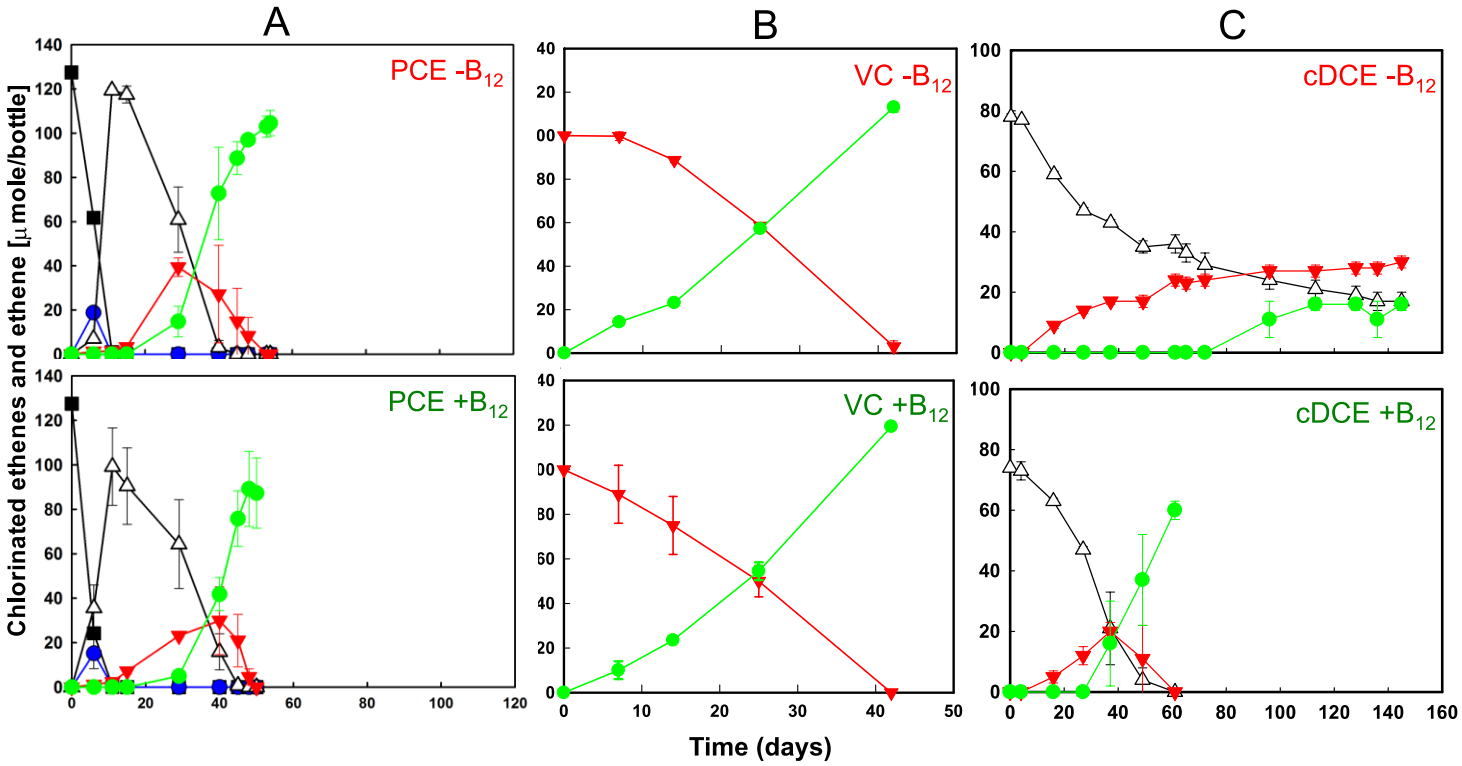

Figure 4.1. Reductive dechlorination in TC-noB_{12} and withB_{12} cultures
Reductive dechlorination of corresponding chlorinated ethenes in **A)** PCE-noB_{12} cultures grown without [upper figure] or with [bottom figure] vitamin B_{12}, **B)** VC-noB_{12} cultures grown without [upper figure] or with [bottom figure] vitamin B_{12}, **C)** cDCE-noB_{12} cultures grown without [upper figure] or with [bottom figure] vitamin B_{12}. Figures present the average data belongs to triplicate cultures, error bars represent the standard error of triplicate cultures. Black squares, PCE; blue circles, TCE; white triangles, cDCE, red inverted triangles, VC; green circles, ethene.

Taxonomic classification of the OTUs at genus level for all samples is shown in Figure 4.2. OTUs belonging to genus *Geobacter* (identified as PCE dechlorinator *Geobacter lovleyi* at species level) contributed to 41 and 19 % of the total sequences in PCE-withB_{12} and PCE-noB_{12} cultures, respectively; however as expected sequences of this group were detected at lower abundances (less than 1% of the total sequences) in cDCE, VC –withB_{12} and –noB_{12} cultures. OTUs assigned to *Dehalococcoides* comprised 9 to 15 % of the total sequences across the all samples. Community structure of PCE, cDCE, VC-withB_{12} and –noB_{12} cultures showed similar profile mostly for the fermenting, acetogenic group of microorganisms, which is probably due to amendment of the same electron donor (i.e., lactate) independent of electron acceptor or vitaminB_{12} amendment conditions. Genus *Clostridium* was the most abundant group in all samples except PCE-withB_{12} and VC-withB_{12} cultures, exceeding abundances of 20% of the total community. In PCE-withB_{12} and VC-withB_{12} cultures *Clostridium* OTUs were also abundant and contributed to 11 and 23 % of the total sequences. OTUs assigned to fermenters *Sedimentibacter* were another abundant group in all of the cultures ranging between 1 to 16 % of the total community. Propionate-oxidizing *Pelotomaculum* OTUs were at high abundances of 11 and 9 % of the total sequences in PCE-withB_{12} and VC-withB_{12} cultures, respectively; however, this group only contributed 1 to 3 % of the total sequences in other cultures. OTUs that could not be identified at genus and family level within the order *Bacteroidales* also showed high abundances (2 to 8 % of the total sequences) in all cultures. In addition, sequences belonged to proteolytic (degrading proteins into smaller polypeptides or amino acids) *Proteiniclasticum* were highly enriched contributing up to 12 % (ranging 2 to 12 %) of the total sequences in cultures grown without vitamin B_{12}-amendment and VC-withB_{12} cultures, whereas this group were detected at lower abundances in other vitamin B_{12}-fed cultures (Figure 4.2). OTUs assigned to genus *Acetobacterium* (showed 99% nucleotide sequence similarity with known cobalamin producers *Acetobacterium woodii* 16S rRNA gene) were contributed to 2 to 5% of the total community sequences in PCE-, cDCE-, VC-noB_{12}; however, this group was at much lower abundances (less than 1% of the sequences) in cultures amended with vitamin B_{12}.

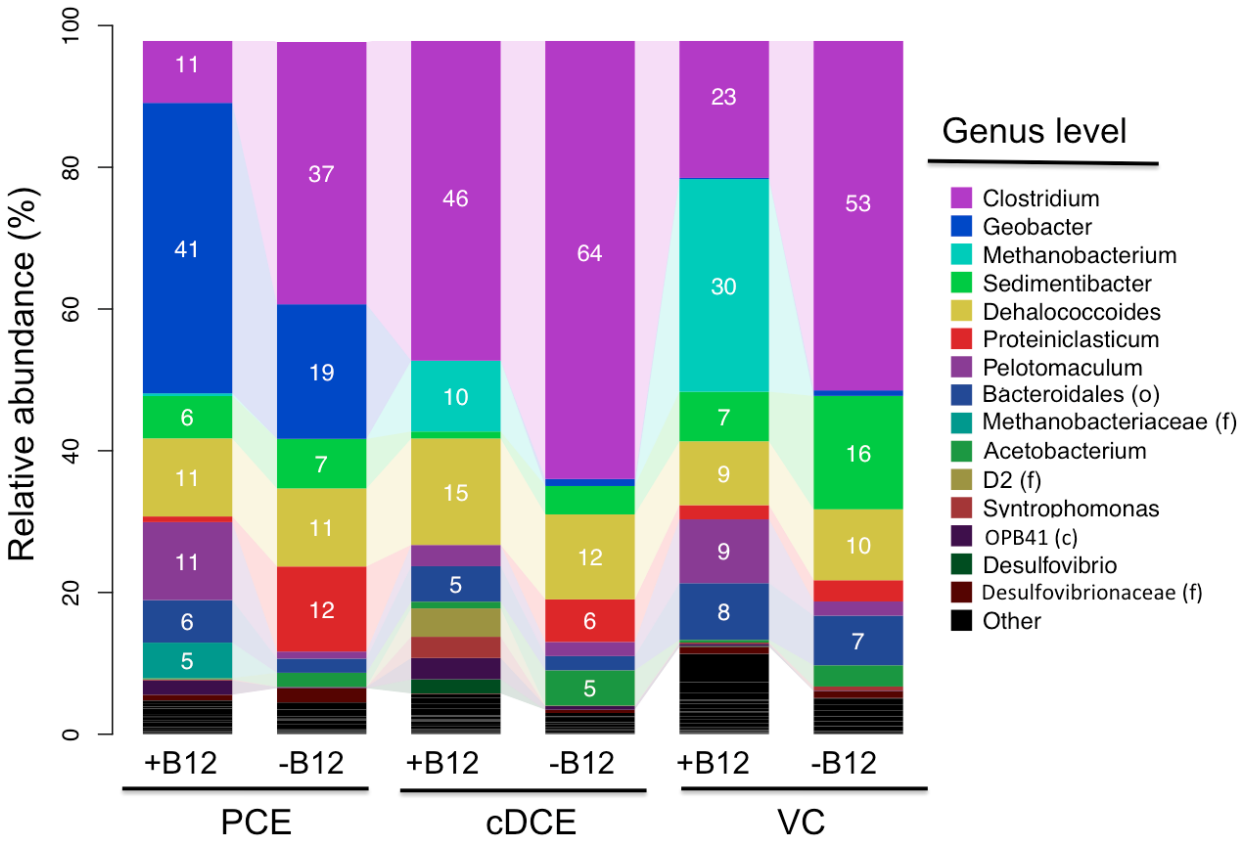

Figure 4.2. Microbial community structures in TC-withB_{12} and -noB_{12} cultures
Microbial community structures in PCE-, cDCE-, VC-withB_{12} and –noB_{12} cultures at the genus level based on 16S rRNA gene amplicon sequence analysis. Top 15 genus were listed, other low abundance genus are grouped in "Others". Numbers on bar graphs represent the relative abundances of each taxonomic OTUs (only >5 % of the total sequences were represented). OTUs, which could not be assigned to any genes, represented with its family or order level classification, and level of classification was indicated in parenthesis [order, (o); family (f)].

Methanogens *Methanobacterium* OTUs comprised the most abundant group (30% of the total sequences) in VC-withB_{12} cultures, and contributed to 10 % of the total sequences from cDCE-withB_{12} cultures; however, *Methanobacterium* OTUs were not detected in PCE-noB_{12}, cDCE-noB_{12}, and VC-noB_{12} cultures, and were in less abundances (<1% of the total sequences) in PCE-withB_{12} cultures. Unclassified methanogens within the family of *Methanobacteriaceae* contributed to 5 % of total sequences recovered from PCE-withB_{12} cultures; but this group was not detected in all other cultures.

4.4.3 Availability of lower bases in dechlorinating microbial communities controls corrinoid production

To explore if the availability of different lower bases control the type of the corrinoids produced by the microbial community in dechlorinating enrichment cultures, PCE-noB_{12} cultures were grown with different lower base amendments, and with or without vitamin B_{12} amendment. HPLC-DAD analysis of corrinoids produced under different lower base amendment-conditions in PCE-noB_{12} cultures, revealed different corrinoid profiles. Figure 4.3 shows HPLC chromatograms of corrinoids produced in PCE-noB_{12} cultures amended with different lower bases

DMB-Cba (that is cobalamin) was the abundant corrinoid produced by the non-dechlorinating microbial community in control cultures received no lower base (PCE-noB_{12}) and also in cultures amended with DMB (Figure 3A, B). By contrast, although DMB-Cba was detected, amendment of lower bases of Bza and 5,6-DiBrBza resulted in the shift from native type of DMB-Cba production to production of corrinoids of Bza-Cba and 5,6-DiBrBza-Cba, respectively in PCE-noB_{12} cultures (Figure 3C, D). These results indicated that corrinoid producers in the cultures either used the available lower bases first, instead of producing DMB lower bases, which could be an energy conservation strategy, or lower base modification (i.e., remodeling the lower base with the available lower bases) was very common in mixed microbial communities.

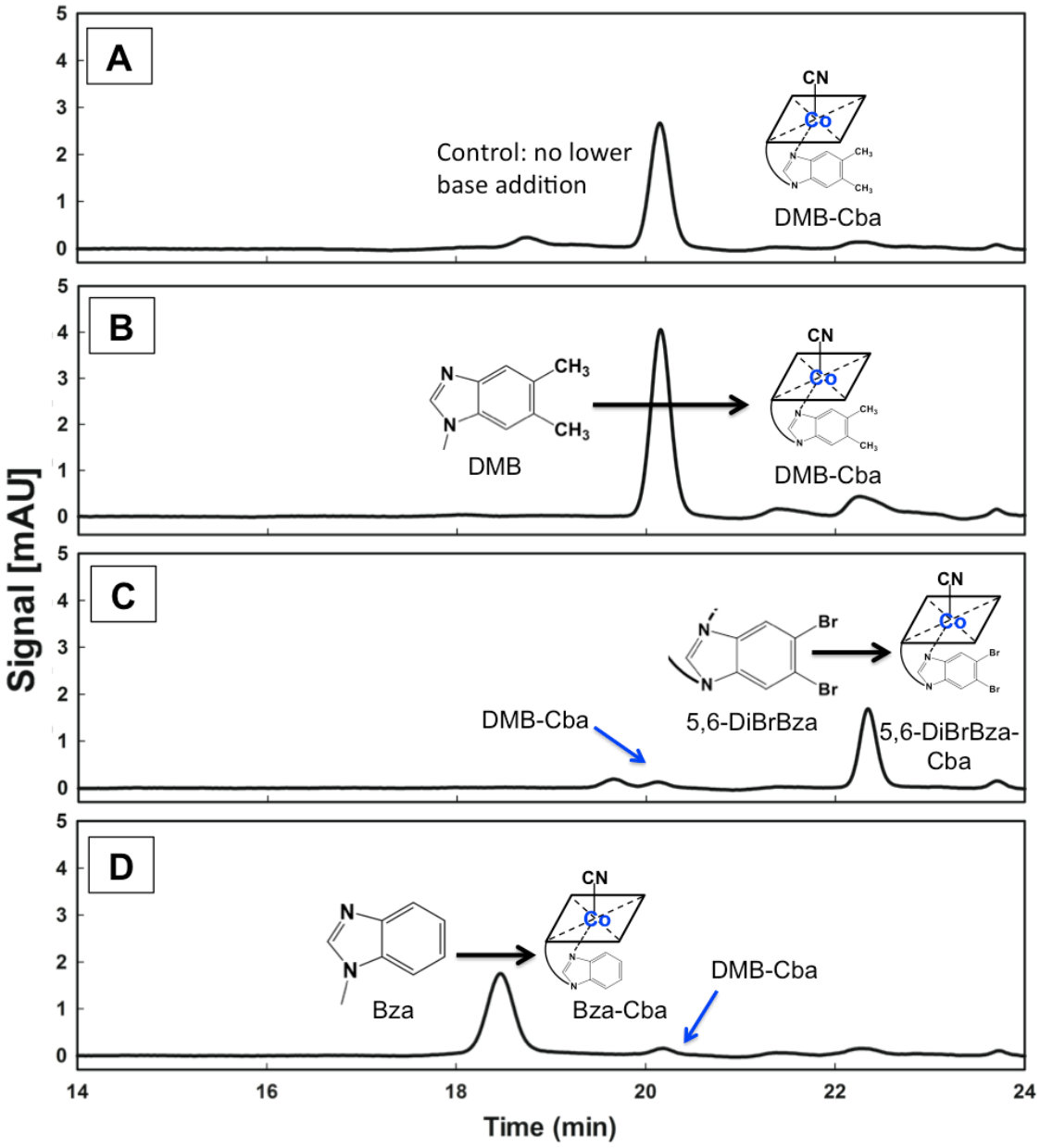

Figure 4.3. HPLC chromatograms of corrinoids produced in PCE-noB$_{12}$ cultures
HPLC chromatograms of corrinoids produced in PCE-noB$_{12}$ cultures, which A) grown without any amendment (control group) or received 50 µM lower bases of B) DMB, C) 5,6-DiBrBza, D) Bza. A) Corrinoid standards retention times: DMB-Cba: 20.18 min; 5,6-DiBrBza-Cba: 22.34 min; Bza-Cba: 18.49 min. Chemical structures on each peak represent the structures of added lower bases and corresponding produced corrinoids. Blue-arrows show the peaks, which were other identified corrinoids detected in cultures.

4.4.4 Impact of different lower bases on dechlorination rates and extents in mixed communities

Native non-dechlorinating microorganisms produced DMB-Cba type of corrinoid abundantly, and sustained *Dhc* reductive dechlorination activity in PCE-noB_{12} cultures. In addition, different lower base-amendment to the cultures made microorganisms to produce corrinoids with amended-lower bases, different from natively produced DMB-Cba. To explore how the dechlorinating communities, especially corrinoid-auxotrophic *Dhc* respond to this shift in available corrinoid, we monitored the reductive dechlorination activity in cultures, which amended with lower bases DMB, Bza and 5,6-DiBrBza.

Figure 4.4 compares the dechlorination profiles of PCE-noB_{12} cultures amended with different lower bases. Consistent with the observation in PCE-noB_{12} cultures grown with vitamin B_{12} or without B_{12} (Figure 4.1), addition of DMB sustained reductive dechlorination activity and initial amount of PCE was completely dechlorinated to ethene in 60 days with a dechlorination rate of 34.3 ± 2.7 μM Cl^- released per day, which is very similar to what we have observed in PCE-noB_{12} and PCE-withB_{12} cultures (Figures 4.1 and 4.5). More than 100 days were required to achieve complete dechlorination of PCE to ethene in PCE-noB_{12} cultures amended with Bza and 5,6-DiBrBza with much lower dechlorination rates of 17.3 ± 2.5 and 20.0 ± 0.5 μM Cl^- released per day, respectively (Figure 4.5). In cultures amended with 5,6-DiBrBza, similar to the observation in DMB-amended cultures, PCE dechlorination product cDCE was completely reduced to daughter products VC and ethene by 40 days. This finding showed that 5,6-DiBrBza-Cba sustained PCE to VC reductive dechlorination as efficient as DMB-Cba does; but the rate-limiting step was actually VC to ethene reductive dechlorination (Figure 4.4). In contrast, in cultures fed with Bza, complete cDCE reductive dechlorination to VC and ethene needed longer time periods (60 days), indicating that Bza-Cba were not effectively functional as 5,6-DiBrBza-Cba and DMB-Bza for the steps of PCE to VC reductive dechlorination.

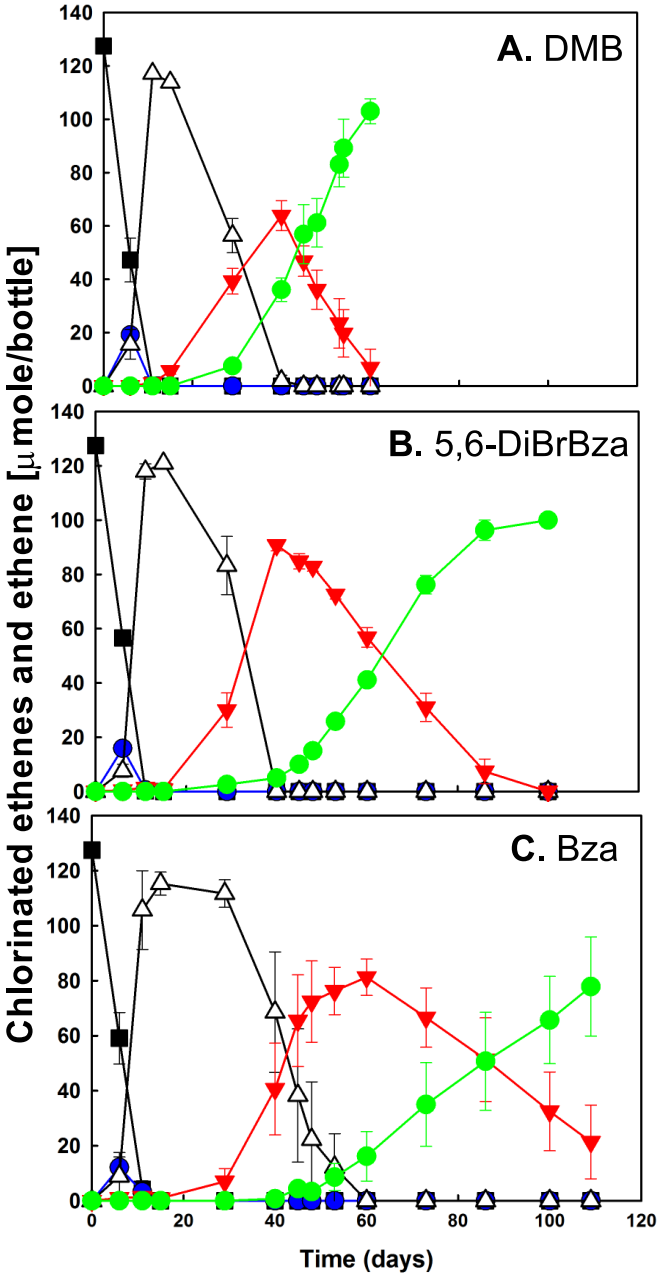

Figure 4.4. Reductive dechlorination of PCE with different lower base amendment Reductive dechlorination of PCE in A) PCE-noB_{12} cultures amended with 50 μM A) DMB, B) 5,6-DiBrBza, C) Bza. Figures present the average data belongs to triplicate cultures, error bars represent the standard error of triplicate cultures. Black squares, PCE; blue circles, TCE; white triangles, cDCE, red inverted triangles, VC; green circles, ethene.

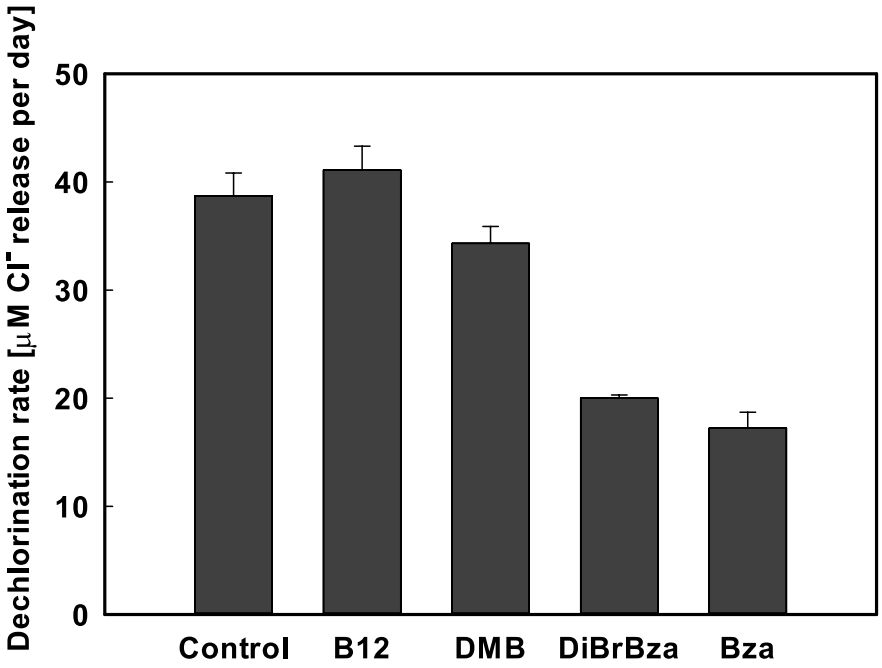

Figure 4.5. Dechlorination rates measured in PCE enrichment cultures
Dechlorination rates measured in triplicate PCE-noB_{12} cultures grown without any vitamin B_{12} or with lower base amendment [Control], with 25 μM vitamin B_{12} [B12], or received 50 μM of DMB [DMB], 5,6-DiBrBza [DiBrBza] or Bza [Bza]. Error bar represent standard error of triplicate cultures.

4.4.5 Addition of DMB to cDCE-noB_{12} cultures did not recover the *Dhc* dechlorination activity

Very low dechlorination rates and incomplete dechlorination activity was observed in cDCE cultures grown without vitamin B_{12}, in contrast to higher dechlorination rates in cultures grown with vitamin B_{12}, showing that corrinoid was limiting factor in no-vitamin B_{12} amended cultures (Figure 4.1) Making the assumption that addition of DMB to cDCE-noB_{12} cultures, will make microbial populations to produce DMB-Cba (favorable corrinoid for reductive dechlorination) or *Dhc* or other populations to remodel unfavorable corrinoid by replacing the lower base with DMB, we added 50 µM DMB to cDCE-noB_{12} cultures. Addition of DMB did not sustain reductive dechlorination activity, and incomplete cDCE dechlorination was monitored with a rate of 2.10 ± 0.7 µM Cl^- released per day (Figure 4. 6). No more than 63 % (46 µmoles) of the total cDCE was further dechlorinated to VC and ethene over a 145-day incubation period. This finding indicated that rather than production of wrong type of corrinoid, cDCE inhibition on the native corrinoid production pathway of corrinoid producers could be a problem in these cultures. This could result in production of non-functional incomplete form of corrinoid (i.e., inhibition on production of cobinamide, which is the a incomplete form of corrinoid without the lower base attachment), which does not function for *Dhc* reductive dechlorination, although it is capable of modifying lower base with DMB.

4.4.1 Corrinoid effect on *Dhc* strain selection

Recently we demonstrated that cDCE dechlorinator *Dhc* strains BAV1 and GT had different preferences for corrinoids with different lower base attachments.[29] In this study, to explore if different *Dhc* strains are selected (become abundant) in PCE-noB_{12} cultures received different lower bases or vitamin B_{12}, we quantified the RDase genes *bvcA*, *vcrA* and *tceA* (representing different strains of *Dhc*) after initial amount of PCE was completely dechlorinated to ethene.

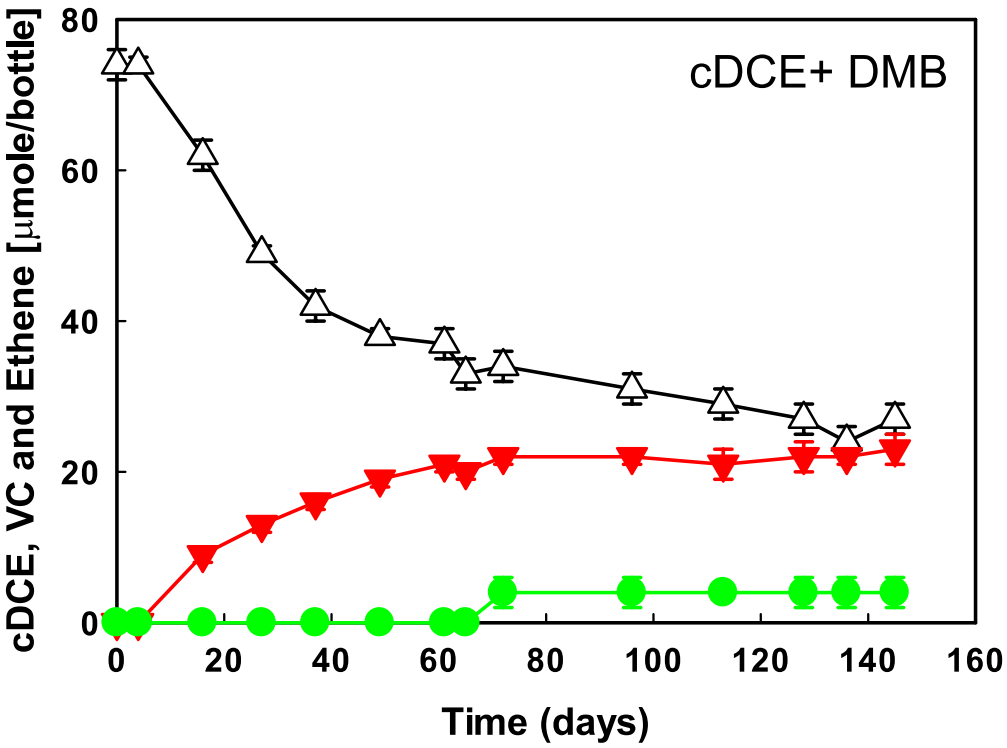

Figure 4.6. Reductive dechlorination of cDCE in cDCE-noB$_{12}$ cultures with DMB amendment

Reductive dechlorination of cDCE in cDCE-noB$_{12}$ cultures amended with 50 μM DMB. Figures present the average data belongs to triplicate cultures, error bars represent the standard deviation of triplicate cultures. White triangles, cDCE, red inverted triangles, VC; green circles, ethene.

Figure 4.7A shows the qPCR enumeration of RDase genes along with *Dhc* 16S rRNA gene. T0 data represented the PCE-noB_{12} *Dhc* population of inoculum cultures (i.e., initial gene copies in each conditions). *Dhc* 16S rRNA genes increased to numbers exceeding 10^8 gene copies/mL in all PCE-noB_{12} cultures amended with/without vitamin B_{12} or one of the lower bases DMB, 5,6-DiBrBza or Bza after complete dechlorination of PCE to ethene. *Dhc* strains carrying *vcrA* gene were the most abundant *Dhc* population in the inoculum (T0), and cultures with different corrinoid/lower base amendments except in cultures amended with Bza. *Dhc* strains with *tceA* gene were the most abundant *Dhc* population in cultures amended with Bza. *bvcA* gene was at much higher numbers exceeding 10^6 gene copies/mL in PCE-noB_{12} cultures amended with or without B_{12} (in which DMB-Cba is natively produced corrinoid); but at lower abundances in other cultures (10^5 gene copies/mL of culture).

Figure 4.7B compares fold increase (i.e., final gene copies/initial gene copies) of each gene under different or lower base or with/without vitamin B_{12} amendments. *Dhc* populations carrying *tceA* gene dramatically increased in 5,6-DiBrBza and Bza amended cultures with fold changes of 194.1 ± 42.8 and 219.0 ± 35.2, respectively, although in the same cultures, total *Dhc* 16S rRNA gene numbers only increased with fold changes of 57.9 ± 3.9 and 61.5 ± 6.6, respectively. *Dhc* populations carrying *bvcA* or *vcrA* genes showed the highest fold increases in cultures amended with vitamin B_{12}, and followed by control cultures, which did not receive vitamin B_{12} or any lower bases.
To further investigate which *Dhc* populations carrying *tceA* gene were in the cultures, we ran qPCR assays targeting *Dhc* strain 195 *pceA* (i.e., detection of *pceA* gene represents *Dhc* 195 *tceA* gene with 1:1 ratio with single gene copies of each gene, and distinguish strain 195 *tceA* from *Dhc* strain FL2, which is another *Dhc* strain carrying *tceA* gene). qPCR failed to detect *Dhc* strain 195 *pceA* gene in PCE enrichment cultures, showing that the populations carrying *tceA* gene in PCE enrichment cultures were either strain FL2 or unidentified *Dhc* strains with *tceA* genes.

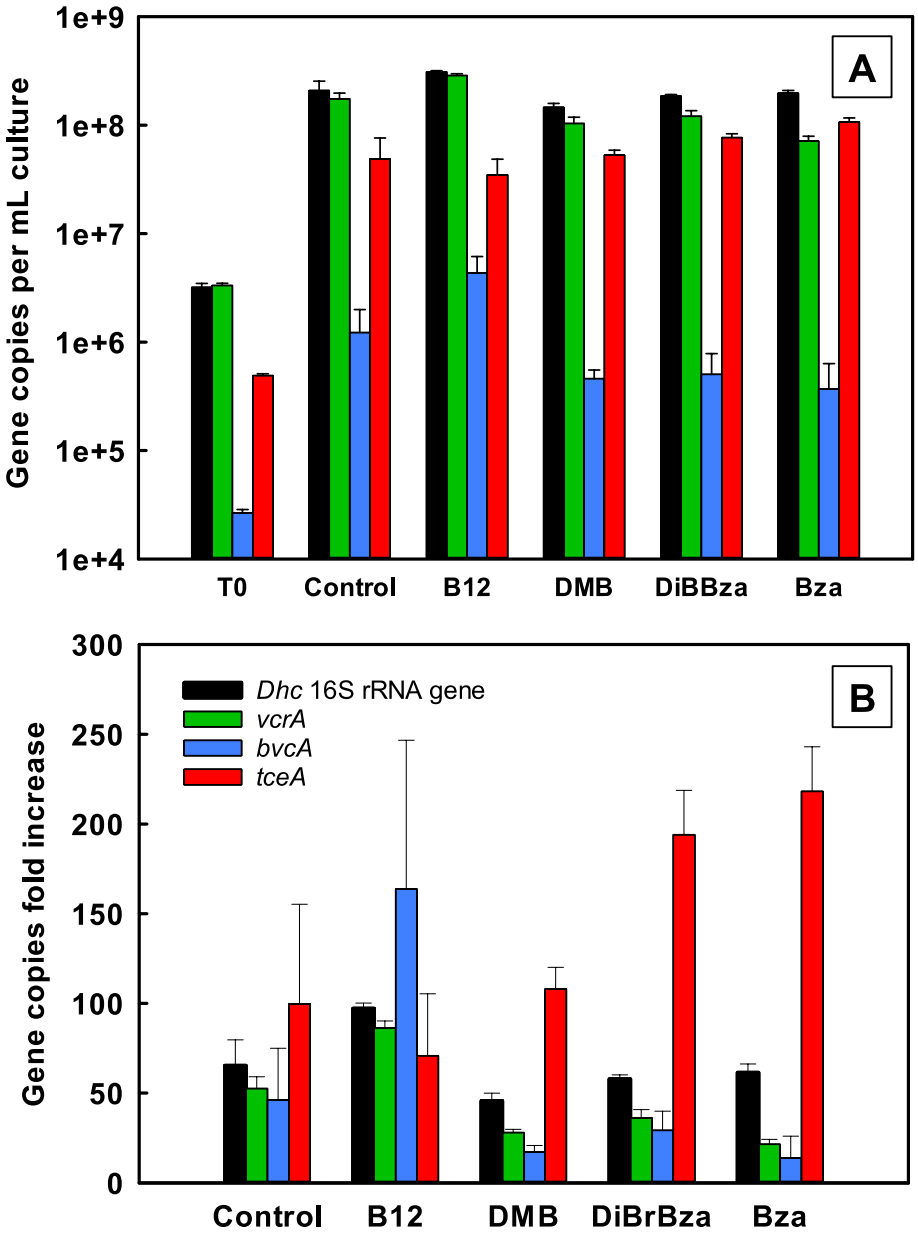

Figure 4.7. Quantification of *Dhc* biomarker genes in PCE-noB_{12} cultures
A) *Dhc* 16S rRNA gene (black bars), *vcrA* (green bars), *bvcA* (blue bars) and *tceA* (red bars) gene copies in samples harvested from PCE-noB_{12} cultures grown without any corrinoid or lower base amendment [Control], or received vitamin B_{12} [B12], DMB [DMB], 5,6-DiBrBza [DiBrBza], or Bza [Bza]. T0 represent the initial gene copies in cultures (i.e., harvested at time 0). Samples were harvested from the cultures after complete PCE-to-ethene cycle or at end of incubation period for the cultures VC was still present. **B)** Fold increase in gene numbers in samples described in A figure.

4.5 Discussion

Corrinoid cofactors play critical roles for specific enzymatic functions, and are required by the majority of organisms in all three domains of life.[45] Although there is a high demand for corrinoid cofactors, only certain subset of prokaryotes are able to *de novo* biosynthesize this crucial molecules in the environments.[46] The exchange of corrinoids among members of microbial communities is therefore crucial in functionally integrated microbial systems. Many *Bacteria* and *Archaea* biosynthesize corrinoids under oxic or anoxic conditions; but only certain anaerobic microorganisms produce corrinoids with different lower bases including DMB derivatives or non-benzimidazole-type.[34, 47] Organohalide-respiring *Dhc* strictly require corrinoids for organohalide-respiration and depend on other microorganisms in the subsurface environments to fulfill its corrinoid-requirement. In subsurface environments, *Dhc* usually co-exist with corrinoid producing microorganisms including methanogens, acetogens, sulfate reducers and fermenters and it is very likely to encounter a variety of different corrinoids in the environment.[12, 31, 48, 49] On the other hand, recent studies revealed that *Dhc* has a very strict corrinoid requirements, and not all naturally occurring corrinoids with different lower base structures support *Dhc* reductive dechlorination activity.[15, 29, 30] For example, *Dhc* co-culture experiments with corrinoid-producer methanogen *Methanosarcina barkeri*, sulfate reducers including *Geobacter sulfurreducens*, or acetogen *Sporomusa* spp. failed to support *Dhc* reductive dechlorination activity, due to production of wrong type of corrinoids (e.g., 5-OHBza-Cba by methanogens), which cannot be utilized by *Dhc*.[13-15, 38] Despite inability to biosynthesize corrinoid, *Dhc* strains possess genes encoding corrinoid salvaging and remodeling. Remodeling of non-functional corrinoids (e.g., 5-OHBza-Cba, phenolic *p*-Cre-Cba) has been observed, and recovery of reductive dechlorination was observed in *Dhc* co-cultures with corrinoid-producers when DMB lower base was provided.[13-15, 30, 38]

Giving the importance of corrinoid for reductive dechlorination, and *Dhc* dependency on other microbial members in subsurface environments, current study explored the *Dhc*

corrinoid-related interactions within the dechlorinating microbial communities and *Dhc* growth responses under different corrinoid or/lower base availability. In PCE, cDCE and VC- noB_{12} dechlorinating cultures, derived from chlorinated-solvent contaminated Third Creek sediment, *Dhc* were able to completely dechlorinated corresponding chlorinated solvents without exogenous vitamin B_{12} amendment, indicating that other microorganisms in the cultures produced corrinoids that *Dhc* could utilize or remodel with available "right" type lower bases (e.g., DMB). VC- and PCE- noB_{12} cultures showed very comparable dechlorination activity with the VC and PCE-dechlorinating cultures amended with exogenous vitamin B_{12}, and corrinoid analyses confirmed that DMB-Cba was produced in cultures grown without B_{12} amendment. In contrast, although DMB was detected in cDCE-noB_{12} cultures (Figure S4.2), incomplete cDCE dechlorination at much lower dechlorination rates were monitored during the 145-day incubation period (Figure 4.1). The cDCE dechlorination occurred when cultures were grown with exogenous vitamin B_{12}, showed that the corrinoid was limiting for *Dhc* growth (i.e., microorganism in the community was producing wrong type of corrinoid, which does not efficiently support *Dhc* growth). Then, we further explored the potential of *Dhc* or other community members to remodel unfavorable corrinoids in cDCE-noB_{12} cultures by modifying the lower bases with exogenously amended DMB. Interestingly, addition of DMB to cDCE-noB_{12} cultures did not sustain *Dhc* reductive dechlorination in cDCE-noB_{12} cultures (Figure 4.6). This unexpected finding indicated that either *Dhc* or other organisms could not remodel the available corrinoids by replacing the lower base with DMB, or the cobinamides (that is upper part of complete corrinoid without lower base attachment) produced by the community members were not in functional form. The first explanation is unlikely, because 1) corrinoid modification is very common in microbial communities and remodeling of corrinoids by *Dhc* and other microorganism in the dechlorinating co-cultures or mixed community by replacing the unfavorable lower base with DMB was demonstrated before.[13, 14, 38] 2) non-dechlorinating microbial community structures of PCE, VC-noB_{12} cultures were very similar to the structure of cDCE-noB_{12} cultures. The same non-dechlorinating community members or *Dhc* strains could remodel the native corrinoids in PCE-noB_{12} cultures with different lower base

amendments, showing that remodeling was not the problem. The alternative explanation seems more possible, since cDCE could be a potent inhibitor of corrinoid producers like acetogens or fermenters in the cultures. Although further experiments are necessary to explore what extent cDCE inhibition on cobinamide production occurs; this observation still has an important value for bioremediation. In practical applications, cDCE inhibition on corrinoid producers may lead to corrinoid shortage for *Dhc* reductive dechlorination, resulting in accumulation of toxic dechlorination intermediates cDCE and VC, which pose a high health risk at contaminated locations. To date, it has not been demonstrated that a lower bioremediation success rate at predominantly cDCE-contaminated sites is due to corrinoid cofactor limitations of *Dhc* populations; but our findings supported this idea. Bioaugmentation with robust cDCE dechlorinating cultures with invulnerable corrinoid-producers could be a solution for such problematic scenarios or cobinamide biostimulation could be an alternative strategy.

Microbial community analysis based on 16S rRNA gene amplicon sequencing revealed co-occurrence of some well-known corrinoid producers in Third Creek enrichment cultures. Fermentation of lactate to acetate and propionate stimulated growth of some known corrinoid producing fermenters and acetogens including *Clostridium*, *Sedimentibacter* and *Acetobacterium.*[34, 45] Among them, *Acetobacterium* possessed the complete DMB-Cba *de novo* biosynthesis pathway and experimentally was shown to produce DMB-Cba,[34, 50], which could explain the DMB-Cba production in cultures enriched without vitamin B_{12} amendment. DMB-Cba production was previously observed in a *Dhc*-containing TCE-dechlorinating ANAS cultures grown without exogenous vitamin B_{12}-amendment, where *Acetobacterium woodii* was one of the community members.[51]

Detection of high abundances of *Clostridium* in Third Creek cultures (Figure4.2), also exhibited a potential role of this group for contribution into corrinoid pool. Some species of genus *Clostridium* were reported to produce 5-methoxy-6-methylbenzimidazolylcobamide (5-OMe-6-MeBza-Cba) or 5-methoxy-

benimindazolycobamide (5-MeOBza-Cba). [34, 48] In addition, PCE to cDCE dechlorinator *Geobacter lovleyi* was highly enriched in PCE-dechlorinating enrichment cultures. A recent study demonstrated that in *Dhc* co-culture experiments, the PCE-to-cDCE dechlorinator *Geobacter lovleyi* strain SZ produced a corrinoid that supported *Dhc* activity,[15] and a later study predicted that *Geobacter lovleyi* produce 5-MeOBza-Cba type of corrinoid based on gene annotation.[50] Although, *Dhc* have less preference for the type of corrinoid that *Clostridium* or *Geobacter lovleyi* produce (i.e., 5-MeOBza-Cba, 5-MeOBza-Cba) over DMB-Cba,[29] recovery of high concentration of DMB-Cba in 1.6-L PCE-noB_{12} cultures (Figure 4.3) indicated likely occurrence of corrinoid-remodeling. Corrinoids and free-DMB, which is natively produced in the culture, are likely acquired and remodeled into DMB-Cba by *Dhc* and/or other members. In another dechlorinating cultures NJ, remodeling of abundantly produced phenolic type corrinoid *p*-Cre-Cba into DMB-Cba in dechlorinating cultures was also observed, and *Dhc* reductive dechlorination was sustained.[51]

In this study, we also explored if available lower bases and corresponding corrinoids affect *Dhc* reductive dechlorination. Interestingly, although Third Creek noB_{12} cultures have high potential to produce DMB-Cba and microorganisms have high preference for DMB-Cba for their enzymatic reactions, addition of Bza and 5,6-DiBrBza shifted native DMB-Cba production by the community to production of corrinoids with lower bases of Bza and 5,6-DiBrBza (Figure 4.3). These results demonstrated that available lower bases determined the corrinoids produced by community members, and corresponding corrinoids significantly affected *Dhc* reductive dechlorination and leaded much lower dechlorination rates (Figure 4.4). This observation has an implication for bioremediation practices. In contaminated sites, where microorganisms producing "wrong" type of lower bases and/or corrinoids are abundant, *Dhc* activity will dramatically be affected with slower rates of cDCE to ethene dechlorination, which may cause a long term problem of cDCE and VC stalls. Therefore, understanding whole community responses to availability of different lower bases, rather than only *Dhc*'s, is necessary to better understand the conditions at contaminated sites and improve bioremediation applications.

Another interesting observation was that *Dhc* and other community members could acquire an artificial lower base (not naturally occurring) 5,6-DiBrBza, and use it to form a functional corrinoid for enzymatic reactions, which has never been demonstrated before. This finding demonstrates that *Dhc* may use more broad lower bases including artificial lower bases for reductive dechlorination activity, which yet to be explored.
In the current understanding of *Dhc* strain diversity, there is still not a very clear answer for what determines *Dhc* strain diversity in subsurface environments. *Dhc* strains have streamlined, small genomes that reflect their highly specialized metabolic capabilities.[52] Each *Dhc* strain contains a unique complement of up to 36 RDase genes, whereas their 16S rRNA gene sequences show 98-100% sequence identity.[40, 52] The presence of such a large variety (up to 36) of RDase genes in *Dhc* genomes indicates that each *Dhc* strain may derive energy from wider yet undiscovered-variety of chlorinated compounds in the environment; however, the relationship between the complement of RDase genes (i.e., different strain) and *Dhc* substrate specificity is still unclear. In PCE-noB_{12} cultures amended with different lower bases or with/without vitamin B_{12}, we observed variation in *Dhc* populations carrying different RDase genes. This observation strongly demonstrates that available corrinoid has impacts on *Dhc* strain diversity and selection. *Dhc* populations carrying *tceA* gene surprisingly increased in cultures amended with 5,6-DiBrBza and Bza (Figure 4.7A, B), indicating *Dhc* populations with *tceA* gene could utilize 5,6-DiBrBza-Cba and Bza-Cba for reductive dechlorination. In contrast *Dhc* cells with *bvcA* or *vcrA* only slightly increased with much lower fold changes under these conditions. Strains with *bvcA* or *vcrA* genes had highest growth fold increase in B_{12}-amended cultures. These observations are also supported by the findings of our recent study with *Dhc* strains BAV1 (*bvcA*) and GT (*vcrA*) such that *Dhc* strains BAV1 and GT showed least preferences for Bza-Cba with lowest dechlorination rates, and highest preference for DMB-Cba with highest dechlorination rates.[29] Our results emphasizes the effect of corrinoid on *Dhc* strain selection; however since these findings based on *Dhc* growth of only one PCE-to-ethene reductive dechlorination cycle, transferring cultures under same lower base amendment needs to be done to further investigate strain selection. Answering this research question promises to advance our understanding of the

evolution of organohalide-respiring *Dhc* and developing better bioremediation strategies based on site specific-conditions (i.e., existing microbial populations, corrinoid type, *Dhc* strains etc.)

To conclude, this study demonstrated the important roles of corrinoid producers in dechlorinating enrichment cultures. Non-*Dhc* microorganisms in Third Creek cultures produce corrinoids and sustain *Dhc* reductive dechlorination without exogenous vitamin-B_{12} amendment. Lower dechlorination rates observed in cDCE-noB_{12} cultures indicated possible cDCE inhibition on corinoid producers, which should be carefully considered while developing bioremediation strategies for contaminated sites. In addition, current research also advanced our understanding about effect of corrinoid on *Dhc* strains selection. Addition of mixture of biostimulants with vitamin B_{12} into chlorinated solvent-contaminated aquifers is a common bioremediation practice; but it should be noticed that all other fast-growing microorganisms (e.g., fermenters, acetogens) need corrinoids as well, and added vitamin B_{12} is very likely consumed very quickly before slow-growing *Dhc* dechlorinates the contaminants. Therefore, corrinoid-flux from other microbial populations is one major control over reductive dechlorination activity and *Dhc* strain selection at contaminated sites, and this research advanced understanding about how *Dhc* behaved as a part of community to different corrinoid/lower bases conditions.

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4.7 Appendix 4

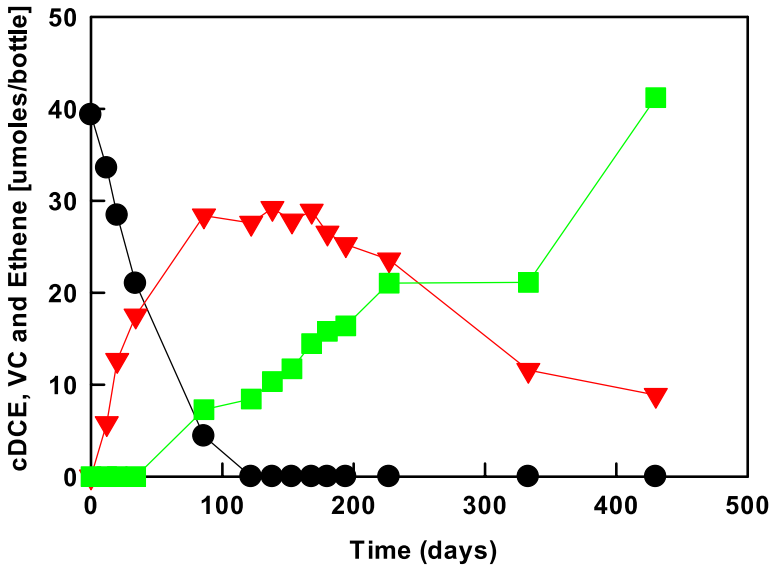

Figure S4.1. Reductive dechlorination of cDCE without B_{12} amendment
Reductive dehclorination of cDCE in early transfer cDCE-noB_{12} cultures grown without any corrinoid or lower base amendment. Figures present the average data belongs to duplicate cultures. Black circles, cDCE, red inverted triangles, VC; green squares, ethene.

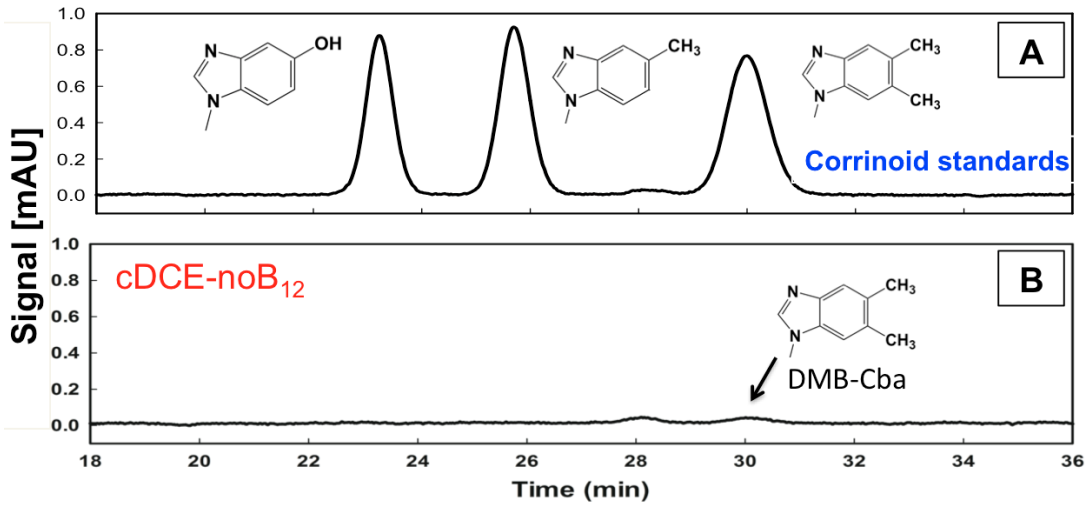

Figure S4.2. HPLC chromatograms of corrinoids in cDCE-noB$_{12}$ cultures
Corrinoids were measured after a complete cDCE-to-ethene dehclorination in cDCE-noB$_{12}$ cultures. **A)** Corrinoid standards [5-OHBza-Cba: 23.21min; 5-MeBza-Cba: 25.69min; DMB-Cba: 29.99min]. **B)** cDCE-noB$_{12}$ cultures, which did not receive any corrinoid or lower base amendment. Chemical structures on each peak represent the lower base structure of corresponding corrinoid.

Table S4.1. Summary of 16S rRNA gene sequencing results

Cultures	Total number of high-quality sequences	Total OTUs/sample
PCE-withB$_{12}$	76,481	90
PCE-noB$_{12}$	54,599	102
cDCE-withB$_{12}$	109,679	102
cDCE-noB$_{12}$	24,495	70
VC-withB$_{12}$	137,362	120
VC-noB$_{12}$	38,227	71

5 Geochemical Conditions Affect Corrinoid Pools That Control *Dehalococcoides mccartyi* Reductive Dechlorination Activity

5.1 Abstract

Dehalococcoides mccartyi (*Dhc*) are the only bacteria that can detoxify toxic and carcinogenic chlorinated ethenes to benign ethene. The key catalysts for breaking the carbon-chlorine bonds are reductive dehalogenases (RDases), which require a corrinoid cofactor. Remarkably, *Dhc* strains lack ability for *de novo* corrinoid biosynthesis, and *Dhc* strains have specific requirements and not all naturally occurring corrinoids support *Dhc* activity. Our work explored if different biogeochemical conditions in the environment and associated microbiology determined the predominant type of corrinoid produced, and hence control over *Dhc* reductive dechlorination activity. Microcosms were established using sediment samples collected from a chlorinated solvent-contaminated site in Knoxville, TN. Sulfate-reducing, iron-reducing, nitrate-reducing, methanogenic/fermentative, acetogenic/fermentative conditions were established by amending the microcosms with sulfate, amorphous FeOOH, nitrate, glucose, lactate + 2-bromoethane sulfonate, respectively. Sediment-free enrichment cultures were obtained after repeated transfers while maintaining the same redox conditions. 16S rRNA gene amplicons sequencing revealed enrichment of different microbial communities under different redox conditions. Corrinoids produced under each condition were characterized and quantified using HPLC system with a UV-diode array detector. Corrinoids with lower bases of benzimidazole type and phenolic type were detected in cultures. Corrinoids with phenolyl lower bases, which does not support *Dhc* reductive dechlorination activity, were abundant in lactate- and glucose-amended cultures. Corrinoids produced by methanogens: 5-hydroxybenzimidazole (5-OHBza), which does not sustain *Dhc* growth was also abundant in glucose-amended cultures. Favorable corrinoid for *Dhc* growth, 5,6-dimethylbenzimidazolyl-comabimde were detected in nitrate-, iron-, and –sulfate-reducing cultures at various concentrations. Our results

revealed differential production of corrinoids with a variety of lower bases under different redox conditions, demonstrating that composition and concentration of bioavailable corrinoid pools are determined by different geochemical conditions. Improved understanding on how geochemical conditions and associated microbiology affect the corrinoid pool, and consequently *Dhc* reductive dechlorination activity will provide valuable information for refined decision-making and the implementation of efficient bioremediation treatment for achieving cleanup goals and protecting humans from exposure to harmful groundwater contaminants.

5.2 Introduction

Chlorinated solvents such as tetrachloroethene (PCE) and trichloroethene (TCE), and their natural degradation products vinyl chloride (VC) and *cis*-dichloroethene (cDCE) are among the most prevalent toxic organic groundwater contaminants found in the shallow subsurface systems.[1-3] PCE and cDCE are probable human carcinogens and VC and TCE are known human carcinogen.[4, 5] Human exposure of chlorinated ethenes through contaminated water and vapor intrusion into dwellings could lead to various health problems, including cancer, neuropathy, cardiovascular defects, respiratory and immune diseases.[6, 7]

Bacterial organohalide-respiration in which chlorinated solvents serve as electron acceptors plays a major role in the detoxification of chlorinated ethenes. Although diverse bacterial strains belonging to different genera have been identified to couple PCE/TCE reductive dechlorination to *cis*-1,2-dichloroethene (cDCE) for energy conservation, *Dehalococcoides mccartyi* (*Dhc*) is the only bacteria capable of complete reductive dechlorination of chlorinated ethenes to benign ethene.[8, 9 10] The key catalysts for organohalide-respiration are reductive dehalogenase enzyme systems (RDases), which require a corrinoid cofactor for the activity.[11-14] Despite their dependence on corrinoid cofactor, *Dhc* strains lack the ability for *de novo* corrinoid biosynthesis[15-19] and the growth of *Dhc* pure cultures strictly depends on exogenous addition of vitamin B_{12}

(cyanocobalamin).[8, 9, 14, 20, 21] All sequenced *Dhc* strains possess the genes involved in corrinoid scavenging pathways, including corrinoid transport, cobinamide salvage/activation, and lower base activation.[22-24].

In various environments including groundwater, subsurface soil, river and marine sediments, *Dhc* commonly co-exist with corrinoid-producing microorganisms such methanogens, acetogens and other bacteria[25-27], which are presumed to provide corrionoid cofactor to corrinoid auxotrophic *Dhc*[28-31]. Naturally occurring corrinoids produced by these microorganisms have been found to be various types with different lower base structures, including benzimidazole, phenolic, and purine types[29, 31, 32]. Recent studies demonstrated that *Dhc* had highly specific corrinoid requirements that some of the naturally occurring corrinoids produced by methanogens, acetogens and iron-reducer *Geobacter sulfurreducens* could not fulfill *Dhc* reductive dechlorination activity in co-cultures [22, 23, 33, 34]. *Dhc* reductive dechlorination and growth only occurred in co-cultures with methanogens, acetogens after addition of 5',6'-dimethylbenzimidazole (DMB), the lower ligand of cobalamin (i.e., vitamin B_{12}) [23, 33, 34]. Studies revealed that different *Dhc* strains (different RDase genes) could have different preferences for different type of corrinoids. Growth of *Dhc* strain 195 harboring *tceA* RDase was only sustained by corrinoids with DMB, 5-methylbenzimidazole (5-MeBza) and 5-methoxybenzimidazole (5-OMeBza) lower bases, but not with benzimidazole (Bza), 5-hydroxybenzimidazole (5-OHBza) or phenolyl lower bases[23, 24]. In another recent study, *Dhc* strain BAV1 harboring *bvcA* RDase dechlorinated VC to ethene by corrinoids with 5-OMeBza, Bza lower bases; in contrast *Dhc* strain GT failed to dechlorinate VC to ethene by these corrinoids[22]. In addition, availability of sufficient amount of corrinoids is also critical for reductive dechlorination activity. Growth of *D. mccartyi* strains BAV1, GT and FL2 with limiting amounts of 1 µg/L exogenous vitamin B_{12} resulted in incomplete reductive dechlorination of polychlorinated ethenes leading to VC accumulation. In contrast, the same cultures amended with 25 µg/L vitamin B_{12} completely degraded chlorinated ethenes with much higher dechlorination rates.[34] These distinct responses of *Dhc* strains to different quantities and type of corrinoids with different lower bases indicate that

availability of right type of the corrinoid and/or lower bases at enough quantities control *Dhc* activity and dechlorination extents in contaminated environments.

A common contaminated site management practice is to continuously supply additional electron donor (e.g., biostimulation) when dechlorination rates and extent following initial bioremediation treatment (i.e., biostimulation alone or combined with bioaugmentation) decrease [35, 36]. In contaminated aquifers, organohalide-respiring *Dhc* depend on corrinoid salvage to acquire growth-essential corrinoid cofactor from the environments. Contaminated sites differ in terms of hydrogeological and biogeochemical conditions[1]. Depending on the site biogeochemical conditions and the type of substrate(s) used for biostimulation (i.e., electron donors), different biogeochemical structures may be formed, which could lead to microorganisms, producing "wrong" lower bases become dominant. In such conditions, continued biostimulation and bioaugmentation will not lead to the most efficient contaminant detoxification. To date, various studies have revealed the crucial role of corrinoid cofactor for *Dhc* reductive dechlorination; but knowledge on availability of right type of corrinoid in different biogeochemical systems, and how biostimulated biogeochemical conditions affects *Dhc* activity are still lacking. To better understand the impacts of redox conditions and electron donor types on the corrinoid pool, we explored the type of cobamides and/or lower bases produced under different geochemical conditions, and their effects on *Dhc* reductive dechlorination activity.

5.3 Material and Methods

Chemicals. Lower bases, DMB (≥ 99%), 5-MeBza (≥ 98%), 5-OMeBza (≥ 97%), Bza (≥ 98%), betaine (≥ 99%) were purchased from Sigma-Aldrich (St Louis, MO, USA). Yeast extract and casitone were purchased from Becton, Dickson and Company (Franklin Lakes, NJ, USA).

5.3.1 Environmental samples

Sediment samples were collected from the chlorinated solvent-contaminated Third Creek sediment in Knoxville. Sediment samples were obtained using direct push tools (AMS, Inc., American Falls, ID). The plastic liners and caps were wiped with 70% ethanol before use. All other materials (spatulas, containers, etc.) were autoclaved and aseptic techniques were applied to the extent feasible. All core samples were immediately transferred to Mason jars, filled completely with creek water to exclude air, capped and placed in a cooler with ice packs. Sediment samples from the same locations were homogenized by mixing inside an anoxic glove box filled with H_2/N_2 (3/97%, vol/vol), and were kept at 4°C till microcosms were established (within 1 week of sample collection).

5.3.2 Microcosms and enrichment cultures under different redox conditions

To explore the impacts of redox conditions and electron donor types on the corrinoid pool, duplicate microcosms under different geochemical conditions, including nitrate-reducing, iron-reducing, sulfate-reducing, fermentative/acetogenic, and fermentative/methanogenic conditions were established. Briefly, inside the anoxic glove box duplicate microcosms were established using sediment samples (6 g, wet weight) in 160-mL glass serum bottles containing 100 mL of vitamin B_{12} (cobalamin)-free, defined, bicarbonate-buffered mineral salt medium with a N_2/CO_2 (80/20 [vol/vol]) headspace as described [37, 38]. The purpose of excluding vitamin B_{12} from the medium was to identify naturally produced corrinoid under different conditions. Microcosms and enrichment cultures set-up conditions are summarized in Table 5.1.Various redox conditions were established by amending the microcosms with amorphous FeOOH (10 mM nominal concentration),[39] 5 mM nitrate (as $NaNO_3$), or 5 mM sulfate (as Na_2SO_4) as electron acceptors and 5 mM lactate as electron donor. To represent commonly used electron donor formulations, additional microcosms were supplemented with lactate (5 mM) and glucose (10mM). BES (2-bromoethane sulfonate)[40] with a concentration of 2 mM was

used to inhibit methanogenesis in all microcosms, except glucose amended condition. Additional microcosms with same amendments were established for each condition, and autoclaved to serve as abiotic controls. Sediment-free enrichment cultures were derived by transferring of 3% (v/v) cultures into fresh 100-mL medium with the same amendments.

Table 5.1. Microcosm and enrichment culture conditions

Condition	Electron Acceptor	Microcosm Electron Donor	Enrichments Electron Donor	BES
Iron-reducing	FeOOH (5 or10 mM)	Lactate (5 mM)	Acetate (5 mM) + H_2 (10 or 160 mL)	+
Sulfate-reducing	Na_2SO_4 (5mM)	Lactate (5 mM)	Acetate (5 mM) + H_2 (10 or 160 mL)	+
Nitrate-reducing	$NO3^-$ (5 mM)	Lactate (5 mM)	Acetate (5 mM) + H_2 (10 or 160 mL)	+
		Substrate		
Fermenting/acetogenic		Lactate (5 mM)		+
Fermenting/ methanogenic		Glucose (10 mM)		-

Lactate as electron donor would have result in lactate fermenting populations become abundant under all conditions. This could have result in underestimation of corrinoid types produced under each redox condition by other microorganisms. To prevent this, after second transfer cultures, iron-, sulfate-and nitrate reducing-enrichment cultures were amended with 5 mM acetate and 10 mL H_2 instead of lactate. All microcosms and enrichment cultures were incubated statically in dark at 30°C. To produce sufficient biomass for corrinoid identification, fifth generation of enrichment cultures growing under different redox conditions were grown in 2-L vessels with 1.6 liter of anoxic, defined barcorbanated medium (B_{12}-excluded). To obtain high biomass yield for corrinoid analysis, 1.6-L cultures were amended with respective electron acceptors and donors second time when complete reduction of electron acceptors and consumptions of substrates were monitored (due to slow growth rates only one feeding cycle was applied to Na_2SO_4- and FeOOH-amended cultures, 5mM each). To sustain defined redox

conditions (i.e., iron-, sulfate-, nitrate-reducing conditions) in enrichment cultures and prevent other conditions (e.g., acetogenic) become abundant due to electron acceptor limitation, corrinoid extraction was proceeded before second time amended-electron acceptors were depleted.

5.3.3 Intracellular corrinoid extraction and purification from enrichment cultures

After sampling for VSS measurements and 16S rRNA amplicon gene sequencing, cells were harvested from the rest of the 1.6-L cultures by centrifugation in 500-mL plastic bottles at 15 000 × g for 20 min at room temperature. Intercellular total corrinoids were extracted in the cyano form and purified following the potassium cyanide (KCN) extraction protocol with following changes.[34] Briefly, following removal of supernatants, cell pellets were suspended in 5 mL of deionized water in sterile 50-mL plastic tubes, the pH was adjusted to 5−6 with 3 % (v/v) glacial acid, and KCN was added at 10 mM final concentration. The suspensions were vortexed, each transferred into sterile 50 mL-plastic tubes and incubated for 20 min in a boiling water bath. Following centrifugation 15 000 × g for 15 min, the supernatant collected, and to increase corrinoid recovery yield, corrinoids were extracted from the cell pellets second time by repeating the previous steps. The combined supernatants were loaded onto Sep-Pak C18 cartridge (Waters Corp, Milford, MA, USA), which was previously equilibrated with 2 mL of 100 % methanol and 40 mL of deionized water. Following sample loading, each cartridge was washed with 10 mL of deionized water and 7.5 mL 10 % methanol. Lastly, corrinoid on the cartridges was eluted with 4 mL of 100 % methanol. The final slightly pink-colored solutions were vacuum dried and residues were suspended in sterile tubes with 0.5 mL deionized water.

5.3.4 Identification and quantification of corrinoids produced under different redox conditions

To use as standards for HPLC measurements, DMB-, 5-MeBza-, 5-OMeBza-, Bza-, 5-OHBza-Cba and phenolic type (Phe-Cba and *p*-Cre-Cba) cobamides were biosynthesized, extracted and purified following the published protocol.[22] Briefly, *Sporomusa* sp. strain KB-1 cultures growing in 1.2 L glass bottles using a defined medium (B12-free) were amended yeast extract and casitone (2 g/L of each) and betaine (50 mM) and supplied with single lower base compounds. After static incubation at 30°, cobamides biosynthesized by *Sporomusa* sp. strain KB-1 were extracted and purified following KCN extraction protocol.[34] Absorbance of purified cobamides were measured with Lambda 35 UV/Vis spectrometer (PerkinElmer, Waltham, MA, USA) at 361 nm, and cobamide concentrations were estimated using a molar extinction coefficient of 28 060 mol/cm.[41] 20 μL of corrinoid samples extracted from 1.6-L redox-enrichment cultures were analyzed with an Agilent (Santa Clara, CA, USA) 1200 high-performance liquid chromatography (HPLC) system with Eclipse XDB-C18 column (5 μm, 4.6 × 150 mm). Following injection onto column, samples were separated with a flow rate of 1mL/min at 30°C using 0.1 % (v/v) formic acid (≥ 88%, w/v) in water (eluent A) and 0.1% formic acid in methanol (eluent B), and a linear change to 25% A/ 75% over 3 min followed by a 5 min hold before the column was equilibrated to initial conditions. Cobamides were detected at 361 nm with an Agilent 1260 Infinity diode array detector and concentrations were quantified by comparing integrated peak areas with 4-point calibration curves, which were generated with purified cobamides.

5.3.5 16S rRNA gene amplicon sequencing and sequence analysis

To perform 16S rRNA gene sequencing, DNA was extracted in triplicates from 5 mL of 1.6 L- enrichment cultures using MO BIO Soil Kit following the manufacturer's protocols. Following extraction, DNA samples were purified using the Genomic DNA Clean and Concentrator Kit (Zymo Research, Irvine, CA). Purified DNA samples were pooled and then samples were PCR amplified by targeting the V4 region of the bacterial

16S rRNA gene using primers F515/R806. [42, 43] Amplification was performed in 50 µL assays consisting of 10 uL of sample DNA, 1 µL of barcoded-primer (10 µM) and 39 µL of a mixture of 22 µL de-ionized water (5 PRIME, Gaithersburg, MD), 5 µL Invitrogen *Pfx50*™ buffer (Invitrogen, Carlsbad, CA), 1 µL CAP 515F primer (10 µM), 5 µL dNTP (Invitrogen, Carlsbad, CA), 1 µL Invitrogen *Pfx50*™ Polymerase (Invitrogen, Carlsbad, CA) and 5 µL of $MgCl_2$ (25mM). Thermo cycling program included denaturation at 94°C for 3 min followed by 35 cycles at 94°C for 45 sec, annealing at 55°C for 60 sec, and extension at 72°C for 90 sec, and final extension at 72°C for 10 min. Quality (size) of produced amplicons was checked using High Sensitive DNA Kit on a model 2100 Bioanalyzer (Agilent, Santa Clara, CA). Relative concentrations of the individual samples were estimated based on the peak height at the appropriate size, and pooled to equal amounts. Pooled samples were purified with SPRI magnetic beads (Beckman Coulter, Inc., Indianapolis, IN). The products from purification step were analyzed again with High Sensitive DNA kit for quality assurance and verification of the removal of primer dimers. Before sequencing, concentrations of pooled amplicons were determined using Illumina Library Quantification kit (KAPA Biosystems, Boston. MA) following the manufacturer's protocol. Quantification of each sample was determined based on amplicon adaptors. The amplicon library was diluted to a starting concentration of 10 nM, followed by paired end sequencing on the Illumina MiSeq sequencer (Illumina, Inc., San Diego, CA).

After sequencing was performed, base calling, demultiplexing and adopter trimming were performed using MiSeq reporter using the default parameters. Raw 16S rRNA gene amplicon sequences were then analyzed using CLC Genomic workbench (v9.0) with CLC Microbial Genomics Module (v1.3.1) from Qiagen. Briefly, all reads were initially subjected to quality control: reads were paired, merged, quality trimmed and filtered based on the presence of the ambiguous nucleotides using the CLC Microbial Genomics default parameters. Following the quality control, sequences were clustered into operational taxonomic units (OTUs) at a threshold of 97% sequence similarity, checked for chimeras with CLC Microbial genomics default parameters and aligned with the

MUSCLE algorithm[44]. The Greengenes database (http://Greengenes.secondgenome.com) was used for taxonomic assignments of OTUs.

5.3.6 Analytical methods

Sulfate and nitrate were quantified with a reagent-free ion chromatograph with eluent generation (RFIC-EG) (Dionex ICS-2100) and a Dionex IonPac® AS18 4x250 mm Analytical column. The eluent was 10 mM potassium hydroxide. Sulfate reduction product sulfide (H_2S) was monitored following the established protocol with following modifications.[45] Twenty µL of aliquots of sulfate-reducing cultures were mixed with 1 mL of 5 mM $CuSO_4$ + 50 mM HCl solution, and the absorbance of the resulting brownish precipitate of CuS was measured on the Thermo Scientific spectronic 20D+ spectrophotometer at a wavelength of 480 nm. Nitrate reduction was confirmed with production of N_2O in nitrate-fed cultures. N_2O production was monitored by analyzing culture headspace samples using on an Agilent (HP) 7890A gas chromatograph equipped with an HP-Plot Q column (30 m by 0.320 mm in diameter; film thickness, 20 µm) and a micro-electron capture detector (µECD) following the establish method.[46] HCl-extractable ferric and ferrous iron were measured using an alteration of the ferrozine assay.[47] Ferrozine buffer was made with 1g/L iron reagent (3-[2- pyridyl]-5,6-bis[4-phenylsulfonic acid]-1,2,4-triazine) hydrate (Acros Organics FerroZine™) in 50 mM HEPES buffer. One hundred µL of culture was incubated in 5 mL of 0.5 M HCl. Twenty µL was then mixed with 1 mL of ferrozine buffer. The absorbance of the resulting purple color was measured on the Thermo Scientific spectronic 20D+ spectrophotometer at a wavelength of 562 nm. Samples were measured quickly after mixing with the ferrozine reagent to avoid possible interference of ferric iron.[48] For total iron measurements, 200 µL of 10% (w/v) hydroxylamine-HCl was added to the 5 mL acid extractions and incubated at room temperature overnight before mixing 20 uL with 1 mL ferrozine buffer. Standard Methods, method 2540[49] were used to determine the solids (TS, TSS, VS, VSS) content of cultures as a measure of total biomass. Lactate and its fermentation products acetate and propionate were analyzed using an Agilent 1200 series HPLC system equipped with and Aminex HPX-97H column (Bio-Rad, Hercules, CA, USA).

Samples were acidified with 1 M H_2S04 with a ratio of 19:1 (v/v) and separated with 4 mM aqueous H_2S04 as the mobile phase at a flow rate of 0.6 mL/min and quantified using a UV detector set to 210 nm. Optical densities (OD) of glucose-fed enrichment cultures were monitored on the Thermo Scientific spectronic 20D+ spectrophotometer at a wavelength of 600 nm as a relative measurement of glucose-consumption in cultures (i.e., no change in OD was relative indication for complete consumption of substrate). Methane production in glucose-amended without methanogen-inhibitor (2-bromoethane sulfonate) was qualitatively confirmed by analyzing 100 μL headspace samples on an Agilent 7890A GC equipped with and DB624 column (30mm × 0.53 mm 1.D., 3 μm) with a flame ionization detector (FDI). The concentrations of electron donor and acceptors were calculated by normalizing the peak area (for measurements from HPLC and IC) and absorbance (measurements on spectrometer) values to standard curves generated with known amount of compounds (0 to 10 mM).

5.4 Results

5.4.1 Established redox conditions in Third Creek microcosms

To identify types of corrinoid produced under different geochemical conditions, anoxic microcosms were established with sediment samples from Third Creek site under various redox conditions including fermenting/acetogenic, fermenting/methanogenic, iron-reducing, nitrate-reducing and sulfate-reducing. In microcosms microbial activity started in a day, and reducing, fermenting/acetogenic and fermenting/methanogenic conditions were obtained. In microcosm amended with lactate or glucose as only substrate, fermentation of these compounds completed in a week. In microcosms amended with various electron acceptors (FeOOH, NO_3^-, SO_4^{2-}), reduction of electron acceptors started in a week following the fermentation of electron donor (i.e., lactate) and electron acceptors were completely reduced in 25 days. In autoclaved abiotic control microcosms, consumptions of amendments were not detected and/or observed [e.g., Initially reddish FeOOH-fed cultures did not turn to black (reduced iron color)]. Sediment free redox

enrichment cultures were obtained by transferring cultures into fresh medium, and enrichment cultures also fermented and/or reduced substrates and/or electron acceptors. Due to the extremely low solubility of FeOOH (Fe^{+3})[50], some portion of FeOOH attached to the surface of the inside vessels. Vigorous shaking did not completely detach the particles. Therefore, total iron ($Fe^{+3/+2}$) and reduced iron (Fe^{+2}) measurements via colorimetric ferrozine assay did not yield 100% mass balance, and complete reduction of iron was confirmed by qualitatively observing the color change in cultures [i.e., oxidized iron (Fe^{+3}) was reddish-orange color, and when it was reduced to Fe^{+2}, it became black).

5.4.2 1.6-L redox enrichment cultures

To identify corrinoid production under each redox condition, 5th generation enrichment cultures were grown in the 2L large vessels. Lactate-fermenting enrichment culture fermented 82 % of initially-amended 5mM lactate in a day and acetate and propionate were detected as fermentation products (Figure 5.1A). Culture was re-amended with 5 mM lactate to increase the biomass yield. Complete fermentation of a total of 10 mM lactate was completed in 3 days, and culture was re-amended with 5 mM glucose on 3rd day (at middle stationary phase). Glucose fermenting culture reached to stationary phase in 4 days, showing complete fermentation of glucose occurred (Figure 5.1B).

In nitrate-reducing enrichment cultures, initially amended 5 mM nitrate (NO_3^-) was reduced in 7 days and max N_2O production of 1.9 ± 0.0 mmole N_2O/bottle production was measured (Figure 5.2A). After amendment of additional 5 mM nitrate on 7th day, slower nitrate-reduction was detected possibly due to electron donor limitation. Addition of 5 mM acetate and 120 mL H_2 enhanced the microbial activity, and nitrate was completely reduced in 13 days. Then cells were harvested for corrinoid analysis. In sulfate-reducing culture, sulfate reduction was much slower compared to other redox cultures. Therefore, cultures were amended with 5 mM $NaSO_4$ only one time. Sulfate reduction started in a day, 4.0 ± 0.28 mM H_2S was detected when 99% of 5 mM sulfate is consumed (50th day, Figure 5.2B).

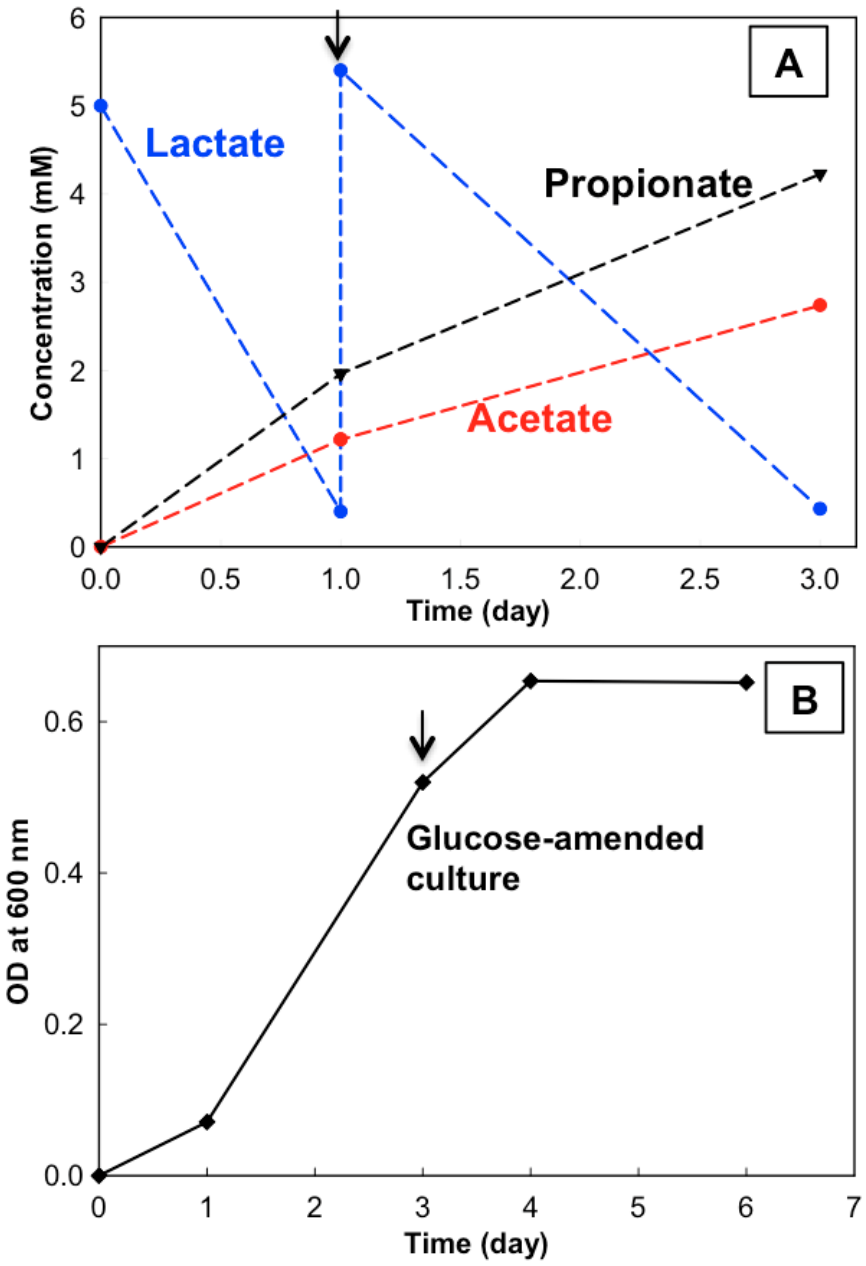

Figure 5.1. Lactate fermentation (A), and growth in glucose-amended culture (B) **A)** Lactate fermentation in lactate-amended cultures. Acetate and propionate are fermentation products**. B)** OD (at 600nm) measurements of glucose-amended cultures. Black arrows represent the time, when cultures were re-amended with substrates. Data represents averaged of duplicate measurements.

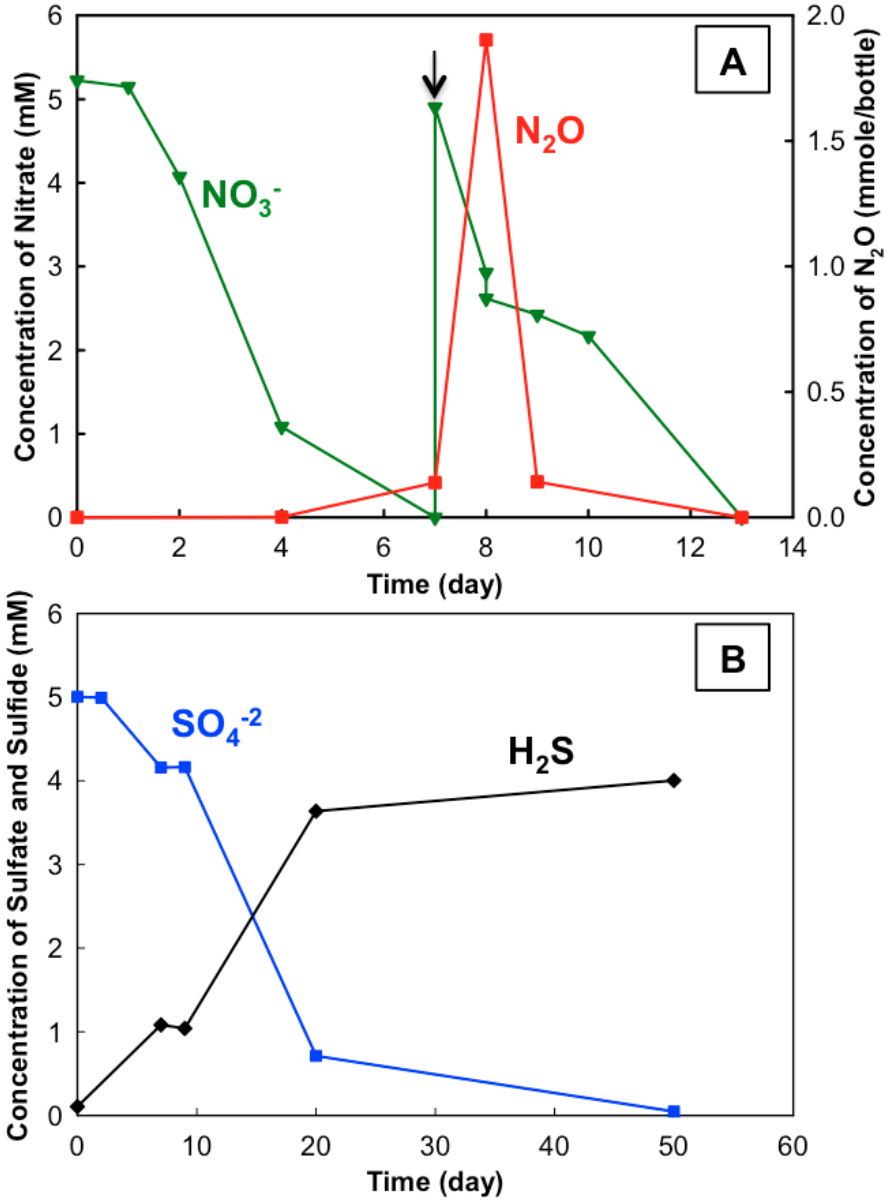

Figure 5.2. Nitrate and sulfate-reduction in redox cultures
A) Nitrate (NO_3^-)-reduction and N_2O production in nitrate-amended cultures. Arrow represents the time, when culture was re-amended with 5 mM NO_3^-. **B)** Na_2SO_4 (represented as SO_4^{-2} in figure) reduction and H_2S production in sulfate-amended cultures. Data represents averaged of duplicate measurements.

In iron-reducing cultures, due to attachment of FeOOH onto the side of the bottles, total iron ($Fe^{+2/+3}$) measurements via ferrozine assay did not yield a 100% mass balance. Therefore, iron reduction was visually observed [i.e., oxidized iron (Fe^{+3}) was reddish, and reduced iron (Fe^{+2}) was black]. When 70 % of initial amount of total iron (5 mM) was detected as reduced form Fe^{+2} (data is not shown) and cultures were almost completely black color, cells were harvested for corrinoid analysis.

5.4.3 Community structures of redox enrichment cultures

Illumina MiSeq 16S rRNA gene amplicon sequencing produced a total of 862,717 16S rRNA gene amplicon sequences after raw pair-reads were merged and trimmed. To improve taxonomic assignment, only reads representing the full 16S rRNA gene V4 region (fixed length 252 bp) were retained for downstream analyses. After further quality filtering (e.g., excluding chimeric reads), a total of 724,114 high-quality 16S rRNA gene amplicon sequences were obtained.The number of sequences originated from different samples varied from 99,290 to 178,451 (Table S5.1). Total of 421 OTUs were assigned by clustering high-quality sequencing at 97% nucleotide sequence identity and taxonomic annotation using Greengenes database (see Table S5.1 for number of OTUs/sample information). Total of twenty bacterial and one archaeal phyla were assigned across all samples. Taxonomic identification of OTUs unrevealed that lactate fermenting, glucose fermenting/methanogenic, iron-, sulfate-, and nitrate-reducing populations were enriched in defined redox conditions. Taxonomic classifications of OTUs for each redox conditions are detailed in Figure 5.3.

Lactate fermenting cultures. Total of 8 bacterial and 1 archaeal phyla were assigned in lactate-fermenting cultures. The majority of OTUs belonged to *Firmicutes* (51% sequences) and *Bacteroidetes* (47% of total sequences), and *Proteobacteria* contributed to 2% of total sequences. Sequences that could not be assigned to a genus or a family with in the order of *Bacteroidales* (22 % of total sequences) constituted the most abundant group (sequences show 100% nucleotide identity with an uncultured bacterium via NCBI blast analysis), followed by *Clostridium* (11% of total sequences, Figure 5.3).

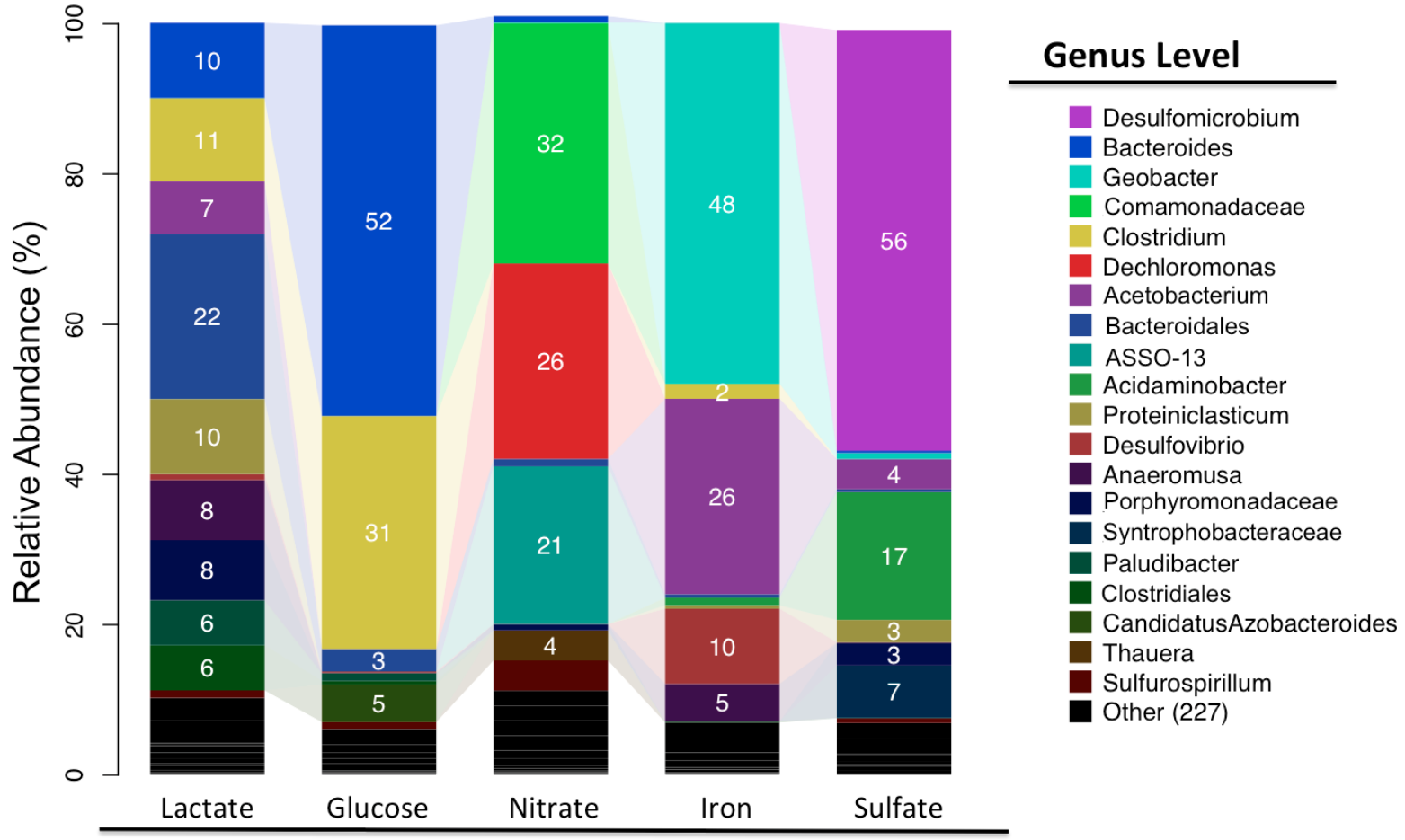

Figure 5.3. Microbial community structures in redox cultures
Microbial communities in lactate- and glucose-fermenting, nitrate-, iron- and sulfate-reducing cultures at the genus level based on 16S rRNA gene amplicon sequence analysis. Top 20 genus were listed, other low abundance genus (227) are grouped in "Others". Numbers on bar graphs represent the relative abundances of each taxonomic OTUs (only >5 % were represented). OTUs, which could not be assigned to any genes, represented with its family or order level classification.

Sequences belonged to proteolytic (degrading proteins into smaller polypeptides or amino acids) *Proteiniclasticum* (10 % of total sequences) was the third most abundant group.*Acetobacterium* within *Eubacteriaceae* and an unclassified genus within the propionate producing family *Porphyromonadaceae* contributed to 7 and 8 % of total sequences, respectively. Sequences assigned to fermenting *Sedimentibacter,* and acetate and propionate producing *Paludibacter* were the other most abundant group of OTUs. Methanogens including *Methanobacterium*, *Methanocelleus*, *Methanocorpisculum* and *Methanospirillum* were also detected at low abundances (<0.02 % of the total sequences recovered from lactate-fermenting culture). 1% of total sequences was assigned to sulfur-reducing *Sulfurispirillum cavolei* (specie level was identified at 100 % nucleotide identity using NCBI blast tool), which can use various organic acids including lactate and acetate as electron donor[51].

Glucose fermenting cultures. A total of 15 bacterial and 1 archaeal phyla was assigned in glucose-fermenting culture. Similar to lactate fermenting cultures the majority of the OTUs were *Bacteroidetes*, *Firmicutes* and *Proteobacteria* with sequence abundances 61%, 38 % and 2 %. Figure 5.3 shows genus level taxonomic classification of glucose-fermenting/methanogenic cultures, and the culture exhibited significant enrichment in *Bacterioides* (52 % of total sequences), *Clostridium* (31 % of total sequences). *Candidatus Azobacteroides* (5%) and an unidentified genus in order *Bacteroidales* (3 %). Different from lactate fermenting cultures, methanogenic group *Methanosaeta* (<0.06 % of total sequences) was enriched.

Nitrate-reducing cultures. Significant portion of the sequences (94 %) recovered from nitrate-reducing cultures belonged to phylum *Proteobacteria*. Phyla *Bacteroidetes* (3 %), *Firmicutes* (2 %), and *Acidobacteria* (2 %) were the other most abundant groups. Denitrifying *Diaphorobacter* within the family *Comamonadaceae* was the most abundant genus (32 %), followed by another denitrifying genus *Dechloromonas* (26 % of total sequences) in nitrate-reducing cultures. Significant portion sequences (21%) that could not be identified at genus and family levels, belonged to order ASSO-13. Similar to lactate-cultures sulfur-reducing *Sulfurospirillum cavolei* (4% of total sequences) were detected in nitrate-reducing cultures.

Iron-reducing cultures. OTUs belonging to phyla *Proteobacteria* (59 % of the total sequences), *Firmicutes* (37 % of the total sequences) were the most abundant groups, followed by *Actinobacteria* (4% of the total sequences) in the sequences recovered from iron reducing cultures. Iron-reducing cultures were enriched in iron reducer genus *Geobacter* (48 % of the total recovered sequences), followed by *Acetobacterium* (which has %99 nucleotide similarity with *Acetobacterium woodii*) within the family *Eubacteriaceae* (26% of the total sequences, Figure 3). OTUs assigned to another iron-reducer *Desulfovibrio* contributed to 10 % of the total OTUs. Amino acid fermenter *Anaeromusa* (5 % of the total sequences) and organic acid fermenter *Clostridium* (2 %) were other most abundant genus in iron-reducing cultures.

Sulfate-reducing cultures. Sulfate-reducing cultures exhibit significant enrichment in sulfate-reducers *Desulfomicrobium* (56% of total sequences, Figure 3), followed by *Acidaminobacter* (17% of the total sequences). Sequences that could not be assigned to any genus within the family *Syntrophobacteraceae* contributed to 7% of total sequences. NCBI-blast analysis with OTUs belong to *Syntrophobacteraceae* gave a 100% nucleotide identity with uncultured *Desulfovirga* sp. 16S rRNA gene sequence, and a sulfate-reducer specie within *Desulfovirga* genus was previously reported.[52] Similar to lactate-amended cultures, proteolytic *Proteiniclasticum* (10 % of total sequences) was also one of the most abundant groups of bacteria in sulfate-reducing cultures. OTUs belong to known corrinoid-producer *Acetobacterium* contributed to 4% of the total sequences.

5.4.4 Native corrinoids produced under different redox conditions

HPLC-DAD analysis of corrinoids produced under different redox conditions (i.e., lactate-, glucose-fermenting, iron-, nitrate, and sulfate-reducing conditions) revealed different corrinoid profiles. To identify the type of the corrinoids, retention times of the corrinoid fractions of the cultures were compared with the retention times of purified corrinoid standards (DMB- , Ben-, 5-MeBza-, 5-OMeBza, 5-OHBza-, *p*-Cre-Cba, and Phe-Cba). Figure 5.4-5.5 show HPLC chromatograms of corrinoids produced in redox cultures.

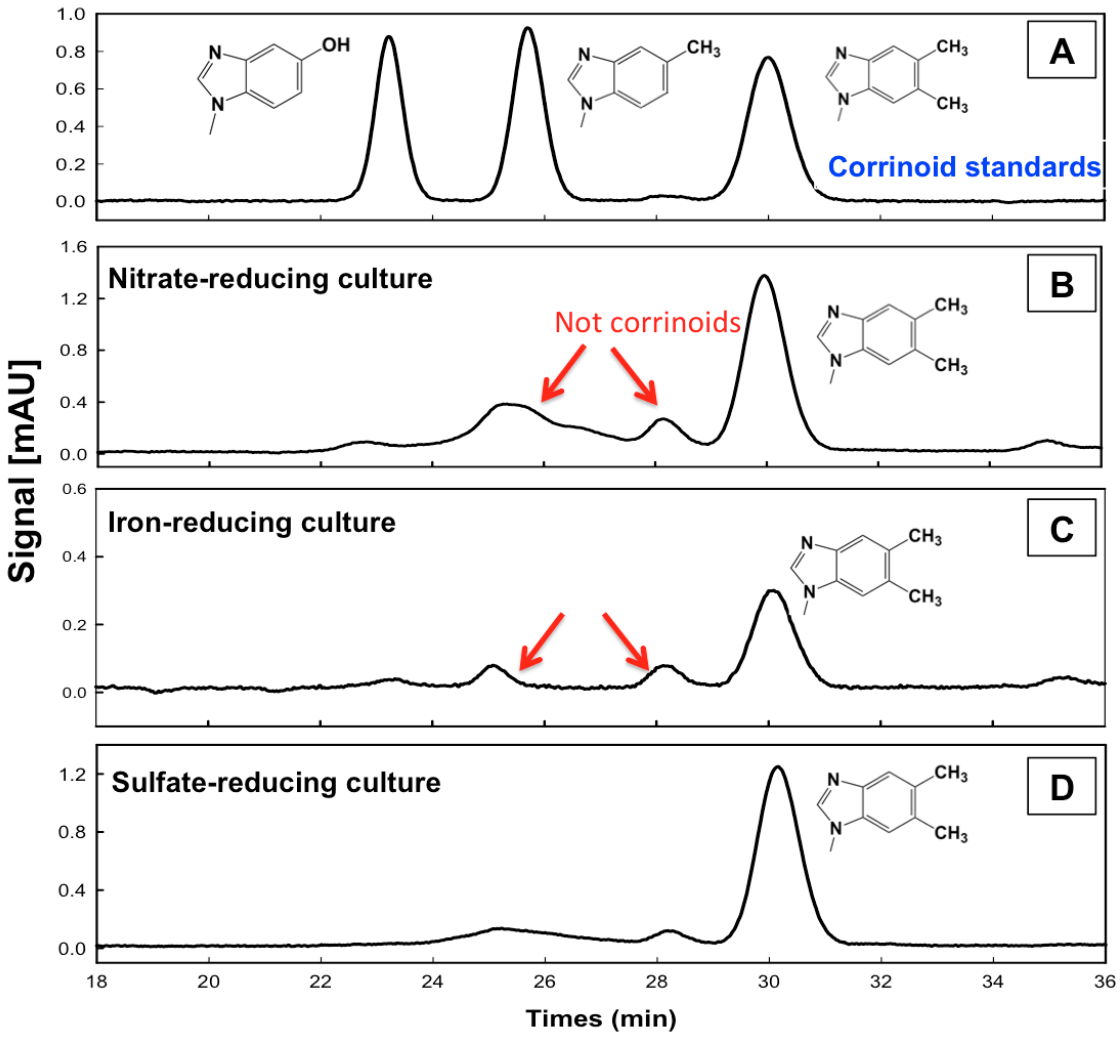

Figure 5.4. HPLC chromatograms of corrinoids produced in redox cultures
A) Corrinoid standards [5-OHBza-Cba: 23.217min; 5-MeBza-Cba: 25.694min; DMB-Cba: 29.99min]. **B)** Nitrate-reducing cultures. **C)** Iron-reducing culture. **D)** Sulfate reducing culture. **E)** Glucose-amended culture. Chemical structures on each peak represent the lowerbase structure of corresponding corrinoid. Red-arrows show the peaks, which are not corrinoids (see Figure S5.1).

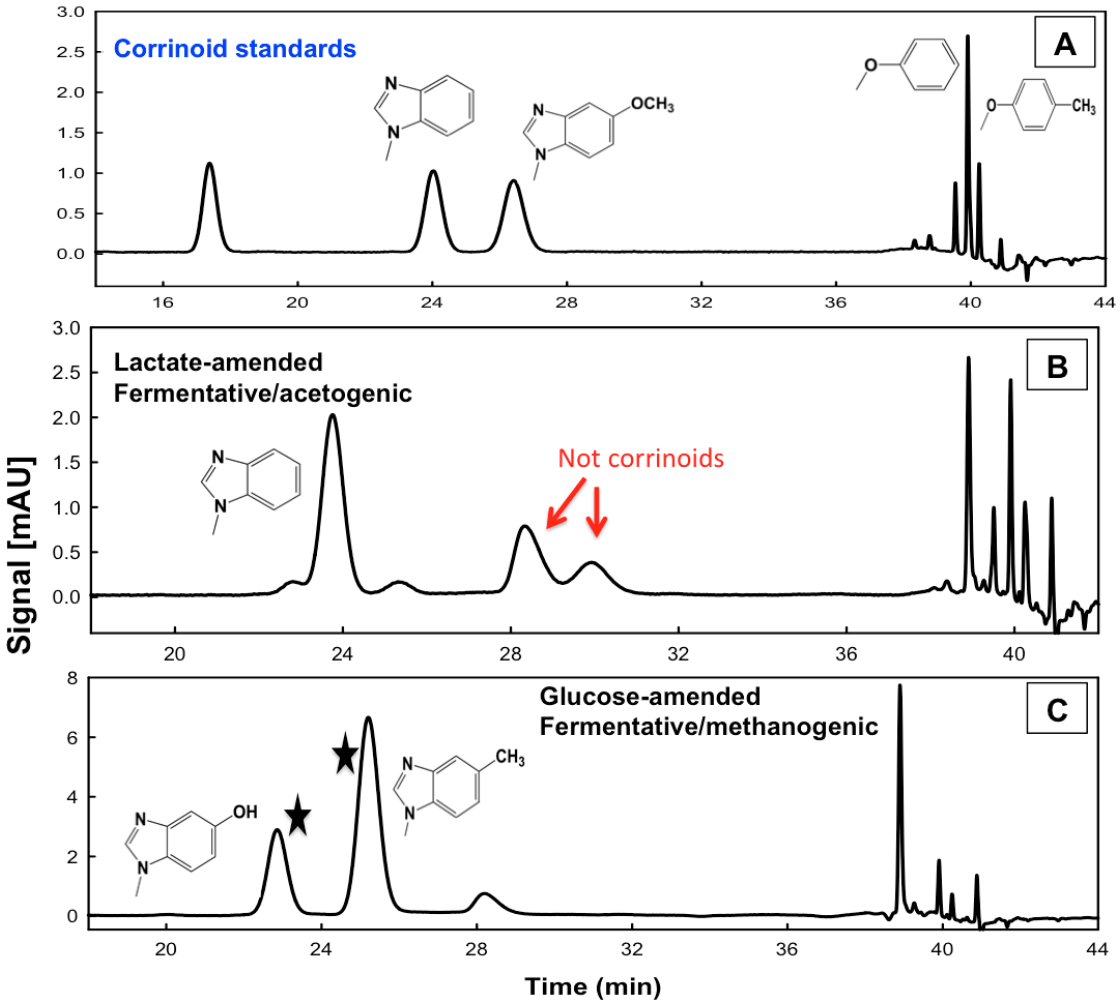

Figure 5.5. HPLC chromatograms of corrinoids produced in redox cultures
A) Corrinoid standards [Bza-Cba: 24.032min; 5-MeOBza-Cba: 26.413min; Phe-Cba: 39.91min: *p*-Cre-Cba: 40.25min]. **B)** Lactate-amended cultures. **C)** Glucose-amended cultures. Chemical structures on each peak represent the lower base structure of corresponding corrinoid. Red-arrows show the peaks, which are not corrinoids (see Figure S3.1).Corrinoid standards for the peaks labeled with black stars are presented in Figure 5.4.

Corrinoids were confirmed by comparing the maximum adsorption wavelength of each fraction in HPLC chromatograph with maximum absorption wavelength of standard vitamin B_{12} solution (361 nm, Figure S5.1). Enrichment of different microbial populations under different redox conditions lead to production of different corrinoids. In lactate-fermenting enrichment cultures, Bza-Cba was the most abundant corrinoid, followed by *p*-Cre-Cba, Phe-Cba and DMB-Cba (cobalamin) (Figure 5.5B). Different from lactate fermenting cultures, 5-MeBza-Cba and (5-OHBza) Factor III type corrinoids were the most abundant corrinoids produced by the native populations in glucose-amended cultures (Figure 5.4E). Phenolic cobamides (*p*-Cre-Cba and Phe-Cba) were also detected in glucose-fed cultures. Interestingly DMB-Cba was the only corrinoid detected in nitrate-, iron-, and sulfate-reducing cultures (Figure 5.4D).

5.4.5 Quantity of corrinoids differs in different redox conditions

Quantities of corrinoids produced under different redox enrichment cultures were estimated by comparing integrated peak areas with standard curves prepared with known amounts of cobamides and normalized by total biomass (g VSS) of redox enrichment cultures. Not only the type of corrinoid but also amount of the corrinoid produced under different conditions showed variations (Table 5.2, Figure 5.6). DMB-Cba was the only corrinoid detected under nitrate-, iron, and sulfate-reducing conditions, amount of DMB-Cba was much lower (1.34 ± 0.02 μg/g VSS) in iron-reducing conditions when compared to amount of DMB-Cba recovered from sulfate and nitrate-reducing cultures (4,67 ± 0.05 and 3.47 ± 0.1 μg/g VSS, respectively). DMB-Cba was also produced by lactate-fermenting cultures but at very low concentrations (0.34 ± 0.01 μg/g VSS). Notably, 5-OHBza-Cba (Factor III type, produced by methanogens) was only produced in glucose-amended cultures at amount 1.72 ± 0.04 μg/g VSS (Table 5.2, Figure 5.6). Bza-Cba was the abundant corrinoid in lactate fermenting culture at amount of 1.54 ± 0.03 μg/g VSS. MeBza-Cba was only detected in glucose-fed fermentative/methanogenic cultures at high amounts of 3.67 ± 0.11 μg/g VSS. Phenolic cobamides *p*-Cre-Cba, Phe-Cba were at concentrations of 1.08 ± <0. 005 and 0.60 ± <0.005 respectively in lactate-amended cultures, but much lower concentrations in glucose-amended cultures.

Table 5.2. Total biomass and specific corrinoid productions in redox cultures

	Lactate	**Glucose**	**Iron**	**Sulfate**	**Nitrate**
VSS (g/L)	2145 ± <0.1	3095 ± 14.1	1245 ± 311.1	580 ± <0.1	873 ± 3.5
Corrinoid (μg/g VSS)					
DMB-Cba	0.34 ± 0.01	-	1.34 ± 0.02	4.67 ± 0.05	3.47 ± 0.10
5-MeBza-Cba	-	3.67 ± 0.11	-	-	-
Ben-Bza	1.54 ± 0.03	-	-	-	-
Phe-Cba	0.60 ± 0.00	0.31 ± 0.00	-	-	-
p-Cre-Cba	1.08 ± 0.00	0.35 ± 0.00	-	-	-
5-OHBza-CBa	1.72 ± 0.04	-	-	-	-

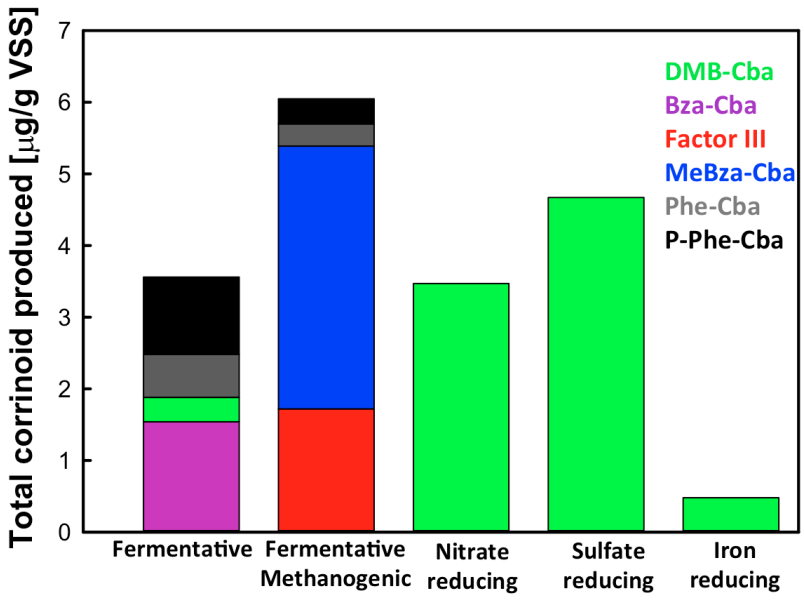

Figure 5.6. Total corrinoid production under different redox conditions. Fermentative condition represents lactate-amended cultures. Fermentative/methanogenic condition represents glucose-amended cultures.

5.5 Discussion

Corrinoids are required cofactors for some enzymes that play critical roles for the majority of the organisms including eukaryotes.[53-55] Since *de novo* corrinoid synthesis only can be performed by several microorganisms that have full pathways for corrinoid production[56], exchange of corrinoids is crucial in functionally integrated microbial systems. Corrinoid-auxotroph *Dhc* strains, which are the only bacteria that can detoxify chlorinated ethenes, have strict requirements for corrinoid cofactors, and rely on the corrinoid salvage pathways to scavenge corrinoids from the environments. In subsurface environments, *Dhc* co-exist with corrinoid producing microorganisms such as methanogens, acetogens, sulfate reducers and fermenters, and it is very likely to encounter a variety of different corrinoids in the environments[25, 27, 57, 58] To date, at least 16 naturally occurring corrinoids with structural variability in lower bases (i.e., benzimidazole, purine or phenol derivatives) have been identified.[59, 60] Although some microorganisms can utilize variety of naturally occurring corrinoids with variability in

lower bases,[22, 61-64] recent studies showed that even corrinoids within one lower base class (that is, either benzimidazole, phenolic, or purine) may not necessarily be functionally equivalent[22, 23, 34, 63]. Recent studies also revealed that not all naturally occurring corrinoids with different lower base structures support *Dhc* reductive dechlorination activity[22, 23, 33]. For example, in *Dhc* strain BAV1 co-cultures with methanogen *Methanosarcina barkeri*, the corrinoid produced by *Methanosarcina barkeri* (presumably 5-OHBza-Cba) did not support *Dhc* reductive dechlorination, and cDCE detoxification was not achieved[34], suggesting that 5-OHBza-Cba, common corrinoid produced by some methanogens could not support *Dhc* reductive dechlorination activity and growth. Another example, acetogenic Bacteria (e.g., *Acetobacterium*, *Clostridium*, *Sporomusa*) are also known as corrinoid-producers; but the corrinoids containing phenolic-type lower bases (*p*-Cre-Cba and Phe-Cba) produced by some *Sporomusa* strains did not support *Dhc* reductive dechlorination activity, [34] Despite of inability to biosynthesize corrinoid, *Dhc* strains possess genes encoding corrinoid salvaging and remodeling. Remodeling of non-functional corrinoids (e.g., 5-OHBza-Cba, *p*-Cre-Cba) has been observed and recovery of reductive dechlorination was detected in *Dhc* cultures when DMB lower base was provided.[23, 33, 34]

Recent studies advanced our understanding of exact corrinoid and/or lower base requirements, and corrinoid metabolism (i.e., salvaging, remodeling) of organohalide-respiring *Dhc.*[22, 23] However, some key questions are still remaining to be answered for improving bioremediation applications: 1) the source/availability of the corrinoid cofactors or the lower bases to sustain *Dhc* reductive dechlorination activity in chlorinated solvent-contaminated aquifers, and 2) how the biogeochemical conditions and corresponding microbial systems shape the corrinoid pools in the environments. One of the most common contaminated site management practice is biostimulation through supply of electron donor (i.e., fermentable carbon substrates), when natural attenuation processes are either non-exit or not sufficient (i.e., cDCE and VC accumulation)[35, 65, 66] The assumption is that electron donor for reductive dechlorination (hydrogen for *Dhc*) is limiting, and supply of fermentable carbon substrates promotes the growth of

dechlorinators. Although continuous addition of fermentable substrates can suffice electron needs of dechlorination activity, the evidence that hydrogen is limiting the dechlorinating activity is difficult to ascertain. A study with long term-monitoring of a contaminated site under low-hydrogen flux indicated that addition of vitamin B_{12} but not hydrogen actually overcame the activity-limiting conditions.[67] In addition, depending on the site biogeochemical conditions (e.g., co-contamination), other redox-processes including methanogenesis, sulfate-, nitrate- and iron-reducing may co-exist, and addition of electron donor may make these processes become favorable, and enhance communities that may produce "wrong" (which does not sustain *Dhc* reductive dechlorination activity) type of corrinoid/ or lower bases. In such scenarios, continued biostimulation will not sustain reductive dechlorination activity, and VC or cDCE stalls may become major problems. Current findings demonstrated that availability of different electron donor and/or acceptors (i.e., different redox conditions) resulted in different microbial populations to become abundant, which leaded to production of different corrinoids. Lactate-amended conditions enhanced growth of acetogens like *Clostridium* and *Acetobacterium*, which are known corrinoid producers (Figure 5.3). Although DMB-Cba (that is, favorable corrinoid for *Dhc* reductive dechlorination) was detected in lactate-amended cultures, Bza-Cba and phenolic corrinoids (*p*-Cre-Cba and Phe-Cba) were at much higher concentrations (Figure 5.6). It was previously showed that corrinoids with phenolyl lower bases did not support *Dhc* strains. In a lactate-amended TCE-dechlorinating cultures, *p*-Cre-Cba was found to be the most abundant corrinoid produced by the community.[68] Although the phenolic corrinoids did not sustain *Dhc* growth, *Dhc* was capable of fulfilling its corrinoid requirement in the TCE-dechlorinating enrichment cultures (in which phenolic corrinoids where the most abundant corrinoids) by corrinoid remodeling, in that case by exchanging lower base of phenolic corrinoid with DMB. The corrinoid Bza-Cba sustain only *Dhc* strain BAV1 reductive dechlorination at very low rates (with reductive dehalogenase (RDase) *vcrA*)[22, 23]. Therefore, at the sites with abundant other strains of *Dhc* (i.e., strain 195 or GT), the complete dechlorination may not be achieved due to production of wrong type of corrinoids. Lactate is one of the most commonly used biostimulation-substrate in contaminated sites to enhance reductive

dechlorination[66]; current findings demonstrated that continuous lactate supply to the contaminated systems may enrich the microorganisms that produce wrong type of corrinoid, which may result in that *Dhc* reductive dechlorination could not be sustained due to corrinoid/lower base limitation. Similarly, Factor III type of corrinoid in glucose-amended cultures, which was first purified from methanogens, also does not sustain *Dhc* reductive dechlorination activity. On the other hand, under glucose-fed conditions MeBza-Cba was also produced at high concentrations (Figure 5.6), which previously was reported to support *Dhc* reductive dechlorination activity. An unexpected finding was that DMB-Cba, favorable corrinoid by *Dhc* were the only detected corrinoids under sulfate-, iron-, and nitrate-reducing conditions (Table 5.2, Figure 5.6). Anaerobic lower base DMB production, the only unknown component of vitamin B_{12} biosynthesis pathway, has recently been identified, and only very few microorganisms (i.e., *Acetobacterium woodii*, *Eubacterium Limosum* and *Thermincola potens*) possess the genes involved in complete DMB biosynthesis (i.e., *bzaABCDE* genes).[69] 16S rRNA gene-base community analysis revealed that among DMB-Cba producers only *Acetobacterium* was detected in lactate-fermenting, iron-, sulfate,- and –nitrate reducing conditions, contributing 7%, 26%, 4% <0.005% of total OTUs (Figure 5.3). Detection of *Acetobacterium* OTUs with relatively high abundance in the cultures can explain the possible role of this bacterium in DMB-Cba production in lactate-amended, iron- and –sulfate-reducing cultures. However, the high concentration of DMB-Cba (Figure 5.6) produced in nitrate-reducing cultures does not match with very low abundances of *Acetobacterium* 16S rRNA (less than 0.005% of the OTUs). These findings indicated that anaerobic DMB lower base and DMB-Cba production may not be limited to recently identified pathway, but may be more diverse and not limited to only three microorganisms. Despite of high abundances *Acetobacterium* (26%) in iron-reducing cultures (Figure 5.3), recovery of the low concentrations of DMB-Cba could be due to corrinoid extraction-error. The black particulates in iron-reducing cultures could cause some limitations during corrinoid extractions, and resulted in low yield of corrinoid. An unexpected finding was that DMB-Cba was the only corrinoid detected in iron-reducing cultures, where 50% of the total microbial populations was iron-reducer *Geobacter lovleyi*, which was predicted to

produce 5-OMeBza-Cba based on gene-anotation[69]. This indicated remodeling of lower bases might be very common in mixed microbial systems. For example, in this case 5-OMeBza-Cba was remodeled and formed DMB-Cba. Sulfate-reducer *Desulfobacterium* was identified as MeBza-Cba producers; however, this group was not detected in the sulfate-reducing cultures derived from TC site material. In contrast, *Desulfomicrobium* was the most abundant sulfate-reducer in the cultures, but analysis of available genomes of *Desulfomicrobium* showed that this group of organism did not possess complete set of genes for corrinoid biosynthesis. Therefore, some other microorganisms must be corrinoid producers in sulfate-reducing cultures.

Our results revealed differential production of corrinoids with variety lower bases under different redox conditions, demonstrating that composition and concentration of bioavailable corrinoid pools are determined by different geochemical conditions. These findings have implications for bioremediation practices. cDCE and VC stalls most of the time considered to be due to electron donor limitations; however our results clearly revealed that availability of corrinoids with wrong type of lower bases has an important effect on the fate of reductive dechlorination. Improved understanding how the type of substrate(s) used for biostimulation depend on site geochemical conditions, and prevailing microbiology affect the corrinoid pool (availability of right type of corrinoid or lower bases), and consequently *Dhc* reductive dechlorination activity can provide valuable information for refined decision-making and the implementation of efficient bioremediation treatment for achieving cleanup goals and protecting humans from exposure to harmful groundwater contaminants.

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5.7 Appendix 5

Table S5.1. Summary of 16S rRNA gene sequencing results

Cultures	Total number of high-quality sequences	Total OTUs/sample
Lactate fermenting/acetogenic	170,661	161
Glucose fermenting/methanogenic	99,290	123
Sulfate-reducing	102,985	111
Nitrate-reducing	178,451	149
Iron-reducing	173,727	204

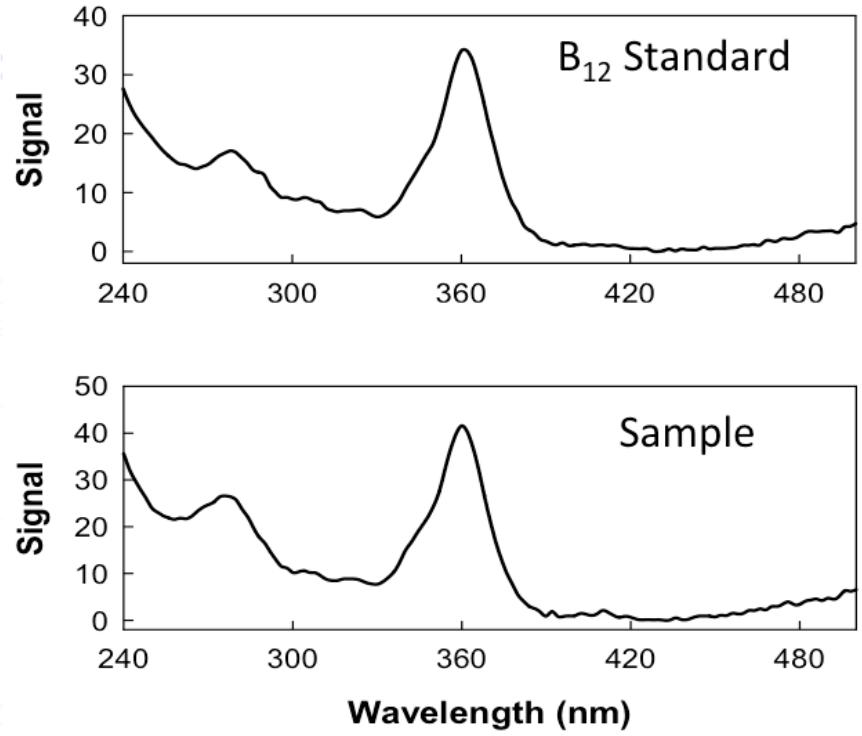

Figure S5.1. UV-Vis absorbance curve comparison for corrinoid confirmation
A corrinoid has a maximum absorbance at wavelength of 361nm.

6 Summary and Conclusion

Chlorinated solvents such as PCE, TCE and their degradation products *c*DCE and VC are among the most prevalent toxic groundwater contaminants. PCE is anticipated to be, and TCE and VC are proven human carcinogens, thus efficient and cost-effective remedies are needed to prevent human exposure through contaminated water or vapor intrusion into dwelling. Biodegradation shows a great promise as an environment-friendly and cost-effective remediation strategy, and bacterial organohalide-respiration in which chlorinated solvents serve as electron acceptors plays a major role in the detoxification of these compounds. Organohalide-respiring *Dehalococcoides* (*Dhc*) are the key players in bioremediation, since only *Dhc* strains have been implicated in complete detoxification of chlorinated ethenes to benign ethene. Recent studies revealed that *Dhc* has highly restricted lifestyle, and depend on other microbial communities in the environment for essential growth-nutrients such as H_2 and corrinoid cofactor. For successful implication of the microbial reductive dechlorination process to remediate chlorinated solvent-contaminated sites, the microbial interactions and environmental parameters, which control *Dhc* reductive dechlorination must be elucidated. The overall objective of this PhD research, therefore, was to address the key gaps in the scientific understanding of the controls over *Dhc* reductive dechlorination and strains selection, and *Dhc* interactions with other microorganisms in the environment. In addressing these goals, a mixed-chlorinated solvent contaminated site adjacent to Third Creek, a Tennessee River tributary in Knoxville, TN (Third Creek site) provided a great opportunity for this PhD research.

At Third Creek site, chlorinated solvents, primarily PCE, TCE, and TCA were released and penetrated the underlying fractured bedrock formation. Remediation of sites like Third Creek site with fractured bedrock formation is particularly challenging, mainly due to difficulties in characterizing fracture networks, and limited microbial transformation of contaminants in fractures. Therefore, as an alternative remedy, the possible role of the fracture-sediment interface as a natural barrier preventing contaminant discharge into Third Creek surface water was evaluated and presented in **Chapter 2**. The detailed hydrogeological, geochemical and microbial characterization of Third Creek site

attributed a critical role to the streambed sediment for contaminant detoxification. Phylogenetically distinct groups of organohalide-respiring bacteria including *Dhc*, *Dehalobacter* (*Dhb*) and *Geobacter* co-exist in the Third Creek sediment, and favorable biogeochemical conditions for organohalide-respiring bacteria established resulting in effective detoxification of toxic contaminants reaching to the sediment. This research presented a promising approach for remediation of contaminated sites, where fractured formation is challenging and sediment-fracture interface can be a barrier for contaminant attenuation.

Naturally well-developed dechlorinating microbial populations at Third Creek sediment formed the basis of the next chapters of this PhD thesis. Chlorinated solvents-dechlorinating Third Creek microcosms, which were established with amendment of various different chlorinated compounds including chlorinated-ethenes, -ethanes, -propanes, and methanes, and derived corresponding enrichment cultures, were used to explore the key microbial community members and specific interactions sustaining *Dhc* reductive dechlorination activity. Community analysis of Third Creek dechlorinating enrichment cultures identified the fermenters and acetogens including *Clostridium*, *Acetobacterium*, *Sedimentibacter*, *Desulfovibrio*, and other dechlorinating microorganisms such as *Geobacter* and methanogenic populations as the key supportive members involved in sustaining *Dhc* reductive dechlorination. Fermenters and/or acetogens in TC cultures sustained *Dhc* reductive dechlorination by providing primarily H_2 and acetate, which required as electron acceptor and carbon source by *Dhc*. In addition, microorganisms in the cultures like fermenters, methanogens encoding systems for oxygen scavenging sustained *Dhc* growth by reducing toxic effect of oxygen. In this research, corrinoid-related interactions were more focused and explored. The key catalyst for organohalide-respiration is the reductive dehalogenase enzyme (RDase), which requires a corrinoid cofactor for the activity. Despite their dependency on corrinoid cofactor, *Dhc* strains lack the ability for corrinoid biosynthesis. *Dhc*'s inability to synthesize growth-essential corrinoid cofactor means that *Dhc* must scavenge this cofactor from the environment, which makes the role of corrinoid-producing populations

very important in dechlorinating communities. Corrinoid related-interactions between *Dhc* and other microorganisms, and environmental factors affecting the corrinoid pools were explored and presented in **Chapters 4 and 5**.

The work presented in **Chapter 3** primarily addressed the question of whether available electron acceptors affects development of dechlorinating communities and dechlorinator strain diversities (selection) especially *Dhc*. Widely-selection of *Dhc* strains carrying *vcrA* gene in TC-enrichment cultures with amendment of various chlorinated-ethenes, demonstrated their broad range-substrate utilization capability; however strains carrying *tceA*, *bvcA*, and *dcpA* genes showed preferences for specific chlorinated solvents. *Dhb* strains were enriched with amendment of chlorinated ethanes and chlorinated methanes. The observation of different strains selection with certain electron acceptors from the same source is relevant for bioremediation. Incomplete or slow reductive dechlorination at sites frequently results in worse scenarios of more toxic intermediates *c*DCE or VC stalls. The reason of incomplete or slow dechlorination could be the lack of the *Dhc* growth factors but also the certain *Dhc* strains. Knowledge of the conditions favoring specific strains of *Dhc* may enable the development of better strategies with strain specific-amendments. In addition, bioremediation strategies where bioaugmentation with specific *Dhc* strains or *Dhc*-containing consortia could be used for certain chlorinated-solvent contamination to overcome limiting/slow dechlorination at sites. From **Chapter 3**, it was also clear that available electron acceptors controlled development of microbial communities in TC cultures. Although microbial communities in enrichment cultures amended with chlorinated ethenes were similar; some variations in community structure were observed with chlorinated-ethanes or methane amendments. Amendment with chlorinated ethanes or CF over three years resulted in reduction of abundances of well-known corrinoid producing *Acetobacterium*, which was attributed to inhibitory effects of these compounds on this population. This is an important observation and could have an implication in bioremediation of co-contaminated sites. Inhibition of chlorinated ethanes or methanes on supportive members would inhibit *Dhc* reductive dechlorination indirectly; this should be taken into account while developing site-specific remediation

strategies. The effect of electron acceptor was most significant on archaeal populations. CF-amendment inhibited the growth of methanogens in the cultures, which could be a case at site co-contaminated with chlorinated ethenes and –methanes. Inhibition of TCA and CF on *Dhc* growth was previously reported. Such inhibition of methanogens would also prevent nutritional support from methanogens, and inhibit *Dhc* activity indirectly. The results of community and strain selection analyses of dechlorinating communities that were selected with different chlorinated acceptor-amendments contributed towards understanding *Dhc* diversity and dynamics of dechlorinating communities, which may enable development of better bioremediation strategies to achieve successful contaminated-site cleanup.

In various environments, methanogens, acetogens and other bacterial groups are corrinoid producers, some of these populations have been shown to abundantly present in TC cultures, indicating their role as corrinoid-producers. Studies revealed that not all naturally occurring corrinoids produced by corrinoid producers supported *Dhc* growth, and our recent study also demonstrated that *Dhc* strains could have different preferences for different corrinoids. These observations raised the questions of the corrinoid-related interactions in the environment, and their controls over *Dhc* reductive dechlorination activity and selection, which were addressed in **Chapter 4**. TC PCE- and VC-noB_{12} cultures, which were derived without exogenous corrinoid (e.g., vitamin B_{12}), dechlorinated amended PCE or VC to ethene. In addition, corrinoid analyses revealed that DMB-Cba (highly-preferred corrinoid) was produced by the native TC supportive community. These results could also explain how the microbial system works well in Third Creek sediment to attenuate the toxic contaminants. However, this is not always the scenario we observed at other contaminated sites, where *c*DCE and VC stalls becoming major problems. In contrast to PCE- and VC-noB_{12} cultures, *c*DCE-noB_{12} cultures extended slow dechlorination rates, resulted in incomplete dechlorination. Addition of exogenous vitamin B_{12} recovered the activity and complete dechlorination of *c*DCE occurred in shorter period of time, demonstrated that corrinoid was the limiting factor for dechlorination in *c*DCE-noB_{12} cultures. Further exploration of these cultures actually

showed evidence for *c*DCE inhibition on corrinoid producers. This was one of the important observations of this research, which could explain incomplete dechlorination occurring at sites, which should be taken into account while developing bioremediation strategies. With the hypothesis of that corrinoid availability is one of the major control affecting *Dhc* reductive dechlorination and causing incomplete or slow dechlorination at contaminated sites, we further explored if available lower bases (that is structure differentiating corrinoids from each other), and corresponding corrinoids control corrinoid pools in the environment, which affect *Dhc* activity and selection. Addition of different lower bases to PCE-noB_{12} cultures, resulted in shift from naturally DMB-Cba production to production of corrinoids with amended lower bases in TC cultures, which affected *Dhc* activity significantly, and leaded much lower dechlorination rates. This observation has an implication for bioremediation practices. In contaminated sites, where microorganisms producing "wrong" type of lower bases (so corrinoids) are abundant, *Dhc* activity will not be sustained, causing a long-term problem of *c*DCE and VC stalls. In **Chapter 4**, we also explored the role of corrinoid on *Dhc* strain selection in subsurface environments. In PCE-noB_{12} cultures amended with different lower bases, we observed variation in *Dhc* populations carrying different RDase genes, showing that available lower bases, thus corresponding corrinoids determined *Dhc* strain selection in TC cultures. Further experiments on question of corrinoid-related *Dhc* strain selection would advance scientific understanding of the evolution of organohalide-respiring *Dhc*. Overall, **Chapter 4** clearly demonstrated that corrinoid-flux from supportive microbial populations is one of the major controls on reductive dechlorination activity and *Dhc* strain selection at contaminated sites.

One of the common contaminated site management practices is to continuously supply electron donor sources (i.e., biostimulation), when incomplete, and slow dechlorination rates occurred with the assumption of that H_2 is limiting. Depending on site biogeochemical conditions and type of the electron donor used for biostimulation, different biogeochemical structures (microbial populations) may be formed, which may lead productions of "wrong" type of corrinoid by the corresponding abundant

populations. In such scenarios, continued biostimulation will not lead effective detoxification of contaminants. This raised the question of controls of existing geochemical conditions on corrinoid pools, thus *Dhc* growth, which was addressed in **Chapter 5**. The aim was to explore the type of corrinoids produced under different geochemical conditions, and their effect on *Dhc* reductive dechlorination. Community and corrinoid analyses of TC redox cultures demonstrated that availability of different electron donor and/or acceptors (e.g., nitrate, lactate, sulfate) resulted in different microbial populations producing different corrinoids at different concentrations. Corrinoids like phenolic types and 5-OHBza-Cba, which does not sustain *Dhc* activity were at high concentrations in lactate-fermenting and methanogenic cultures, respectively. An unexpected finding was that DMB-Cba, favorable corrinoids, were detected at very high concentrations in sulfate-, iron- and nitrate-reducing conditions. Anaerobic DMB-lower base biosynthesis pathway has been very recently identified, and only three organisms, *Acetobacterium woodii* and *Eubacterium Limosum* and *Thermincola potents* have been identified to possess complete DMB lower base biosynthesis genes. Low abundances of *Acetobacterium* OTUs (only detected DMB-producers in the cultures) recovered from nitrate-reducing cultures, despite of the production of DMB-Cba at high concentrations indicated that anaerobic DMB production may not be limited to recently identified pathway and three organisms, rather it is more diverse through other pathways. Overall, the worked presented in **Chapter 5** demonstrated that the composition and concentration of bioavailable corrinoid pools are determined by different geochemical conditions. These findings can provide valuable information for refined-decision making and the implementation of efficient bioremediation strategies for achieving cleanup goals to prevent human exposure to harmful groundwater contaminants.

Vita

Burcu Şimşir was born in Ankara. She spent all of her time in Ankara till her graduate school years. While growing up, she played basketball from late middle school through years of undergraduate, and was a basketball player in several teams including the university basketball team. Burcu attended Middle East Technical University to pursue her undergraduate degree in Fall, 2006. During the last two years of the undergraduate Burcu worked with Dr. Göksel Demirer in his anaerobic-laboratory as an undergraduate research assistant, and involved in several projects, through which she gained her interest in anaerobic microbiology. In July, 2010, she received a Bachelor of Envioronmental Engineering Magna Cum Laude. Burcu's undegraduate research experiences inspried and encouradged her to pursue graduate degree, and in Fall, 2010, she entered graduate school in Department of Environmental Engineering at the University of Tennessee, Knoxville, where she worked with Frank Löffler. During six years of graduate school, Burcu gained a lot of experiences and knowledge on environmental microbiology and molecular biology. Upon completion of her doctoral degree, Burcu intends to pursue an academic career.