Management of Microbial Communities to Improve Growth of Chloroethene-Respiring *Dehalococcoides*

by

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ABSTRACT

Reductive dechlorination by members of the bacterial genus *Dehalococcoides* is a common and cost-effective avenue for *in situ* bioremediation of sites contaminated with the chlorinated solvents, trichloroethene (TCE) and perchloroethene (PCE). The overarching goal of my research was to address some of the challenges associated with bioremediation timeframes by improving the rates of reductive dechlorination and the growth of *Dehalococcoides* in mixed communities.

Biostimulation of contaminated sites or microcosms with electron donor fails to consistently promote dechlorination of PCE/TCE beyond *cis*-dichloroethene (*cis*-DCE), even when the presence of *Dehalococcoides* is confirmed. Supported by data from microcosm experiments, I showed that the stalling at *cis*-DCE is due a H_2 competition in which components of the soil or sediment serve as electron acceptors for competing microorganisms. However, once competition was minimized by providing selective enrichment techniques, I illustrated how to obtain both fast rates and high-density *Dehalococcoides* using three distinct enrichment cultures. Having achieved a heightened awareness of the fierce competition for electron donor, I then identified bicarbonate (HCO_3^-) as a potential H_2 sink for reductive dechlorination. HCO_3^- is the natural buffer in groundwater but also the electron acceptor for hydrogenotrophic methanogens and homoacetogens, two microbial groups commonly encountered with *Dehalococcoides*. By testing a range of concentrations in batch experiments, I showed that methanogens are favored at low HCO_3^- and homoacetogens at high HCO_3^-. The high HCO_3^- concentrations increased the H_2 demand which negatively affected the rates and extent of

dechlorination. By applying the gained knowledge on microbial community management, I ran the first successful continuous stirred-tank reactor (CSTR) at a 3-d hydraulic retention time for cultivation of dechlorinating cultures. I demonstrated that using carefully selected conditions in a CSTR, cultivation of *Dehalococcoides* at short retention times is feasible, resulting in robust cultures capable of fast dechlorination. Lastly, I provide a systematic insight into the effect of high ammonia on communities involved in dechlorination of chloroethenes. This work documents the potential use of landfill leachate as a substrate for dechlorination and an increased tolerance of *Dehalococcoides* to high ammonia concentrations ($\geq$2 g L^{-1} NH_4^+-N) without loss of the ability to dechlorinate TCE to ethene.

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CHAPTER 1

INTRODUCTION AND SIGNIFICANCE

1.1 The legacy of chlorinated solvents as environmental pollutants

Contamination with anthropogenic chemicals from the industry-driven progress of civilization has bared heavy effects on land and water resources worldwide. The ‘70s and ‘80s were pivotal in the realization of the effect of environmental contaminants on human health and the need to establish laws and agencies to better protect people. A prominent example is the founding of the U.S. Environmental Protection Agency (EPA) in 1970, which set to ensure environmental protection to the American people through a range of federal research, monitoring, standard-setting, and enforcement activities (US EPA 1992). This was followed in 1972 by the Clean Water Act (based on the Federal Water Pollution Control Act of 1948) and by the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA) in 1980, which established the Superfund program, an environmental program addressing abandoned hazardous waste sites (US EPA 2013b). As a result of these actions and the continuing update of environmental policies and regulations in the latest decades, we have achieved better environmental practices and have taken action towards remediating sites affected by pollutants.

However, decades of improper disposal practices of chemicals, careless handling, accidental spills, and the continuous generation of waste by all communities, industries, technologies and military, have taken a heavy toll on the environment. To date, there are ~1300 Superfund sites and hundreds of thousands of sites polluted with organic and

inorganic compounds requiring decontamination (US EPA 2013b). In fact, the EPA estimates that a quarter of American populations resides within four miles of a Superfund hazardous waste site (US EPA 2012). Some of the most common organic pollutants at Superfund sites, U.S. National Priorities List (NPL) and groundwater sources are the chlorinated ethenes, trichloroethene (TCE) and perchloroethene (PCE) (McCarty 1997; Moran et al. 2007; Rittmann and McCarty 2001; US EPA 2013b).

1.2 Prospects for bioremediation

Both TCE and PCE were used extensively in the past century as organic solvents for multiple industrial processes (summarized in section 2.1). Most of the environments containing chlorinated ethenes are the result of activities that took place many years ago, although accidental spills still occur nowadays (Office of Response and Restoration 2012). Chlorinated ethenes are of major concern to the public as they have serious health effects. According to the Agency for Toxic Substances and Disease Registry (ATSDR), the reported health effects include liver and kidney toxicity and depression of the nervous system (ATSDR 2011), while prolonged exposure can cause cancer (National Research Council 2006). Because of their frequent presence in groundwater, toxicity and potential for human exposure, the EPA and the ATSDR have placed TCE and PCE in the 2011 Priority List of Hazardous Substances at number 16 and 33, respectively, out of 275 substances (ATSDR 2011). Vinyl chlorinde (VC), a monochlorinated ethene synthesized as a precursor for polyvinyl chloride plastic and a product of biological transformation of polychlorinated ethenes (reviewed in section 2.1 and 2.2), is the first organic pollutant of the ATSDR list, ranked at number 4, after arsenic, lead, and mercury (ATSDR 2011).

Between 2000 and 2009, the EPA allocated over $200 million/year for remediation, which includes remediation of chloroethenes, and estimated that remediation for fiscal years 2010 to 2014 would be from $335 to $681 million each year (United States Government Accountability Office 2010). Towards these remediation efforts, a multitude of physical, chemical and biological treatments have been employed for cleanup of chlorinated solvents. The EPA website lists the following: pump-and-treat systems, bioremediation, electrokinetics, flushing technologies (cosolvent/alcohol flooding, surfactant flushing, *in situ* oxidation), monitored natural attenuation, phytoremediation, thermal processes (steam injection, electrical heating, *in situ* vitrification), volatilization technologies (soil vapor extraction, air sparging, in-well stripping), and treatment walls (US EPA 2013c).

Among the biological methods, bioremediation using *Dehalococcoides via* reductive dechlorination is an accepted, environmentally benign, and cost effective approach for decontaminating water and soil polluted with chlorinated ethenes (Ellis et al. 2000; Hendrickson et al. 2002; Lendvay et al. 2003; Major et al. 2002). *Dehalococcoides* can utilize PCE and TCE, and the lesser chlorinated ethenes, dichloroethene (DCE) and VC, as electron acceptors for energy metabolisms, generating ethene, as the non-toxic end product (He et al. 2003b; Maymo-Gatell et al. 1997; Sung et al. 2006b). At contaminated sites, bioremediation using *Dehalococcoides*-containing cultures occurs through biostimulation or bioaugmentation. Biostimulation entails establishing the appropriate conditions (pH, redox potential) and providing nutrients for growth (electron donor, carbon source, and vitamins) to stimulate growth of the endogenous *Dehalococcoides* microbial populations. Bioaugmentation involves the same

steps as for biostimulation, plus the addition of a microbial consortium capable to dechlorination of PCE and TCE to ethene. To this day, all effective bioaugmentation consortia for chlorinated ethenes contain the bacterial genus *Dehalococcoides*.

1.3 Dissertation framework

Because of *Dehalococcoides*' unique ability to transform chlorinated ethenes to ethene, hundreds of sites have been restored *via* biostimulation or bioaugmentation (Lyon and Vogel 2012). Moreover, bioaugmentation with *Dehalococcoides*-containing cultures for remediation of chlorinated solvents is now the emblematic example of bioaugmentation due to some clearly documented successes (Ellis et al. 2000; Lendvay et al. 2003; Lyon and Vogel 2012; Major et al. 2002). Nevertheless, *in situ* bioremediation using *Dehalococcoides*-containing cultures is many times hindered by factors leading to undesired or unpredictable outcomes. Some of these include difficulty of aquifer preconditioning, pH management, choice of electron donor as H_2 precursor, *in situ* transport and distribution of microbial cultures and nutrients, composition and efficacy of bioaugmentation cultures, declines due to not well understood microbial interactions, and the very long time often needed to achieve targeted site cleanup (Stroo et al. 2012).

The overarching goal of my dissertation research is to address some of the challenges associated with bioremediation timeframes by improving growth of *Dehalococcoides* in mixed communitied and the rates of reductive dechlorination. My four main dissertation objectives were to 1) propose and validate a laboratory enrichment method that consistently results in fast rates of TCE dechlorination and mixed cultures with high-densities of *Dehalococcoides*, 2) evaluate the role of bicarbonate as a pH buffer

and electron sink in the microbial dechlorination of chloroethenes, 3) develop and optimize a continuous bioreactor for fast cultivation of *Dehalococcoides* in mixed cultures, and 4) assess the effect of high ammonia concentrations on TCE dechlorination and the microbial communities directly and indirectly involved in the dechlorination process.

To accomplish these objectives, I applied a combination of fundamental principles of microbiology and engineering. The laboratory research experiments discussed herein complement important needs in the field of bioremediation and can be utilized to draft strategies to advance bioremediation of chlorinated ethenes. Moreover, my research expands our fundamental understanding of the physiology, kinetics, and ecology of *Dehalococcoides*-based mixed cultures. The structure of the dissertation is as follows:

Chapter 2. I first provide a background on chlorinated ethenes, reductive dechlorination, *Dehalococcoides*, and mixed communities. In addition to the relevant literature review, in Chapter 2 I also summarize a line of research (where I was directly involved) that played a significant role towards the motivation and the research approaches in Chapters 3-6. This is the characterization of DehaloR^2, the principal dechlorinating culture containing *Dehalococcoides* enriched in the Krajmalnik-Brown Laboratory with sediment samples retrieved and provided by Dr. Rolf Halden. The work on DehaloR^2 was published in *Applied Microbiology and Biotechnology* (Ziv-El et al. 2011; Ziv-El et al. 2012a).

Chapter 3. Biostimulation with electron donor sometimes fails to promote the growth of *Dehalococcoides* and significant reductive dechlorination beyond *cis*-DCE in microcosm experiments or at contaminated sites. I hypothesized that, often times, the discrepancy between the presence and the activity of *Dehalococcoides* is not due to their metabolic potential, but to the inherent intricacies driven by the variety of alternate electron acceptors in soils or sediments. In this chapter, I investigated selective enrichment and culturing techniques to abridge microbial diversity in order to yield ethene, the desired end-product of reductive dechlorination of chloroethene, and robust growth of chlorinated ethene-respiring *Dehalococcoides* in mixed cultures. The research in this chapter also describes the enrichment and characterization of three additional dechlorinating cultures, ZARA-10, LINA-09, and ISLA-09. This chapter will be submitted for publication.

Chapter 4 Buffering to achieve pH control is crucial for successful TCE anaerobic bioremediation. Bicarbonate (HCO_3^-) is the natural buffer in groundwater and the buffer of choice in the laboratory and at contaminated sites undergoing biological treatment with organohalide respiring microorganisms. However, HCO_3^- also serves as the electron acceptor for hydrogenotrophic methanogens and hydrogenotrophic homoacetogens, two microbial groups competing with *Dehalococcoides* for H_2. I studied the effect of HCO_3^- as a buffering agent and the effect of HCO_3^--consuming reactions in a range of concentrations (2.5-30 mM). My hypothesis was that the presence of excess HCO_3^- would channel electrons towards methanogenesis and homoacetogenesis, and, hence, this

competition for H_2 would decrease the rates of reductive dechlorination. This chapter was published in an altered format in *Microbial Cell Factories* (Delgado et al. 2012).

Chapter 5. With the knowledge acquired on enriching, growing, and managing *Dehalococcoides*-containing cultures, I next address the need for continuous production of dense cultures in larger volumes in the laboratory. In this chapter, I report on the successful growth of a representative *Dehalococcoides*-containing culture in a continuous-flow stirred tank reactor (CSTR) at a 3-d hydraulic retention time using feed concentrations of 1 and 2 mM TCE, respectively. Typically, *Dehalococcoides* cultures are grown in batch-fed reactors. Batch systems can be cumbersome, as self or competitive inhibition on dechlorination, and toxicity on *Dehalococcoides* and other community members prevents feeding TCE or PCE in high concentrations. Therefore, batch cultivation of *Dehalococcoides* entails receiving (and reducing mostly to ethene) several non-inhibitory, successive feeds of electron acceptors. A CSTR theoretically could overcome these limitations. However, based on the findings from Chapter 3 and 4, I hypothesized that, for successful cultivation of *Dehalococcoides*-containing cultures, I must minimize the excessive proliferation of microorganisms competing with *Dehalococcoides* for H_2. This chapter will be submitted for publication.

Chapter 6. A large majority of soil and groundwater environments containing PCE or TCE are also impacted by other co-contaminants, e.g., other organic chlorinated solvents, petroleum hydrocarbons, heavy metals, inorganic, and organic nitrogen. Under appropriate conditions, the latter represents a potential nutrient source. Prime examples

of environments where chloroethenes and nitrogen-containing compounds such as ammonium/ammonia are found as co-contaminants are landfills. A large number of cases of groundwater contamination with landfill leachate have been documented. Therefore, in this chapter I assessed the impact of total ammonia nitrogen on mixed microbial communities driving the reductive dechlorination of TCE and the possibility of using leachate as electron donor for dechlorination or ammonia-nitrogen as specific inhibitor of the microbial community members. This chapter will be submitted for publication.

Chapter 7. Here, I present key findings and some concluding remarks from the research studies described in Chapters 3-6. I also make recommendations for studies that would either be a natural progression or alongside the research from this dissertation. These include constructing and deconstructing reductively dechlorinating communities, finding a "natural" electron acceptor for *Dehalococcoides*, and modeling reductive dechlorination in a CSTR.

CHAPTER 2

BACKGROUND

2.1 Chlorinated ethenes

PCE and TCE have accumulated in the environment as a consequence of their broad commercial and industrial usages and historically careless disposal. In fact, before 1972, encouraged by the preconception that "dilution is the solution to pollution", these solvents were commonly disposed in the ground or down the drain (Loffler et al. 2012). Because of their low solubility and higher density than water, PCE and TCE migrated in the subsurface where they often persist as dense nonaqueous phase liquids (DNAPLs). Table 2.1 summarizes relevant properties and facts for chlorinated ethenes, which range from a tetra-chlorined ethene (PCE) to a monochlorinated ethene (VC), and ethene, the non-chlorinated backbone compound. The dichloroethene (DCE) isomers (*cis*-, *trans*-, and 1,1-) and VC also have industrial application or serve as intermediates in chemical processes (Table 2.1). Contamination with DCEs and VC from industry manufacturing has been reported (Bradley 2003; Office of Response and Restoration 2012). However, unlike PCE and TCE, the large majority of contamination with DCEs and VC has arisen from biological and abiotic transformation of the higher chlorinated ethenes and trichloroethane (TCA), another priority pollutant and industrial organic solvent.

Table 2.1 Chlorinated ethenes factsheet

Compound	Molecular weight	Aqueous solubility (mM)	Main industrial usage	ATDSR 2011 rank	Health effects	EPA maximum contaminant level (μg L^{-1})
PCE	165.83	1.2	Dry cleaning agent, metal degreaser, solvent, chemical intermediate	33	Probable human carcinogen	5
TCE	131.39	8.4	Metal degreaser and cleaning agent, chemical intermediate	16	Human carcinogen	5
cis-DCE	96.94	36.1	Waxes and resins solvent, rubber extraction agent, refrigerant, chemical intermediate	213	Probable human carcinogen	70
trans-DCE	96.94	64.9	Waxes and resins solvent, rubber extraction agent, refrigerant, chemical intermediate	177	Probable human carcinogen	100
1,1-DCE	96.94	25.8	Production agent for adhesives and synthetic fibers, refrigerant, food packaging and coating resins agent	81	Probable human carcinogen	7
VC	62.49	43.2	Chemical intermediate	4	Human carcinogen	2
Ethene	28.05	4.7	Fruit ripening agent, chemical intermediate	-	-	None

2.2 Microbiology at work: reductive dechlorination by organohalide respiring bacteria

All chlorinated ethenes can be transformed by microbes under anaerobic conditions through reductive dechlorination, which is the core process of my research. Reductive dechlorination has also been termed halorespiration, chlororespiration, dechlororespiration, and organohalide respiration. Throughout this dissertation, I use reductive dechlorination and organohalide respiration. A schematic of reductive dechlorination of PCE to ethene is presented in Figure 2.1. Reductive dechlorination entails removing a chlorine atom and replacing it with a hydrogen atom in a stepwise fashion, as depicted in Figure 2.1. Two electrons are required to drive the replacement of each chlorine atom; therefore, dechlorination of PCE to ethene is an eight electron process. Additionally, one proton (H^+) and one Cl^- are released at each dechlorination step.

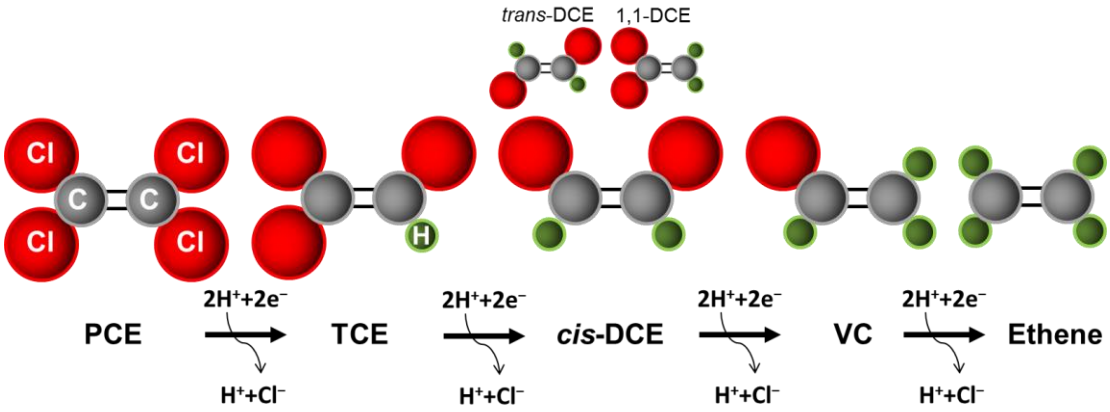


Figure 2.1 Schematic of reductive dechlorination. Sequential reductive dechlorination of PCE, TCE, DCE, and VC to ethene and the corresponding oxidation of H_2 at each step in the pathway. The most common DCE congener from reductive dechlorination is *cis*-DCE. The smaller size of *trans*- and 1,1-DCE in the schematic is meant to denote the lower abundance of these products.

Biological reductive dechlorination of PCE to VC was first described by Bouwer and McCarty (Bouwer and McCarty 1983), and the revelation that VC, the most toxic intermediate from reductive dechlorination, can be detoxified to ethene under methanogenic conditions followed several years later (Distefano et al. 1991; Freedman and Gossett 1989). These works were pivotal to establishing a line of research on microorganisms with the potential to transform chlorinated solvents to an environmentally harmless product, thus becoming important for biotechnological applications. Bacteria capable of organohalide respiration using halogenated compounds have been reviewed in several publications (Hug et al. 2013; Loffler and Edwards 2006; Loffler et al. 2005; Smidt and de Vos 2004; Tas et al. 2010). The phylogenetic relationship between the multiple groups of organohalide respirers is shown in Figure 2.2.

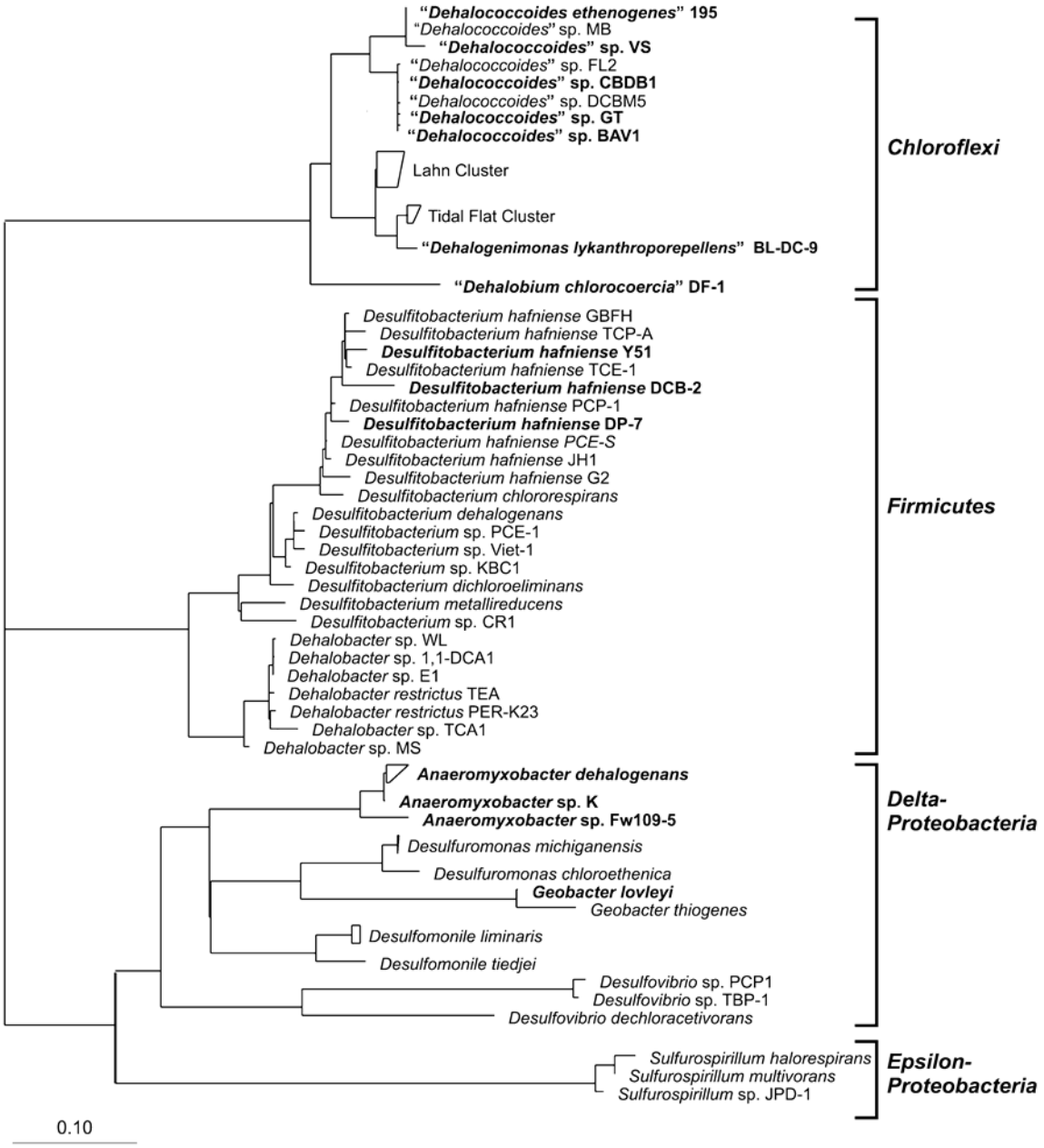


Figure 2.2 Phylogenetic tree of organohalide respiring bacteria based sequences of 16S rRNA gene. The bacterial names and taxa in bold letters indicate already completed or in progress genome sequencing. This figure is from Tas et al. (2010).

2.3 *Dehalococcoides,* the prominent bioremediator

Chlorinated ethenes dechlorinators are found within the Gram-negative and Gram-positive groups and belong to diverse taxa: *Chloroflexi* (class *Dehalococcoidetes*), *Firmicutes* (class *Clostridia*) and *Proteobacteria* (class *δ*- and *ε*-*Proteobacteria*) (Figure 2.2). Multiple genera are capable of partial reduction of PCE and TCE to *cis*-DCE, e.g. *Geobacter* (Sung et al. 2006a), *Desulfuromonas* (Loffler et al. 2000), *Desulfitobacterium* (Gerritse et al. 1996), *Sulfurospirillum* (Luijten et al. 2003), *Dehalogenimonas* (Manchester et al. 2012) , and *Dehalobacter* (Holliger et al. 1998). *Dehalococcoides* is the only genus capable of complete reduction of PCE to ethene (Maymo-Gatell et al. 1997) and the focal point of my studies. *Dehalobacter, Dehalogenimonas*, and *Dehalococcoides* are strictly organohalide respiring bacteria; however, most other genera perform alternate metabolic reactions using non chlorinated electron acceptors, including sulfur reduction (*Desulfuromonas* (Loffler et al. 2000) and *Geobacter* (Sung et al. 2006a)), fermentation (*Desulfitobacterium* (Villemur et al. 2006) and *Sulfurospirillum* (Luijten et al. 2003)), metal reduction (*Geobacter* (Sung et al. 2006a) and *Desulfitobacterium* (Villemur et al. 2006)), and denitrification (*Sulfurospirillum* (Luijten et al. 2003)).

In the survey of contaminated groundwater from multiple North American and European sites by Hendrickson et al., *Dehalococcoides* were found in all site samples where ethene formation was observed from the reductive dechlorination of PCE or TCE (Hendrickson et al. 2002). To date, ethene formation from this process has not been proven to occur in any other bacterium; hence, a paradigm exists exclusively linking

ethene production to *Dehalococcoides*. The isolation and characterization a microorganism responsible of PCE detoxification to ethene, *D. mccartyi* strain 195, in 1997 was received with great interest and enthusiasm by the research community (MaymoGatell et al. 1997) and have since put *Dehalococcoides* at the heart of bioremediation of chlorinated solvents.

For bioremediation of chlorinated ethenes using *Dehalococcoides*, the main processes implemented in the field are biostimulation and bioaugmentation (Lyon and Vogel 2012). A simple schematic of the combined processes is provided in Figure 2.3. Biostimulation entails establishing the appropriate conditions (pH, redox potential) and providing nutrients for growth (electron donor, carbon source, and vitamins) to stimulate the endogenous microbial populations at a contaminated site. Bioaugmentation usually involves the same steps as for biostimulation, plus the addition of a microbial consortium containing *Dehalococcoides* capable to dechlorination of PCE and TCE to ethene.

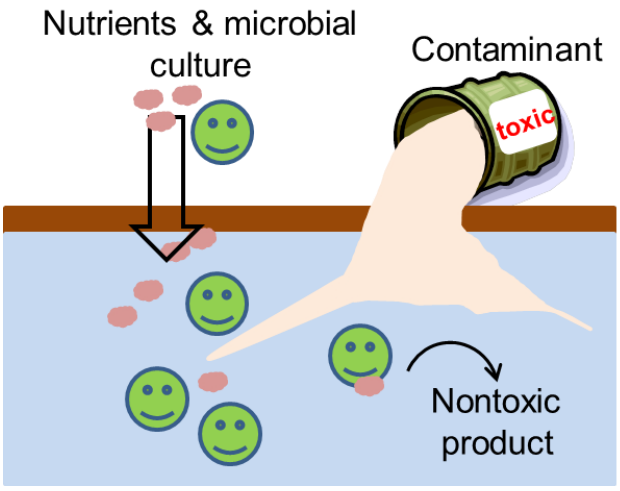


Figure 2.3 Schematic of bioremediation via biostimulation and bioaugmentation.

2.3.1 Dehalococcoides, an overview

Phylogenetically, *D. mccartyi* species are part of the phylum *Chloroflexi* (green non-sulfur bacteria), class *Dehalococcoidetes*, order *Dehalococcoidales*, family *Dehalococcoidaceae*, in the genus *Dehalococcoides* (Loffler et al. 2013). They are mesophilic, neutrophilic, and strictly anaerobic microorganisms. Exposure to as little as 4 mg L^{-1} O_2 results in cell death (Amos et al. 2008). *Dehalococcoides* are small in size (one of the smallest bacteria characterized) with a diameter of 0.4-1 µm and a cell thickness of 0.1 µm (He et al. 2005; MaymoGatell et al. 1997). They have an irregular coccus-like morphology (hence, the name coccoid) that resembles a doughnut or a red blood cell (MaymoGatell et al. 1997).

Isolation of these bacteria has been reported to be difficult, often requiring years to obtain isolates (He et al. 2005; Loffler et al. 2005; Loffler et al. 2012; Loffler et al. 2013; Magnuson et al. 2000). Loffler et al. (2005) detailed the steps involved in the enrichment, cultivation, and isolation of *Dehalococcoides.* Some of the inherent difficulties in the isolation of these bacteria come from the fact that they need strictly anaerobic conditions, are not culturable on agar plates, require multiple dilution-to-extinction procedures to ensure purity, exhibit low biomass and turbidity cannot be measured using optical density, and cannot be viewed effectively using light microscopy (Loffler et al. 2012; Loffler et al. 2005). Despite of these challenges, several characterized isolates exist and are compiled in Table 2.2.

Table 2.2 Dehalogenation capabilities of isolated *D. mccartyi* strains

***D. mccartyi* strain**	**Electron acceptors**[a,b]	**Reference**
195	PCE, TCE, *cis*-DCE, 1,1-DCE, *trans*-DCE, VC, 1,2-dibromoethane	(MaymoGatell et al. 1997)
	HCB	(Fennell et al. 2004)
	2,3-DCP, 2,3,4-TCP	(Adrian et al. 2007)
	1,2-DCA	(Maymo-Gatell et al. 1999)
BAV1	*cis*-DCE, *trans*-DCE, 1,1-DCE, VC, vinyl bromide, 1.2-DCA	(He et al. 2003a)
CBDB1	HCB	(Adrian et al. 2000)
	PCE, TCE	(Adrian et al. 2007)
	2,3-DCP; 2,3,4-TCP, , 2,3,5-TCP, 2,3,6-TCP, 3,4,5-TCP, 2,3,4,6-TeCP, pentachlorophenol	(Adrian et al. 2007)
	polychlorinated dioxins	(Bunge et al. 2003)
	polychlorinated biphenyls	(Adrian et al. 2009)
VS	TCE, *cis*-DCE, 1,1-DCE, VC	(Cupples et al. 2003; Muller et al. 2004)
FL2	TCE, *cis*-1,2-DCE, *trans*-1,2-DCE, PCE, VC	(He et al. 2005)
GT	TCE, *cis*-DCE, 1,1-DCE, VC	(Sung et al. 2006b)
DCMB5	1,2,4-trichlorodibenzo-*p*-dioxin 1,2,3-TCB	(Bunge et al. 2003)
MB	PCE, TCE, octa-BDEs	(Cheng and He 2009)
ANAS1	TCE, *cis*-DCE, 1,1-DCE	(Lee et al. 2011)
ANAS2	TCE, *cis*-DCE, 1,1-DCE, VC	(Lee et al. 2011)

[a]The list of electron acceptors for each strain includes those metabolized and cometabolized.
[b]Abbreviations: DCA, dichloroethane; HCB, hexachlorobenzene; TCB, trichlorobenzene; DCB, dichlorobenzene; TeCP, tetrachlorophenol; TCP, trichlorophenol; DCP, dichlorophenol; MCP; monochlorophenol; BDE, bromodiphenyl ether.

2.3.2 Metabolism and nutritional requirements

A central effort in my dissertation work is the targeted improvement of reductive dechlorination rates and growth of *Dehalococcoides*. In all of my research chapters, I address this effort through multiple avenues, including optimization of nutrients, nutrient concentrations, and other components in the growth medium. Therefore, I next present an overview of *Dehalococcoides* metabolism and nutritional requirements.

Electron acceptors. As seen in Table 2.2, in the laboratory, growth of *Dehalococcoides* isolates has only been proven *via* organohalide respiration using an array of halogenated and polyhalogenated electron acceptors, mainly chlorinated or brominated, with varying carbon backbones, including ethenes (Cheng and He 2009; He et al. 2005; He et al. 2003a; Lee et al. 2011; Maymo-Gatell et al. 1997; Sung et al. 2006b), ethanes (Maymo-Gatell et al. 1999), dioxins (Bunge et al. 2003), biphenyls (Adrian et al. 2009), benzenes (Adrian et al. 2000; Fennell et al. 2004), and phenols (Adrian et al. 2007). Based on the current knowledge, some of the strains (e.g., strain 195 and CBDB1) seem to be more metabolically diverse, while others (e.g., strain GT, ANAS1, and ANAS2) are limited (based on the current library of tested acceptors) to only one type of halogenated compound (Table 2.2). None of the isolated *Dehalococcoides* strains tested was able to ferment or respire the following non-halogenated electron acceptors: oxygen, sulfate, sulphite, thiosulphate, sulphur, fumarate, nitrate, ferric iron, or 3-chloro-4-hydroxy benzoate (Adrian et al. 2000; He et al. 2005; He et al. 2003b; Lee et al. 2011; Maymo-Gatell et al. 1997). The process of organohalide respiration, through which *Dehalococcoides* derive energy, is mediated by a class of enzymes called reductive dehalogenases (RDases). I include a discussion on these enzymes in section 2.4.

Electron donor and carbon source. Whereas diverse in terms of the halogenated electron acceptors they can utilize, thus far *Dehalococcoides* are restricted to H_2 as their obligate electron donor. All *D. mccartyi* strains grow by organohalide respiration when H_2 was provided as a gas, and none were able to derive reducing equivalents from

formate, acetate, lactate, pyruvate, glycerol, fumarate, citrate, glucose, methanol, ethanol, or yeast extract (He et al. 2005; He et al. 2003b; Maymo-Gatell et al. 1997). *Dehalococcoides* contain multiple hydrogenase complexes, membrane-bound (Hup, Hyc, Ech, Hym) as well as cytoplasmic (Vhu), to oxidize H_2 to protons and electrons (Schipp et al. 2013). Insights from the sequenced genomes of strain 195 (Seshadri et al. 2005), VS (McMurdie et al. 2009), BAV1 (McMurdie et al. 2009), GT (Stroo ch2), and CBDB1 (Kube et al. 2005) reveal five separate hydrogenase gene clusters conserved between the strains in term of nucleotide sequence and organization. In pure cultures of *D. mccartyi* and in mixed cultures grown on PCE, the Hup hydrogenase was highly expressed at the transcriptional level (Morris et al. 2006). On the other hand, Ech and Hyc had lower expression levels and were proposed to generate low-potential electrons for biosynthesis rather than for respiration (Morris et al. 2006). The hydrogenase functional redundancy was hypothesized to have evolved as a consequence of varying fluxes of hydrogen in the environment (Loffler et al. 2012).

Unlike for other non-*Dehalococcoidetes* classes of organohalide respirers, energy generation and cellular synthesis (carbon metabolism) are not linked in *Dehalococcoides*. All strains utilize acetate for anabolism (Loffler et al. 2013; Maymo-Gatell et al. 1997); yet, the extent of *Dehalococcoides* carbon sources has not been fully investigated. Several substrates tested that did not support growth include fumarate, malate, lactate, pyruvate, glucose, succinate, propionate, and glutamate (Cheng and He 2009; Lee et al. 2011). It was previously suggested from genomic data that *Dehalococcoides* might also utilize CO_2 as a carbon source (Islam et al. 2010). Recent publications state acetate/CO_2

as carbon sources for *Dehalococcoides* (Lee et al. 2011; Schipp et al. 2013). However, further experimental data are needed to confirm the need for this substrate as multiple research groups, including the Laboratory of Dr. Krajmalnik-Brown, have been able to successfully cultivate pure cultures of *D. mccartyi* using acetate only (as the carbon source) in medium without CO_2/HCO_3^-.

Other required nutrients. All *Dehalococcoides* utilize ammonia through glutamate and glutamine, which donate nitrogen for synthesis of cellular components (He et al. 2007). Commonly, the concentration of ammonia (added as NH_4Cl) in the medium to derive growth of *Dehalococc*oides is 6 mM (Loffler et al. 2005). The effect of ammonia at concentrations above those required for growth was investigated for the first time in this dissertation (Chapter 6). Interestingly, *D. mccartyi* strain 195, MB, ANAS1, and ANAS2 possess a nitrogenase-encoding operon (*nif*) for fixing atmospheric N_2 to ammonia (Lee et al. 2012; Lee et al. 2009). Strain 195 is the only that has been grown as a diazatroph; however, the N_2-fixing strain grows poorly and dechlorinates TCE at slower rates compared to the 195 strain cultured with ammonia as the source of nitrogen (Lee et al. 2009; Maymo-Gatell et al. 1999).

Vitamins are essential nutrients for *Dehalococcoides*. These are typically provided at a final concentration per liter: biotin, 0.02 mg; folic acid, 0.02 mg; pyridoxine hydrochloride, 0.1 mg; riboflavin, 0.05 mg; thiamine, 0.05 mg; nicotinic acid, 0.05 mg; pantothenic acid, 0.05 mg; vitamin B12, 0.05 mg; *p*-aminobenzoic acid, 0.05 mg; thioctic acid, 0.05 mg Vitamin B_{12} (cyanocobalamin) is of utmost importance as it is a cofactor for the reductases carrying out the reductive dechlorination. Even though required for

core metabolic reactions, *Dehalococcoides* do not possess genes for *de novo* synthesis of vitamin B_{12}. They do, however, encode genes in their genome for acquisition and transport (Yan et al. 2012).

2.4 Reductive dehalogenase: core enzymes for organohalide respiration

A comprehensive overview of reductive dehalogenase (RDase) enzymes from *Dehalococcoides* and from other organohalide respirers and a proposal for a classification systems was published by Hug et al. (Hug et al. 2013). RDases are oxygen-sensitive proteins located in association with the cytoplasmic membrane. They are monomeric and contain prosthetic corrinoid cofactors and two Fe_4S_4 clusters (Magnuson et al. 2000; Magnuson et al. 1998). The large subunit, A, of the enzyme is the reactive center and contains a Tat signal sequence. The presence of the Tat sequence suggests that this protein is exported across the cytoplasmic membrane (Wickner and Schekman 2005). The smaller subunit, B, is proposed to function as the anchor for subunit A into the outside of the cytoplasmic membrane (Krajmalnik-Brown et al. 2004; Muller et al. 2004).

Protein purification and the subsequent characterization of RDases from *Dehalococcoides* have been limited by the low biomass yields obtained from these microbes. Therefore, the large majority of our knowledge of *Dehalococcoides* RDases stems from genomic data. Currently, there are several hundred RDase gene sequences in *Dehalococcoides* (NCBI). Each sequenced strain contains a multitude of putative RDase genes, ranging from 17 to 36 genes (Loffler et al. 2012; Loffler et al. 2013). This functional surplus could be an indication of unrevealed metabolic capabilities and an

adaptation to metabolizing electron acceptors beyond those shown in the laboratory. Out of the large number of putative RDase genes, only four have been assigned a function with respect to PCE to ethene dechlorination: *pceA*, *tceA*, *vcrA*, and *bvcA*. The gene products catalyze the following reactions:

PceA: PCE → TCE (Fung et al. 2007; Magnuson et al. 1998)

TceA: TCE → VC (Magnuson et al. 2000)

VcrA: DCEs, VC → ethene (Muller et al. 2004)

BvcA: VC → ethene (Krajmalnik-Brown et al. 2004)

The genes encoding these reductases are also important genetic markers for *Dehalococcoides*. In the laboratory or during bioremediation scenarios, quantitative real-time PCR tracking the RDase genes, as well as the 16S rRNA gene of *Dehalococcoides*, make it possible to correlate *Dehalococcoides* presence to chemical measurements (Ritalahti et al. 2006).

2.5 Importance of mixed cultures containing *Dehalococcoides*

The original bioremediation applications in environments contaminated with the lesser chlorinated ethenes relied on aerobic cometabolism by methane, toluene, phenol, and ammonia oxidizers (Ely et al. 1997; Pant and Pant 2010; Rasche et al. 1991). In practice, this strategy was challenging from multiple aspects, including the fact that bacteria cometabolizing chlorinated ethenes were not growing on these substrates, were producing toxic intermediates, were limited by the availability of oxygen, and could not degrade the fully chlorinated ethene, PCE (Pant and Pant 2010; Rasche et al. 1991;

Steffan and Vainberg 2012). In the early 2000s, bioremediation took a new turn of events with Ellis et al. (2000) and Major et al. (2002) documenting successful bioaugmentations of contaminated sites with *Dehalococcoides* mixed cultures that grow on PCE and TCE. Hence, various sediment-free, chlorinated ethene-respiring communities have been developed and characterized for application in bioaugmentation and for fundamental laboratory studies (Duhamel and Edwards 2006; Macbeth et al. 2004; Richardson et al. 2002; Schaefer et al. 2009).

Table 2.3 contains a comprehensive list of dechlorinating cultures, their origin, and enrichment conditions. As shown in Table 2.3, the overwhelming majority of the microbial inocula for these chloroethenes bioaugmentation cultures was obtained from environments with contaminated soil, sediment, or groundwater. Evidently, these contaminated environments provide a unique niche for growth of organohalide-respirers, as they contain chlorinated electron acceptors in abundance. Thus far, studies on bioremediation of chlorinated ethenes have established a strong correlation between robust or improved growth of *Dehalococcoides* and their rates of reductive dechlorination in communities, compared to strains in isolation. For this reason, as seen in Table 2.3, *Dehalococcoides* bioaugmentation cultures are cultivated using fermentable substrates. The choice of fermentable for the enrichment and cultivation of these consortia differ between research labs, resulting in diverse communities, with lactate and methanol as the most commonly used fermentable compounds.

Table 2.3 Inocula sources and enrichment conditions of chlorinated ethenes-dechlorinating cultures

Culture	Inoculum source	Contamination and/or anthropogenic activity	Enrichment		
			Chlorinated e^- acceptor	e^- donor and carbon source	Reference
Unnamed[1]	Sludge, Ithaca wastewater treatment plant, NY	Wastewater	PCE	Methanol and acetate	(Distefano et al. 1991)
Pinellas	Soil and groundwater, Department of Energy's Pinellas site, Largo, FL	Chlorinated solvents (mostly TCE)	TCE	Lactate	(Harkness et al. 1999)
ANAS	Soil, Alameda Naval Air Station, CA	Chlorinated solvents (mostly TCE) and waste oil	TCE	Lactate	(Richardson et al. 2002)
KB1®	Soil and groundwater, Southern Ontario contaminated site, Canada	TCE	TCE	Methanol	(Duhamel et al. 2002)
Unnamed[2]	Aquifer material, Bachman Road Residential Wells site, Oscoda, MI	PCE	*cis*-DCE	Lactate	(Lendvay et al. 2003)
Victoria[3]	Aquifer material, Victoria contaminated site, TX	PCE	PCE	Benzoate	(Cupples et al. 2003)
Unnamed[4]	Sediment, Red Cedar River, MI	No contamination	PCE	H_2 and acetate	(He et al. 2005)
PM	Aquifer material, Point Mugu Naval Air Weapons Station, CA	TCE	TCE	Butanol	(Yu et al. 2005a)
EV	Groundwater, Evanite site, Corvallis, OR	TCE	PCE	Butanol	(Yu et al. 2005a)

Enrichment originating *D. mccartyi* strains [1]195,[2] BAV1, [3]VS, and [4]FL2

Table 2.3 (Cont.) Inocula sources and enrichment conditions of chlorinated ethenes-dechlorinating cultures

Culture	Inoculum source	Contamination and/or anthropogenic activity	Enrichment		
			Chlorinated e^- acceptor	e^- donor and carbon source	Reference
SDC-9™	Aquifer material, contaminated site, Southern CA	Chlorinated solvents	PCE	Lactate	(Schaefer et al. 2009)
Hawaii-05™	Aquifer material, Hickam Air Force Base, HI	Chlorinated solvents	TCE	Lactate	(Vainberg et al. 2009)
PKJS™	Aquifer material, Air Force Plant PJKS, CO	TCE	TCE	Lactate	(Vainberg et al. 2009)
DehaloR^2	Estuarine sediment, Chesapeake Bay, MD	Wastewater effluent	TCE	Lactate and methanol	(Ziv-El et al. 2011)
ZARA-10	Garden soil, Cuzdrioara, Romania	No contamination	TCE	Lactate and methanol	Chapter 3
LINA-09	Mangrove sediment, Carolina, Puerto Rico	No contamination	TCE	Lactate and methanol	Chapter 3
ISLA-08	Groundwater sediment, Parris Island Marine Corps Recruit Depot, SC	PCE	TCE	Lactate and methanol	Chapter 3

.

The differences in growth substrates or other growth-medium components and the origin of microbial inocula have yielded both similar and distinguishable features in the microbial composition of these cultures. The common community members in most chloroethene-dechlorinating cultures in Table 2.3 are *Dehalococcoides* (usually more than one strain), other organohalide respirers performing reduction of PCE/TCE to *cis*-DCE (e.g., *Geobacter*, *Dehalobacter*, and *Desulfuromonas*), fermenting Bacteria, and methanogenic Archaea. Fermentation of complex substrates provides *Dehalococcoides* with growth macronutrients (i.e., H_2, their electron donor and acetate, their carbon source) and with micronutrients (i.e., specific amino acids (Zhuang et al. 2011), and vitamin B_{12} (co-factor cobalamin required for their reductive dehalogenase enzymes (He et al. 2007)). However, fermentation products also sustain the growth of other microbial groups that directly compete with *Dehalococcoides* and other community members for some of the very same resources.

For example, historically, methanogens have been considered a sink of H_2 in bioaugmentation cultures or in communities biostimulated at contaminated sites. The H_2 fed to dechlorinators, either directly or indirectly (through fermentation reactions), can be spent by hydrogenotrophic methanogens, especially in HCO_3^- abundant conditions as they reduce HCO_3^-/CO_2 to produce methane. In terms of the energetics of H_2-consuming reactions, hydrogenotrophic methanogens gain less energy than dechlorinators (Loffler et al. 1999). Similarly, the affinity for H_2, dictated by the half-saturation concentration (K_s), is lower for hydrogenotrophic methanogens compared to dechlorinators (Cordruwisch et al. 1988; Kotsyurbenko et al. 2001).

Many methanogenic microorganisms produce corrinoids (Mazumder et al. 1987; Silveira et al. 1991; Stupperich and Krautler 1988; Stupperich et al. 1987; Yan et al. 2013), including variants of vitamin B_{12} (Factor III and pseudo vitamin B_{12}). Recent coculture experiments revealed that the corrinoids synthesized by *Methanosarcina barkerii* strain Fusaro containg the lower α-ligand, 5'-hydroxybenzimidazole, failed to support growth and reductive dechlorination by *D. mccartyi* strain BAV1 (Yan 2013). However, when the α-ligand, 5',6'-dimethylbenzimidazole, was added to these cocultures, growth of *D. mccartyi* was enhanced when compared to axenic cultures (Yan et al. 2013). In fact, similar observations were documented with *Sporomusa* sp. strain KB-1 and *Geobacter sulfurreducens*. These bacteria also do not synthsize the "right" corrinoids, but by processing 5',6'-dimethylbenzimidazole, they can stimulate improved growth of *D. mccartyi* strains (Yan et al. 2013; Yan et al. 2012). Therefore, understanding and managing the dualistic relations (synthrophic versus competitive) in mixed microbial communities dechlorinating PCE or TCE is essential for effective bioremediation using *Dehalococcoides*, and is a recurring theme throughout this disseration.

2.6 DehaloR^2, a model *Dehalococcoides*-containing dechlorinating culture performing rapid dechlorination of TCE to ethene

DehaloR^2 is a sediment-free, anaerobic microbial culture initially developed in 2008 (Yao 2009) and stably maintained since 2009 in the Krajmalnik-Brown Laboratory. The microbial inoculum for DehaloR^2 was core sediments from a brackish tributary of

the Chesapeake Bay near Baltimore, MD, provided by Dr. Rolf Halden. The development and characterization of DehaloR^2 was published by Ziv-El et al. (2011).

2.6.1 *From sediment microcosm to sediment-free culture*

The dechlorination activity in the microcosms established with Chesapeake Bay estuarine sediment is presented in Figure 2.4. TCE was predominantly converted to *trans*-DCE and *cis*-DCE by day 40, after which reductive dechlorination stalled at a *trans*-to-*cis*-DCE mole ratio of 1.67±0.15. After 160 days of incubation, the microcosm stalled at DCE was transferred to fresh medium, and complete dechlorination was attained when sediment was precluded from the culture (Figure 2.4). This was a first and unusual report of achieving complete dechlorination to ethene after transferring an incompletely dechlorinating microcosm. The immediate onset and rapid complete dechlorination to ethene in ~10 days (Figure 2.4) suggested that *Dehalococcoides* capable of dechlorination to ethene were present in the microcosm. However, as hypothesized, they may have been inhibited by sediment constituents, including the antimicrobial agents, triclosan and triclocarban, and triclocarban dechlorination products, which were detected in the sediment from Chesapeake Bay (Miller et al. 2008). The cause for this inhibition and the accumulation of DCE isomers will be investigated with other sediment and soil materials in Chapter 3.

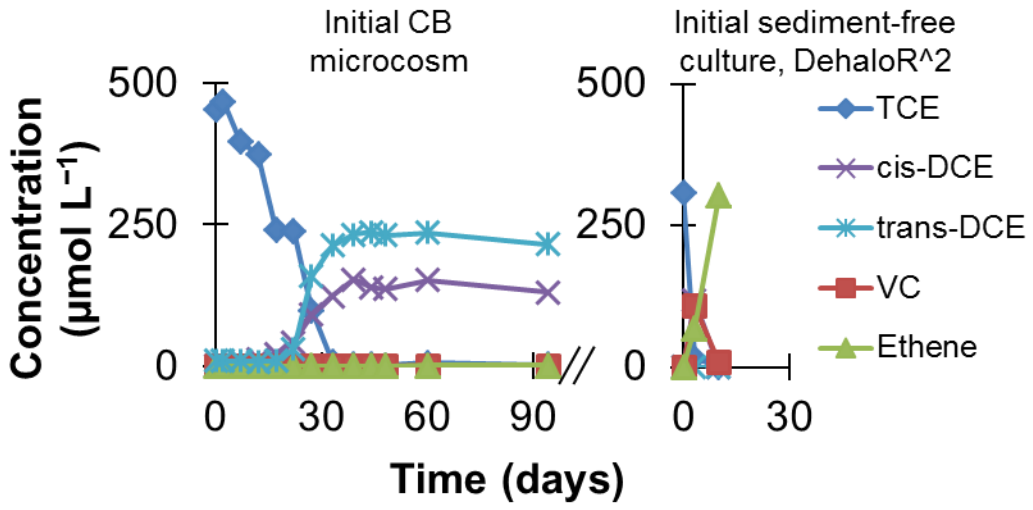


Figure 2.4 Chemical conditions in culture vessels showed a shift from incomplete reductive dechlorination of TCE to DCE (*trans*-to-*cis* isomer ratio of 1.67±0.15) in the initial sediment microcosm to complete and much more rapid dechlorination to ethene with negligible accumulation of *trans*-DCE in the first transfer to a sediment-free culture, designated DehaloR^2. Shown are measurements for a representative microcosm and averages of triplicate cultures of DehaloR^2. This figure was regenerated and modified from Ziv-El et al (Ziv-El et al. 2011).

2.6.2 *Enriched microbial communites in DehaloR^2*

The microbial community enriched in DehaloR^2 was investigated through 454 pyrosequencing, a clone library, and qPCR targeting specific bacterial and archael members. The structure of the microbial communities as determined by pyrosequencing is shown in Figure 2.5. In the sediment microcosm, *Proteobacteria* was the dominant phylum (72% of all sequences), which decreased after enrichment. In the duplicate sediment-free culture samples, *Firmicutes* became the major phylum with 78-86% of the total sequences. The genus *Dehalococcoides* and its corresponding phylum, *Chloroflexi*, were non-detect (zero sequences) in the sediment and increased to 9-16% in DehaloR^2 culture. In Chapter 3, the microbial communities of three additional soil/sediment-free dechlorinating cultures, enriched under the same growth conditions as Dehalor^2, were investigated using 454 pyrosequencing.

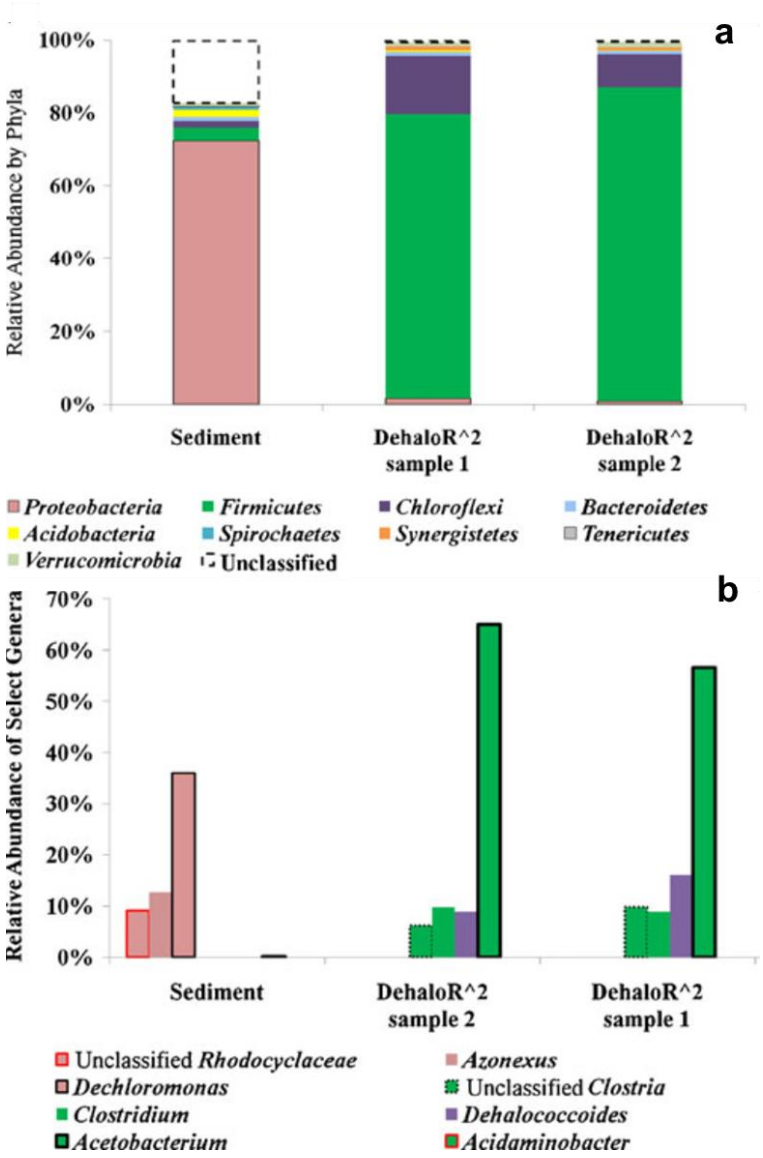


Figure 2.5 Bacterial community diversity by phylum in the microcosm sediment and duplicate DehaloR^2 enrichment cultures. Pyrosequencing targeted the V4 region of the 16S rDNA for the sediment and the combined V2 and V3 regions for DehaloR^2. This figure is from Ziv-El et al. (Ziv-El et al. 2011).

The findings from the constructed clone library were complementary to the pyrosequncing data. Of the 73 sequenced clones, 73% were fermenters, with homoacetogens constituting 48% (31 *Acetobacterium* and 4 *Spirochaetes* clones). *Dehalococcoides* sp. were represented by 19 clones (26.0%) and mutliple strains, some of

which may be novel, according to sequencing data. The actual copy number of the 16S rRNA genes of *Dehalococcoides* as measured by qPCR in the enriched sediment-free culture were $1.54 \pm 0.27 \times 10^{11}$ copies L^{-1}, while those of *Geobacter* were $2.67 \pm 0.38 \times 10^{10}$ copies L^{-1}. The abundaces of *Dehalococcoides* and *Geobacter* in DehaloR^2 compared favorably to reports from other enrichment cultures in the literature.

2.6.3 *Reductive dechlorination – a rate comparison in mixed microbial cultures*

One of the key findings in the DehaloR^2 study was its maximum rates of dechlorination of TCE to ethene. These rates were determined by consecutively feeding the electron acceptor, TCE, and electron donor in batch serum bottles. A time-course experiment used to calculate the dechlorination rates is in Figure 2.6a, when lactate and methanol were the electron donors, and Figure 2.6b, when lactate only was used as electron donor. The maximum rate of TCE reductive dechlorination to ethene was 0.92 ± 0.1 mM Cl^{-} d^{-1} when the concentration of *Dehalococcoides* had also reached a maximum (Table 2.4).

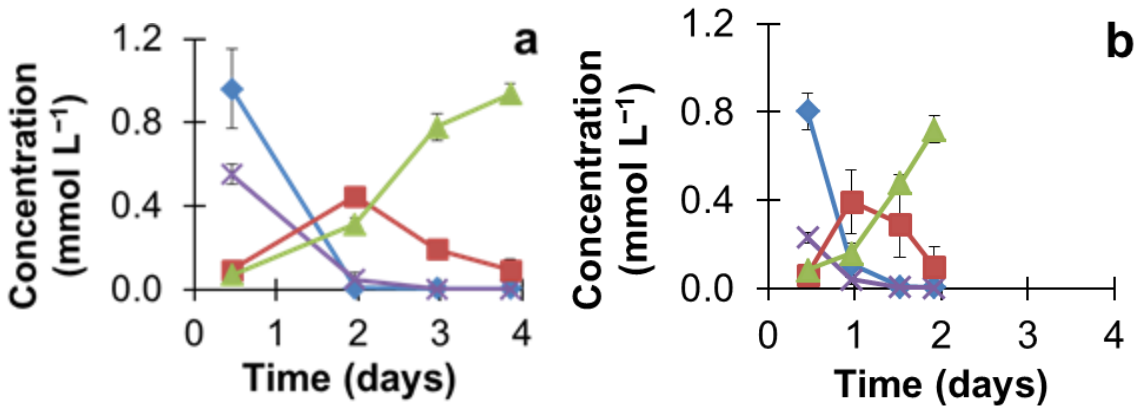


Figure 2.6 Dechlorination of TCE to ethene by DehaloR^2 sediment-free cultures when the electron donors were (a) lactate and methanol upon 3rd consecutive addition, and (b) lactate only upon 5th consecutive addition. The error bars are standard deviation of triplicate cultures.

Table 2.4 Comparison of maximum chlorinated ethene turnover rates $(\Delta C\ \Delta t^{-1})_{max}$ to ethene and the corresponding concentration of *Dehalococcoides* (X_{Dhc}), for select chlorinated ethene mixed microbial communities in batch serum bottles. This table is adapted from Ziv-El et al (Ziv-El et al. 2011; Ziv-El et al. 2012a)

Culture	$(\Delta C\ \Delta t^{-1})_{max}$ [mM $Cl^-\ d^{-1}$]	*Dehalococcoides* [cells L^{-1}]
DehaloR^2	0.92 ± 0.1 (TCE to 90 % ethene)	$1.54 \pm 0.27 \times 10^{11}$
SDC-9	2.9 (PCE)	1.4×10^{11}
Unnamed	0.96 (PCE)	N/A
VS	0.31 (VC)	4.0×10^{11}
KB1	0.16 (TCE)	8×10^{10}
ANAS	0.006 (TCE), 0.05 (TCE)	$1.0 \pm 0.29 \times 10^{10}$
BDI	0.03 (TCE)	1×10^{11}

Reporting and comparing the maximum rate of reductive dechlorination can be of practical value when selecting potential cultures for bioaugmentation. As seen in Table 2.4, DehaloR^2 is one of the fastest cultures reported in the literature (Table 2.4) (Amos et al. 2008; Cupples et al. 2004; Richardson et al. 2002; Vainberg et al. 2009; Xiu et al. 2010). The cultures tabulated in Table 2.4 were enriched under different conditions and contained vaying microbial communities. In Chapter 3, I performed an examination to determine whether the fast rates of dechlorination and high densities of *Dehalococcoides*

observed in DehaloR^2 are related to the environmental source of the microbial inocula or to the laboratory enrichment techniques provided. Moreover, in Chapter 5, I testeded whether the fast rates of DehaloR^2 can be improved by better managing microbial communites and by changing the growth conditions from batch-fed to continuously-fed.

CHAPTER 3

SELECTIVE ENRICHMENT TECHNIQUES ABRIDGE SOIL OR SEDIMENT MICROBIAL DIVERSITY TO YIELD ROBUST CHLORINATED ETHENES-RESPIRING *DEHALOCOCCOIDES* CULTURES[1]

3.1 Introduction

Dehalococcoides mccartyi is a newly classified genus and species belonging to the *Dehalococcoidia* class in the phylum *Chloroflexi* (Loffler et al. 2013). The members of this genus respire halogenated compounds with an array of carbon backbones of biogenic and anthropogenic origin (i.e., ethenes, ethanes, benzenes, phenols, and biphenyls) (Adrian et al. 2009; Adrian et al. 2007; Adrian et al. 2000; Bunge et al. 2003; He et al. 2003b; Loffler et al. 2013; Maymo-Gatell et al. 1999; Maymo-Gatell et al. 1997; Wang and He 2013). The environmental distribution of *Dehalococcoides* spans across a wide range of habitats. They have been detected in the soil, sediment, and groundwater of numerous contaminated sites (Hendrickson et al. 2002; Tas et al. 2010; van der Zaan et al. 2010) and in an array of uncontaminated environments, including freshwater river sediments (He et al. 2005), saltwater and freshwater lake sediments (Krzmarzick et al. 2013), forest and state park soils (Krzmarzick et al. 2012), estuarine sediments (Kittelmann and Friedrich 2008b), and marine subsurface sediments (Futagami et al. 2009). Whereas their "natural" role in the cycling of halogens has only been recently investigated (Krzmarzick et al. 2012), *Dehalococcoides* have been largely explored in the past two decades in the context of bioremediation of contaminated environments.

[1] This chapter was prepared as a manuscript and will be submitted for publication.

Of particular importance for bioremediation are *Dehalococcoides mccartyi* strains that utilize the soil and groundwater contaminants perchloroethene (PCE) and trichloroethene (TCE) and transform them to the non-toxic, non-chlorinated end product, ethene (Cupples et al. 2003; He et al. 2003b; Maymo-Gatell et al. 1997; Sung et al. 2006b). These strains couple the reductive dechlorination of PCE, TCE, and the daughter chlorinated products, *cis*-dichloroethene (*cis*-DCE) and vinyl chloride (VC), to growth using H_2 as electron donor and acetate as carbon source (Loffler et al. 2013). Hence, stimulation of endogenous *Dehalococcoides* (biostimulation) or addition of laboratory-cultivated consortia containing *Dehalococcoides* (bioaugmentation) are avenues utilized to decontaminate and restore sites polluted with chlorinated ethenes (Ellis et al. 2000; Lendvay et al. 2003; Major et al. 2002).

The capacity to detoxify chlorinated ethenes is, to date, unique to *Dehalococcoides*; on the other hand, the potential for partial reduction of PCE and TCE to cis-DCE extends to multiple bacterial genera (Hug et al. 2013). Thus, it is puzzling when *Dehalococcoides* are present, yet, dechlorination of PCE/TCE stalls at *cis*-DCE and VC. This outcome was reported in soil and sediment microcosm studies and in bench-scale bioremediation scenarios (Futagami et al. 2009; Harkness et al. 1999; Kittelmann and Friedrich 2008a; van der Zaan et al. 2010; Ziv-El et al. 2012a). The inability to biostimulate *Dehalococcoides* in order to promote reductive dechlorination beyond *cis*-DCE and VC was also documented at contaminated sites undergoing biological remediation (Ellis et al. 2000; Shani et al. 2013; Stroo et al. 2012). A unifying explanation across studies for this inability to achieve reductive dechlorination of *cis*-DCE or VC to ethene is absent; the most commonly proposed explanation in the above

mentioned works was the absence of *Dehalococcoides* strains with DCE- and VC-respiring metabolic capabilities (Ellis et al. 2000; Futagami et al. 2009; Harkness et al. 1999; Kittelmann and Friedrich 2008a; Sleep et al. 2006). However, this unpredicted outcome was also noted even when *Dehalococcoides mccartyi* genes coding for the VC reductive dehalogenase enzymes, *vcrA* and *bvcA*, were detected (van der Zaan et al. 2010). Yet, neither VC reduction nor increases in *Dehalococcoides mccartyi* occurred in microcosms biostimulated with a fermentable substrate (van der Zaan et al. 2010).

We hypothesize that, often times, the discrepancy between the expected and the observed activities of *Dehalococcoides* in microcosms or in the environment is not due to their metabolic potential but to the intrinsic competition for H_2, driven by the variety of alternate electron acceptors in soils and sediments. Common electron acceptors, including nitrate, Fe (III), sulfate, sulfur, and bicarbonate (HCO_3^-) foster the growth of diverse, H_2-oxidizing microorganisms. These terminal electron accepting microbial processes were previously shown to affect the reductive dechlorination of chlorinated ethenes in enrichment cultures containing *Dehalococcoides* (Berggren et al. 2013a; Delgado et al. 2012; Fennell and Gossett 1998; Yang and McCarty 1998). In fact, recently, Fe (III) reduction and VC dechlorination were deemed antagonistic reactions (Shani et al. 2013). Therefore, in the presence of alternate electron acceptors, biostimulation of *Dehalococcoides* may be impeded or minimized, resulting in prolonged lag times before the onset of dechlorination and/or incomplete dechlorination of PCE and TCE.

Our study investigates selective enrichment and culturing techniques to abridge microbial diversity in soil and sediment microcosms in order to yield microbial

communities which completely dechlorinate PCE/TCE to ethene and to obtain robust growth of chlorinated ethene-respiring *Dehalococcoides*. For this investigation, we used geographically distinct, microbially-diverse soil and sediment from uncontaminated environments and compared them against a less diverse, contaminated sediment. Our findings support competition for the electron donor as the underlying factor for the inability to biostimulate *Dehalococcoides* in soil and sediment microcosms stalled at *cis*-DCE. Furthermore, using three soil/sediment-free enrichment cultures, we bring evidence linking fast rates of TCE to ethene dechlorination and high densities of *Dehalococcoides* to the culturing protocol, independent of the origin of the microbial inocula, which brings about potential implications for improving bioremediation in chloroethene-contaminated environments.

3.2 Materials and methods

Environmental sources

The soil and sediment samples originated from the following geographic locations: Cuzdrioara, Cluj County, Romania (47.17°N, 23.92°E), Carolina, Puerto Rico, USA (18.34°N, 65.95°W), and Parris Island Marine Corps Recruit Depot, Beaufort County, South Carolina, USA (32.33°N, 80.69°W). The Cuzdrioara soil was collected from an uncontaminated vegetable garden from a depth of ~15 cm. The Carolina sediment was sampled from an uncontaminated, tropical mangrove with a shallow water table (10-15 cm). The sediments from Parris Island were core samples collected from a 5 m depth in an area of the military base contaminated with PCE. The source of PCE contamination was an accidental spill from a dry-cleaning store in 1994 (Krug et al.

2010). Once brought to the laboratory, all soils and sediments were stored at 4 °C until the establishment of microcosms.

Microcosms and enrichment of soil/sediment-Free, chloroethene-respiring *Dehalococcoides* cultures

We established the following microcosms: Cuzdrioara soil, n = 3; Carolina sediment, n = 3; and Parris Island sediment, n = 20 (duplicates from 10 core sections evenly dispersed throughout the 5 m depth profile) in HCO_3^--buffered, reduced anaerobic mineral medium. The salts and trace mineral concentrations in the medium were previously described (Delgado et al. 2012). Each microcosm consisted of 5 g soil or sediment in 160-mL glass serum bottles with 100 mL medium. The initial pH of the medium was 7.8. We added to each microcosm 0.2-0.3 mmol L^{-1} TCE (nominal concentration) as the chlorinated electron acceptor. Additionally, we added the fermentable substrates lactate (5 mM) and methanol (12 mM) as H_2 and acetate precursors, 1 mL ATCC vitamin mix, and 50 μL of vitamin B_{12} from a 1 g L^{-1} stock solution. The microcosm bottles were incubated statically at 30° C. Cuzdrioara and Carolina microcosms were incubated for 200 days, during which time 5 mM lactate was re-added on two separate occasions (days 46 and 180).

We performed serial transfers (10% vol/vol) into same size serum bottles using the same medium compositions to remove the soil or sediment. The microcosm bottles were shaken vigorously and allowed to settle for 15 minutes so that the supernatant was mostly devoid of soil or sediment when transferred over into the new bottles. Upon each transfer, we supplied additional TCE, lactate, and methanol. After three consecutive transfers, we named the enrichment culture from Cuzdrioara soil, ZARA-10, from

Carolina sediment, LINA-09, and from Parris Island sediment, ISLA-08. We maintained the three enrichment cultures by feeding them 3-4 times consecutively with 0.5 mmol L^{-1} TCE, 5 mM lactate, and 12 mM methanol or 1 mmol L^{-1} TCE, 5 mM lactate, and 24 mM methanol. Each addition of TCE was allowed to proceed to ≥80% ethene. We flushed the headspace of the bottles with ultra-high purity N_2 gas to remove headspace gases that accumulated as a result of dechlorination (ethene and VC), fermentation (CO_2) and methanogenesis (CH_4) before adding a new dose of TCE and fermentable substrates. Removal of CO_2 from the headspace would also raise the pH of the medium, which decreased as a result of dechlorination and fermentation. When not actively used in experiments, we keep stock cultures of the soil/sediment-free enrichments in a 4°C refrigerator. No significant loss of activity is observed in these cultures even after several months of storage at 4°C.

ZARA-10 and LINA-09 bioaugmentation experiments

Bioaugmentation experiments were carried out to evaluate whether ZARA-10 and LINA-09 soil/sediment-free cultures could dechlorinate TCE to ethene in the soil and sediment from which they originated. We setup glass serum bottles (four bottles per culture) with 2.5 g soil or sediment, 50 mL reduced anaerobic medium, 0.25 mmol L^{-1} TCE, 5 mM lactate and 12 mM methanol, and 1% inoculum vol/vol (0.5 mL) ZARA-10 or LINA-09 culture, respectively. We measured the dechlorination of TCE to ethene in time-course experiments.

Gas and liquid chemical analyses

Gas samples were extracted from the headspace of bottles to measure chlorinated ethenes (TCE, *cis*-DCE, and VC), ethene, and methane using a Shimadzu GC-2010

(Columbia, USA) instrument with a flame ionized detector (FID). Details on the column type and properties, GC temperature and pressure profiles, calibration curves, and detection limits for each compound were published elsewhere (Delgado et al. 2012).

1 mL liquid samples were prepared for high performance liquid chromatography (HPLC; Shimadzu LC-20AT) through filtration using a 0.2 μm polyvinylidene fluoride membrane syringe filter (Pall Corporation, USA). We measured lactate, acetate, propionate, and methanol using the method outlined by Parameswaran et al. (Parameswaran et al. 2011), except the elution time was 40 minutes and the column temperature was constant at 50°C.

Microbial community analyses

We extracted genomic DNA from 0.25 g of soil or sediment using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., USA) following the protocol recommended by the manufacturer. For soil/sediment-free consortia, we used pellets formed from 1.5 mL liquid culture. Before DNA extraction, we pretreated the pellets as noted in Ziv-El et al. (Ziv-El et al. 2011). Then, we followed the protocol for Gram-positive bacteria outlined in the DNeasy® Blood and Tissue Kit (QIAGEN, USA).

We enumerated the 16S rRNA genes of *Dehalococcoides mccartyi* and their reductive dehalogenase genes, *tceA*, *vcrA*, and *bvcA* via quantitative PCR (qPCR). Triplicate TaqMan® assays were setup for assayed samples and standard curve points and contained the following per 10 μL reaction: 4 μL of 2.5X MasterMix solution (5 PRIME MasterMix), 0.3 μL F' and R' primers from 10 μM stocks, 0.03 μL probe from 100 μM stock, 1.37 μL PCR water, and 4 μL DNA template (diluted 1:10). The primers for the *Dehalococcoides mccartyi* 16S rRNA gene, *tceA*, and *vcrA* were published in

Holmes et al. (Holmes et al. 2006) and for *bvcA* in Ritalahti et al. (Ritalahti et al. 2006). We used an Eppendorf Realplex 4S realcycler with a PCR temperature profile and cycles as outlined by Ziv-El et al. (Ziv-El et al. 2011).

We determined the relative community structure using 454 pyrosequencing in soils, sediments, and soil/sediment-free enrichment cultures. The DNA samples were analyzed at the Research and Testing Laboratory (Lubbock, TX). The targets were the combined V2 and V3 regions of the Bacterial 16S rRNA gene using the primers 104F (5'-GGCGVACGGGTGAGTAA-3') and 530R (5'-CCGCNGCNGCTGGCAC-3'). Amplicon pyrosequencing was performed with 454 GS-FLX Titanium protocols (Wolcott et al. 2009). We qualified raw sequences by trimming off low-quality bases and removing low-quality, chimeric sequences, and singletons using the QIME software. After quality control, aligning and clustering as described (Kang et al. 2013), we obtained bacterial identification by using the Ribosomal Database Project classifier with a 50% confidence threshold (Cole et al. 2009). We obtained the following number of qualified sequences: Cuzdrioara soil, 6,924; Carolina sediment, 7,904; and Parris Island sediment, 1,486; ZARA-10 enrichment culture, 3,141; LINA-09 enrichment culture, 2,847; and ISLA-08 enrichment culture, 2,205. We calculated Phylogenetic Diversity (PD) in QIME using the PD Whole Tree estimator. Before proceeding with these calculations, we trimmed the sequencing data such that all samples had equal sampling depth (1486). We also performed Principal Component Analysis (PCA) to evaluate similarity among soil and sediment samples and soil/sediment-free enrichment cultures.

3.3 Results and discussion

Biostimulation of endogenous *Dehalococcoides* in microcosms from distinct environments

A range of (mostly) contaminated environments has served as microbial inocula for *Dehalococcoides* enrichment cultures throughout the two decades of research on reductive dechlorination. These environments were thought to be most fitting for finding microorganisms that can metabolize a specific pollutant as they have already been exposed to that pollutant. Table 2.3 contains a comprehensive collection of cultures employed in fundamental studies and in bioaugmentation research/applications for PCE or TCE dechlorination. Development of these enrichment cultures is a lengthy process (Loffler et al. 2005), as the enrichments must be actively fed and transferred to ensure the desired biological activity. Therefore, careful consideration is given to any environmental sample (soil, sediment, or groundwater) before pursuing this labor- and time-intensive work. Evidence of reductive dechlorination to VC and ethene is often a crucial decision factor in choosing to pursue this laboratory work. This evidence can also play an important role in deciding whether to proceed with *in situ* biostimulation. VC and ethene are measured either directly during contaminated site analyses or in laboratory microcosm experiments (Aziz et al. 2012; Stroo et al. 2012). For the majority of the enrichment cultures in Table 2.3 there was evidence of desired biological activity through one or both assessment methods.

In this study, reductive dechlorination of TCE and the subsequent enrichment of *Dehalococcoides* respiring chlorinated ethenes were evaluated in a total of 26 microcosms. Figure 3.1 (left panels) shows the dechlorination profile in microcosms

established with the three geographically-distinct environmental sources as inocula and amended with TCE and the fermentable substrates, lactate and methanol. As depicted in Figure 3.1A-B (left panels), *cis*-DCE was the end product from TCE reduction in all microcosms set up with uncontaminated soil from Cuzdrioara and uncontaminated sediment from Carolina. Moreover, we biostimulated two additional times with 5 mM lactate (days 46 and 180) to ensure the availability of electron donor and we incubated these microcosms for up to 200 days. However, the additional electron donor did not further advance *cis*-DCE reduction. We tracked production of methane in the microcosms stalled at *cis*-DCE (Figure 3.2). Methane concentrations increased throughout the incubation period, reaching up to 7.2 mmol L^{-1} and 8.8 mmol L^{-1} in Cuzdrioara and Carolina microcosms, respectively (Figure 3.2). Although multiple additions of fermentable substrates and the extended incubation did not generate VC and ethene, as presented in Figure 3.1A-B (left panels), the methane data strongly indicated that methanogenesis was one of the biological processes benefiting from biostimulation.

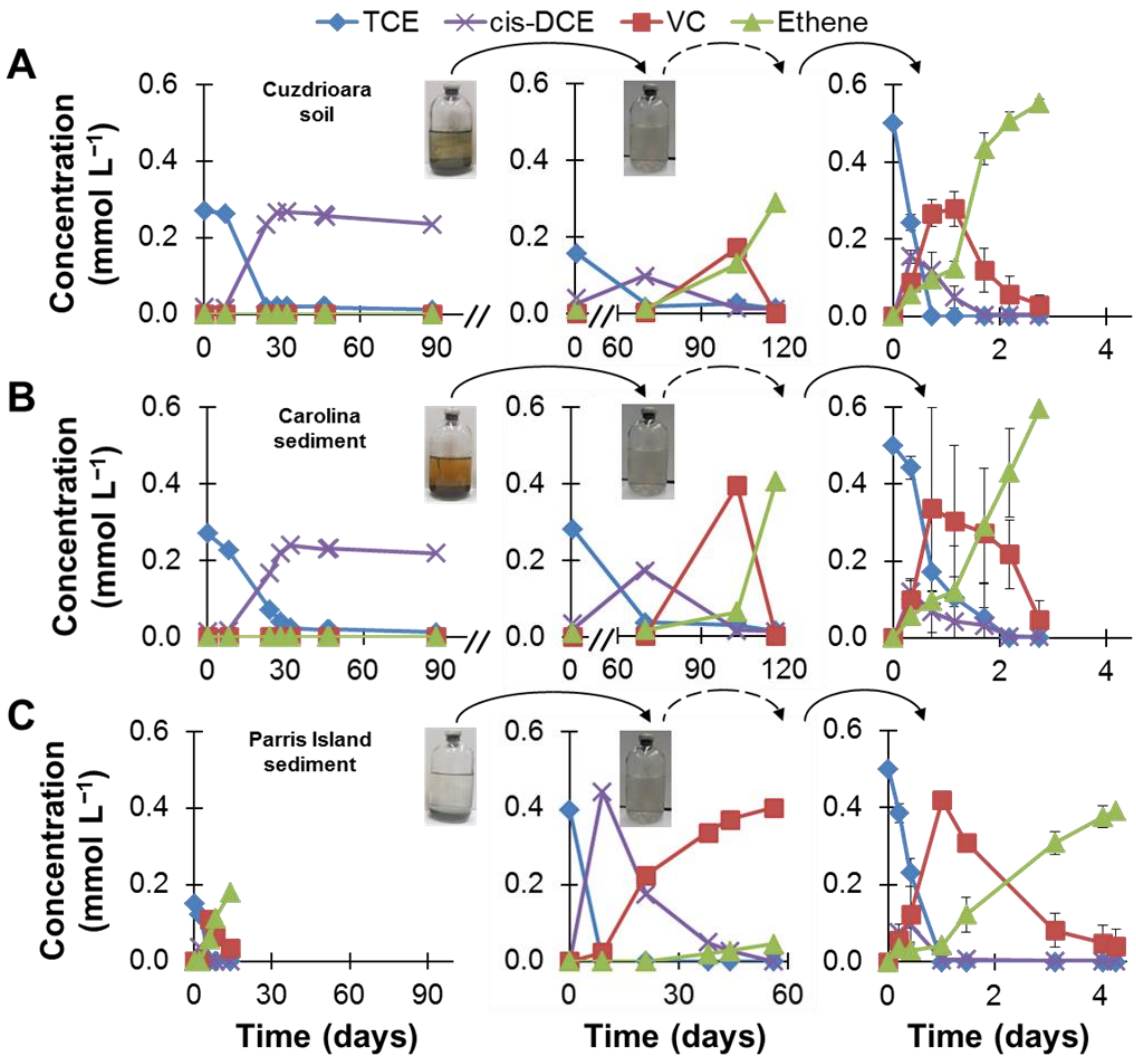


Figure 3.1 Biostimulation of organohalide-respiring communities containing *Dehalococcoides*. (A)-(C) Dechlorination of TCE in microcosms (left panels), first transfers from microcosms (middle panels), and enriched soil/sediment-free cultures (right panels). The microcosms (left panels) were established with (A) uncontaminated garden soil, (B) uncontaminated mangrove sediment, and (C) PCE-contaminated groundwater sediment. A total of 26 microcosms were established and one replicate is shown from each environmental enrichment. The time-course experiments from the right panels assessing dechlorination of TCE to ethene are from the third consecutive addition of 0.5 mmol L^{-1} TCE. The error bars are standard deviation of triplicate cultures. Note the time scale differences between left, middle, and right panels.

Contrary to the observations from Figure 3.1A-B (left panels), we achieved complete dechlorination of TCE to ethene by transferring microcosm supernatant devoid of soil or sediment into fresh medium from each microcosm bottle stalled at *cis*-DCE

(Figure 3.1A-B, middle panels). This outcome confirmed that endogenous *cis*-DCE- and VC-respiring *Dehalococcoides* could indeed be biostimulated. The fact that VC and ethene were produced in the absence of the soil or sediment brought about two possibilities: 1) compounds inhibiting *Dehalococcoides* were present in the soil or sediment or, in accordance with our hypothesis, 2) the electrons from the fermentable substrates were being utilized by H_2-competing microorganisms growing on components of the soil and sediment serving as electron acceptors.

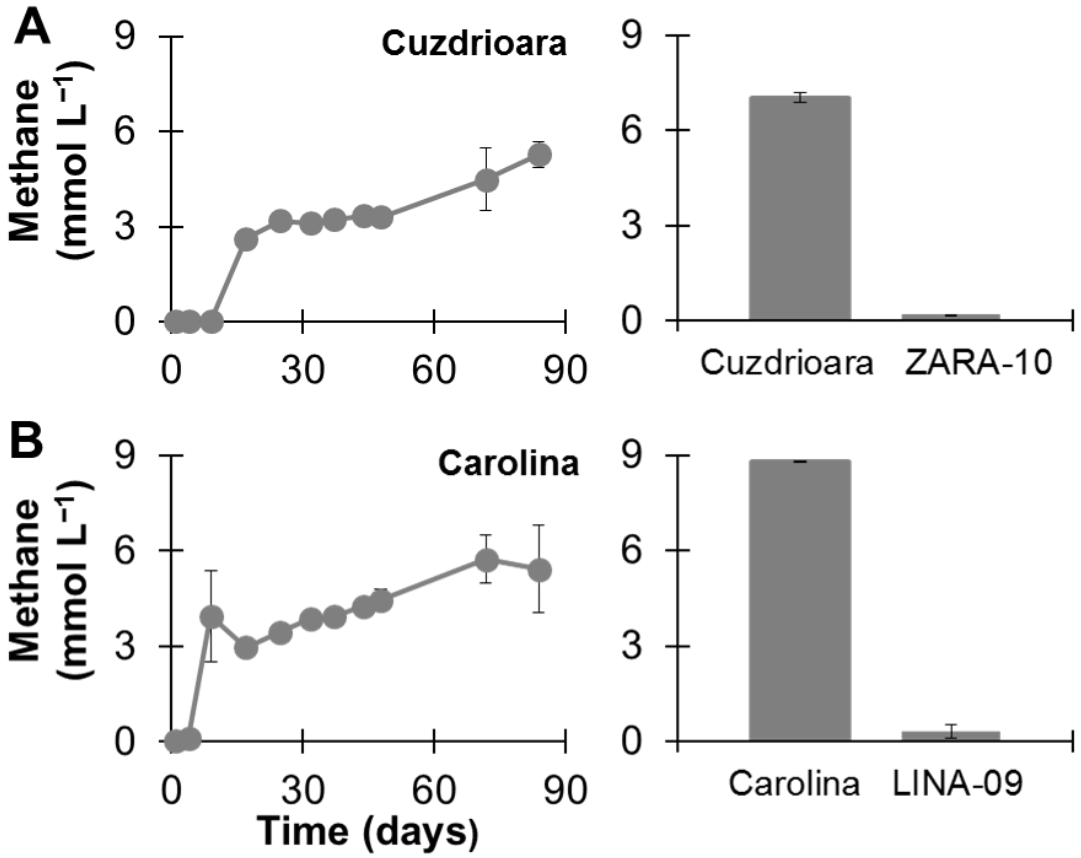


Figure 3.2 Methane production in microcosms and subsequent enrichment cultures. (A)-(B) Left panels: time-course methane measurements in Cuzdrioara and Carolina microcosms biostimulated with fermentable substrates. (A)-(B) Right panels: final methane concentrations recorded in Cuzdrioara and Carolina microcosms (end of experiments from Figure 3.1A-B, day 200) and final methane concentrations in ZARA-10 and LINA-09 enrichment cultures (end of experiments from Figure 3.1A-B, day 2.8).

With the lactate and methanol provided upon establishing the microcosms with Parris Island contaminated sediment, dechlorination proceeded beyond *cis*-DCE (Figure 3.1C, left panel). VC and ethene formed within 30 days in 40% of the microcosms containing sediment from this location. Furthermore, VC and ethene production was obtained in microcosms from across the different core depths. The dechlorination activity in our laboratory microcosms and microcosm transfers (Figure 3.1C left and center panels) mirrored the endogenous dechlorinating activity at the Parris Island site, where TCE, *cis*-DCE, VC, and ethene were detected from dechlorination of PCE after biostimulation with emulsified vegetable oil (US EPA 2013a).

Characterization of soil/sediment-free cultures enriched in *Dehalococcoides*

As shown in Figure 3.1 A-B (left panels), microcosm data failed to predict the "true" reductive dechlorination potential in the Cuzdrioara soil and the Carolina sediment. In spite of this, we showed that ethene could be obtained as the end-reduced product of TCE dechlorination through the enrichment process. We then further sought to evaluate the impact of selective enrichment and culturing techniques on the soil/sediment-free microbial communities developed from the three distinct environments. The enrichment cultures ZARA-10, LINA-09, and ISLA-08 were maintained under identical batch-fed growth conditions. Figure 3.1A-C (right panels) reveals that, as a result of the growth protocol, fast reduction of TCE to ethene was achieved in all three enrichment cultures, regardless of the environment where the microbial inocula originated. Moreover, the culture performance parameters tabulated in Table 3.1 show similarly high maximum observed transient conversion rates (on the

order of mM Cl^- released per day) in ZARA-10, LINA-09, and ISLA-08 enrichment cultures, and dechlorination of 0.5 mmol L^{-1} TCE to ≥80% ethene in as short as 1.7 days.

Table 3.1 Conversion rates of TCE to ethene, days to complete dechlorination, *Dehalococcoides* concentrations, and *Dehalococcoides* yields in the sediment-free enrichment cultures developed in this study. The values reported are averages from triplicate cultures

	Enrichment culture		
	ZARA-10	**LINA-09**	**ISLA-08**
Highest transient conversion rate[1] (mmol Cl^- released L^{-1} d^{-1})	2.67 ± 0.34	2.37 ± 0.43	2.51 ± 0.08
Conversion of 0.5 mmol L^{-1} TCE to ≥80% ethene[2,3] (days)	1.7	2.8	4.0
Conversion of 1 mmol L^{-1} TCE to ≥80% ethene[2] (days)	2.3	5.9	8.8
Final *Dehalococcoides* densities (cells mL^{-1}) at 0.5 mmol L^{-1} TCE[3]	9.6×10^8	1.4×10^9	1.9×10^9
(cells mL^{-1}) at 1 mmol L^{-1} TCE[3]	2.3×10^9	1.8×10^9	2.3×10^9
Yield *Dehalococcoides*[4] (cells µmol^{-1} Cl^- released)	2.6×10^8	1.8×10^8	2.3×10^8

[1]Rate calculated between two consecutive sampling points. The transient rates were highest for all cultures on the third addition of 1 mmol L^{-1} TCE.
[2]Conversion times reported for the third addition of TCE
[3]Final densities after three consecutive additions of TCE
[4]Yields were calculated from the change in the 16S rRNA gene copies measured by qPCR and the change in concentration of TCE reduced to ethene as described (Duhamel and Edwards 2007).

Insights from the composition of microbial communities

To gain insights into the differences between the microcosms that could or could not be biostimulated beyond *cis*-DCE, we took advantage of high throughput sequencing. The larger pie graphs in Figure 3.3 illustrate the 454 pyrosequencing data at class level from Cuzdrioara soil, Carolina sediment, and Parris Island sediment. The class of interest for TCE to ethene respiration is *Dehalococcoidia*, containing the genera *Dehalococcoides* (Loffler et al. 2012), *Dehalogenimonas* (Moe et al. 2009), and *Dehalobium* (May et al. 2008). *Dehalococcoidia* was in low abundance in all soil and sediment samples (<1% of total sequences), and hence, it is not shown in the large pie graphs. As seen in Figure 3.3

(large pie graphs), the Cuzdrioara and Carolina environments were more diverse than the Parris Island sediment and were predominantly populated by *α*-, *β*-, *γ*-, *δ*-, and *ε*-*Proteobacteria*. The microbial communities in Cuzdriora soil and Carolina sediment (Figure 3.3, large pie graphs) concur with previously described microbial communities in environments where sulfate (Hansel et al. 2008; Purdy et al. 2001), sulfur (Hansel et al. 2008), iron (Miceli et al. 2012), nitrate (Van Nostrand et al. 2011; Van Trump et al. 2011), and HCO_3^- (Kotsyurbenko et al. 2001) were abundant. Compared to Cuzdrioara and Carolina samples, the Parris Island sediment contained fewer classes (Figure 3.3, large pie graph), and had a higher relative abundance of *Dehalococcoidia* (0.4%), potentially pointing towards an opposed relationship between high microbial diversity and successful biostimulation of VC- and ethene-producing *Dehalococcoidia.*

While in low abundance in the soils and sediments, relative to the other bacterial classes, *Dehalococcoidia* became one of the most prevalent taxa in all three enrichment cultures. This is depicted in the small overlaid pie charts in Figure 3.3: ZARA-10, 9%; LINA-09, 3%; ISLA-08, 21%. Furthermore, the predominant classes in all three originating environments, *α*-, *β*-, *γ*-, and *ε*-*Proteobacteria* were absent (zero sequences) in the enrichment cultures (Figure 3.3, small overlaid pie graphs). The family *Geobacteraceae* within the *δ*-*Proteobacteria*, containing bacterial members known to respire TCE (Sung et al. 2006a), was maintained in low abundance (<1%) in the enrichment cultures ZARA-10 and LINA-09. These findings are opposite to previously published data by Miceli et al. who employed the same soil and sediment samples collected from Cuzdrioara and Carolina for the enrichment of anode-respiring communities in microbial electrochemical cells (Miceli et al. 2012). In the study by

Miceli et al., when garden soil from Cuzdrioara was used as a microbial inoculum, the resulting enrichment was dominated by *δ-Proteobacteria* (~90% relative abundance) (Miceli et al. 2012). When the source of microbes was Carolina mangrove sediment, ~60% of the enriched biofilm was comprised of *α-*, *γ-*, and *δ-Proteobacteria* (Miceli et al. 2012). The elimination or minimization of these classes in the enrichment cultures from this study is attributed to the selective conditions provided in the medium, with TCE and HCO_3^- as the sole electron acceptors. HCO_3^--reducing methanogens continued to be active in all enrichment cultures; however, as shown in Figure 3.2, methane production was drastically diminished when compared to the activity in the microcosms.

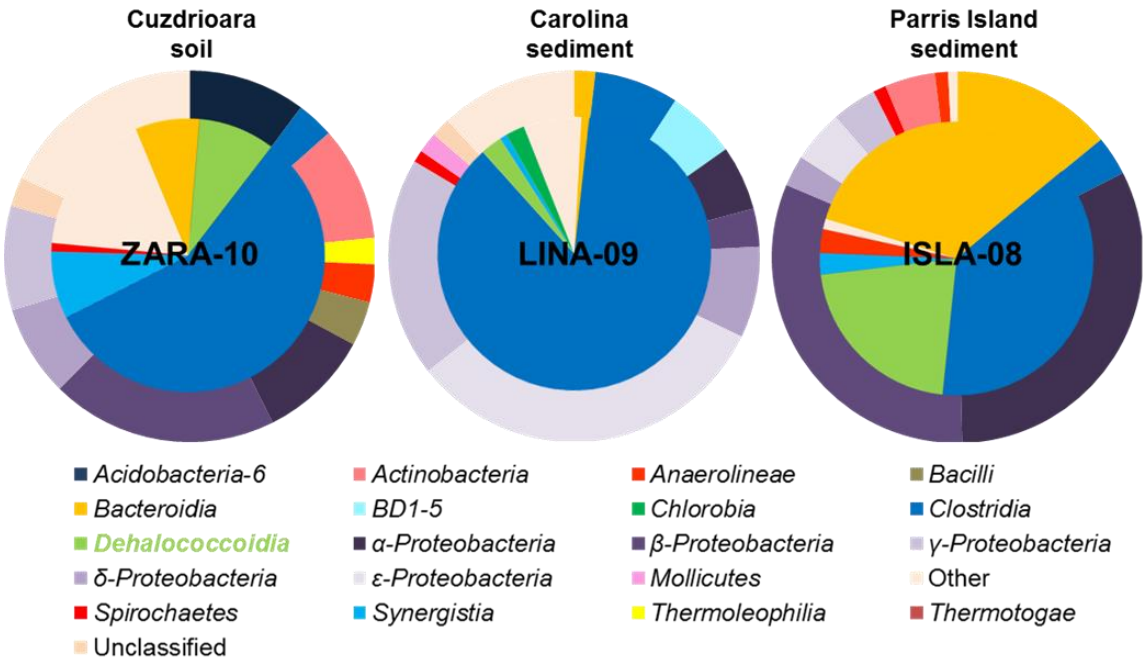


Figure 3.3 Bacterial diversity at class level as determined by 454 pyrosequencing of the V2-V3 region of the 16S rRNA genes. The large pie charts represent the relative abundance of select classes in the Cuzdrioara uncontaminated soil, Carolina uncontaminated sediment and Parris Island contaminated sediment. The small overlaid pie charts show the five most abundant classes in the soil/sediment-free enrichment cultures, ZARA-10, LINA-09, and ISLA-08. The classified taxa presented contributed to at least 1% of the total relative abundance and are organized in alphabetical order.

Diversity analyses reveal convergent enrichment cultures

We conducted alpha diversity analyses using phylogeny-based metrics (Phylogenetic Diversity (PD) index) to estimate microbial diversity. According to the PD analyses depicted in Figure 3.4A-C, the microbial diversity in the three enrichment cultures resulted in comparable, low PD values. This was achieved in the Cuzdrioara soil and the Carolina sediment (Figure 3.4A-B), which had PD values approximately four fold higher than that of Parris Island (Figure 3.4C). Furthermore, principal component analyses (PCA) between samples (beta diversity) reveals that heterogeneous soil and sediment samples (blue symbols, Figure 3.4D) converged to very similar, highly efficient TCE-respiring microbial communities after the enrichment (green symbols, Figure 3.4D).

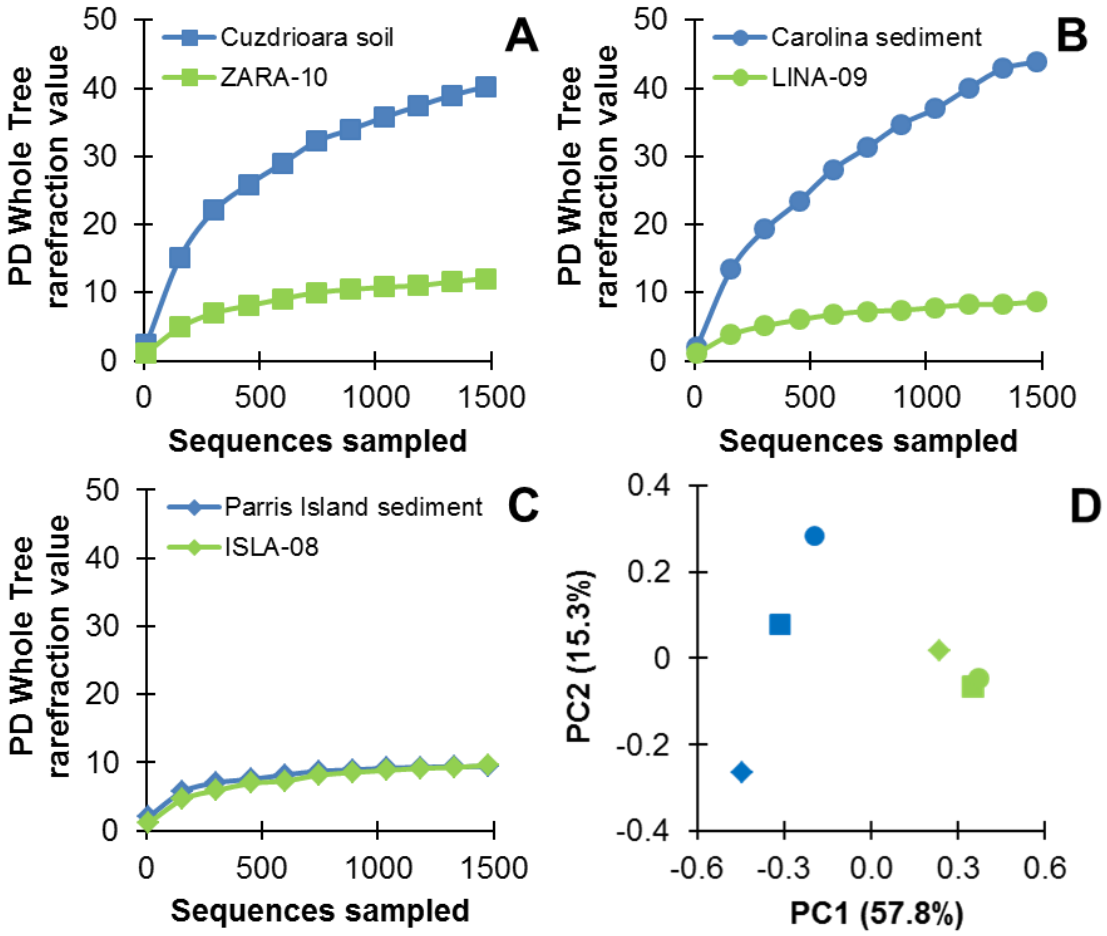


Figure 3.4 Alpha and beta microbial diversity analyses. (A)-(C) Phylogenetic Diversity (PD) Whole Tree measurements from the 454 analysis using trimmed, equal sequencing depth OTUs (1486) per sample. (D) Weighted UNIFRAC distance calculated after trimming the samples to equal sequence depth in QIIME. The Principal Component Analysis (PCA) was generated by grouping the samples into two categories (soils/sediments vs. enrichment cultures). The color blue corresponds to the soil/sediment samples, while green corresponds to the soil/sediment-free enrichment cultures.

Effect of enrichment techniques on growth of *Dehalococcoides mccartyi*

Enumeration of *Dehalococcoides mccartyi* was achieved through qPCR targeting the 16S rRNA genes (Table 3.1 and Figure 3.5) and their reductive dehalogenase genes, *tceA*, *vcrA*, and *bvcA* (Figure 3.5). Reductive dehalogenase genes coding for enzymes involved in dechlorination of TCE to VC (TceA) (Magnuson et al. 2000), *cis*-DCE and VC to ethene (VcrA) (Muller et al. 2004), and VC to ethene (BvcA) (Krajmalnik-Brown

et al. 2004) were all enriched in ZARA-10 and LINA-09 soil/sediment-free cultures (Figure 3.5). The fact that *Dehalococcoides mccartyi* strain 195-/FL2-like (containing *tceA*), strain GT-/VS-like (containing *vcrA*), and strain BAV1-like (containing *bvcA*) were present in these enrichment cultures from *cis*-DCE stalled microcosms is further evidence that the potential for complete dechlorination (with overlapping functional redundancy) was present in the environmental samples.

The three enrichment cultures exhibited some microbiological differences with respect to the *Dehalococcoides mccartyi* strains present (*vrcA* was not detected in ISLA-08). However, common to all enrichment cultures were the very high (and similar) densities of *Dehalococcoides mccartyi* of ~10^9 *Dehalococcoides* cells mL^{-1} (or 10^{12} cells L^{-1}) (Table 3.1 and Figure 3.5). These densities were the result of the culturing protocol, where we fed high doses of TCE (1.5-3 mmol L^{-1} over three additions). To our knowledge, this is the first study to report such high densities of *Dehalococcoides mccartyi* in multiple enrichment cultures and in batch-fed cultures cultivated in serum bottles.

Taken together, the rates and the times required for complete reduction to ethene of the TCE supplied (Figure 3.1, right panels), and the resulting *Dehalococcoides mccartyi* concentrations (Table 3.1 and Figure 3.5) compare favorably to previously published values, tabulated by Ziv-El et al. (Ziv-El et al. 2011; Ziv-El et al. 2012a). The dechlorination rates and *Dehalococcoides* densities, two important and interconnected factors for successful bioremediation, varied sometimes by orders of magnitude (Ziv-El et al. 2011; Ziv-El et al. 2012a) between the cultures reported. A contributing factor to these variances could be the enrichment techniques employed, as the compared studies

used different conditions and different stimulation techniques for the development and cultivation of reductively dechlorinating cultures. Our results support the idea that an "optimal' microbial community, where *Dehalococcoides* thrive, can be achieved under the enrichment conditions described herein.

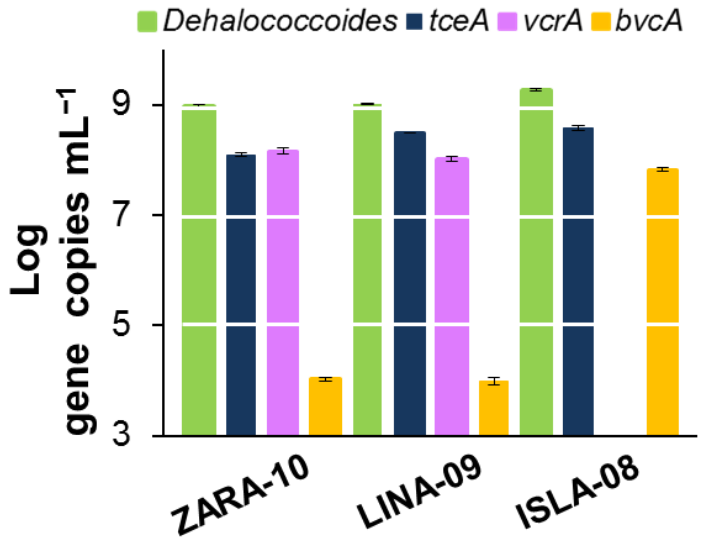


Figure 3.5 Enumeration of *Dehalococcoides mccartyi* in enrichment cultures. qPCR tracking *Dehalococcoides* 16S rRNA genes and their reductive dehalogenase genes, *tceA*, *vcrA*, and *bvcA* using qPCR in the enrichment cultures after three consecutive additions of 0.5 mmol L^{-1} TCE. The plot is representative of triplicate cultures and the error bars are standard deviations of triplicate qPCR reactions.

Outlook on bioremediation using *Dehalococcoides*

One important aspect of our study, shown with the Cuzdrioara soil and Carolina sediment, is the ability to stimulate the production of VC and ethene and the growth of *cis*-DCE and VC-respiring *Dehalococcoides*. We believe this outcome was obtained by removing or diluting the soil or sediment components and the subsequent microbial guilds competing for the electron donor. To strengthen this point, we designed a simple experiment to show that, once *Dehalococcoides* are enriched and in high abundance, they could better compete in the complex soil or sediment environments from which they

originated. For this, we re-established microcosms containing Cuzdrioara soil and Carolina sediment and bioaugmented the microcosms with a 1% vol/vol inoculum of ZARA-10 or LINA-09 enrichment culture, respectively, to more appropriately reflect the dilution factor in bioremediation scenarios at contaminated sites. As seen in Figure 3.6A-B and supportive of our hypothesis, with both enrichment cultures dechlorination of TCE proceeded to ethene and we achieved close to complete dechlorination of 0.25 mmol L^{-1} TCE to ethene in ~30 days. The rates of reductive dechlorination obtained with this smaller inoculum also exclude the possibility that the *cis*-DCE stall resulted from components in the soil or sediment inhibiting the native *Dehalococcoides*.

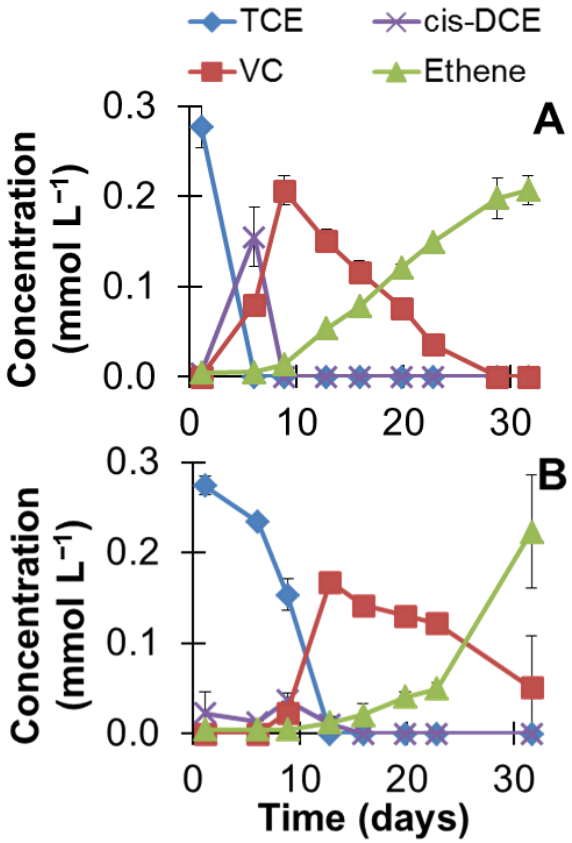


Figure 3.6 Bioaugmentation of microcosms with their respective enrichment cultures. Dechlorination of TCE in (A) in Cuzdrioara soil microcosms bioaugmentated with ZARA-10 enrichment culture and in (B) Carolina sediment microcosms bioaugmented with LINA-09 culture. The inoculum used for these experiments was 1% vol/vol.

For bioremediation of PCE or TCE contaminated sites, microcosm experiments have historically been utilized as indicators of endogenous microbial biological activity (Stroo et al. 2012). The results of microcosm experiments help researchers and bioremediation practitioners decide on whether biostimulation or bioaugmentation is the appropriate treatment for decontamination of environments polluted by chlorinated solvents (Stroo et al. 2012). In cases where incomplete dechlorination was observed in microcosms, this has been attributed to the presence of inhibiting conditions or the lack of *Dehalococcoides* capable of complete dechlorination. Our findings clearly show that neither result from Cuzdrioara or Carolina biostimulated microcosms could be explained by those two hypotheses.

Instead an electron donor competition is proposed, supported by our data, in which components of the soil or sediment serve as electron acceptor for competing H_2-oxidizing microorganisms. Therefore, our results bring experimental evidence towards a new possible explanation to "unsuccessful" microcosm experiments. If indeed microbial competition for electron donor is a major determining factor in the success of the microcosms, it will certainly be a determining factor in bioremediation as well. Our work provides a new perspective to better understand site assessment and possibly improve the bioremediation process.

CHAPTER 4

ROLE OF BICARBONATE AS A PH BUFFER AND ELECTRON SINK IN MICROBIAL DECHLORINATION OF CHLOROETHENES[2]

4.1 Introduction

Organohalide respiring microorganisms represent a unique, efficient, and sustainable approach to detoxifying chloroethenes contamination from soil, water, and groundwater (Ellis et al. 2000; Marzorati et al. 2010; Maymo-Gatell et al. 1997). These microbes are important because they can use priority pollutants such as trichloroethene (TCE), dichloroethene (DCE) and vinyl chloride (VC) as electron acceptors for energy metabolism (Tas et al. 2010). *Dehalococcoides* bacteria hold a prominent role among the organohalide respirers isolated to date, as these are the only ones having the proven ability to detoxify chloroethenes to the innocuous end product, ethene (He et al. 2003b; Maymo-Gatell et al. 1997). *Dehalococcoides* have a constrained metabolism; they strictly utilize hydrogen (H_2) as the electron donor and acetate as the carbon source (Loffler et al. 2013). The most common method for delivery of H_2 and acetate at bioremediation sites is the addition of fermentable substrates as precursors (Ellis et al. 2000; Major et al. 2002; Schaefer et al. 2010). H_2 gas has also been supplied for groundwater field applications (Edstrom et al. 2005) and in engineered *ex situ* treatment technologies for chloroethenes remediation (Ma et al. 2003; Villano et al. 2011; Ziv-El et al. 2012b). In systems fed with H_2, the pH tends to rise as a result of competing

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biological reactions, whereas dechlorination and/or fermentation of H_2-releasing compounds decrease the pH. As a consequence, buffering and pH management are important parameters for assessing *in situ* and *ex situ* remediation approaches, and are crucial for sustained dechlorination (Robinson and Barry 2009; Robinson et al. 2009; Ziv-El et al. 2012b).

In groundwater, dissolution of carbonate (CO_3^{2-})-containing minerals serves as the natural pH buffer. Among the CO_3^{2-} species, bicarbonate[3] (HCO_3^-) is the most abundant at neutral pH, and it contributes substantially to the alkalinity of groundwater. Typical HCO_3^- concentrations in groundwater are in the range of 0.7-10 mM (Abdelouas et al. 1998; Wilkin and Digiulio 2010). Additionally, HCO_3^- is supplemented to groundwater as a common strategy when biostimulation or bioaugmentation are employed in order to buffer the protons produced by the biological reactions (Ellis et al. 2000; Schaefer et al. 2010).

In laboratory settings, pH management is also commonly achieved through the addition of HCO_3^- buffer in the form of $NaHCO_3$ or $KHCO_3$. HCO_3^- has been used for growth of *Dehalococcoides* strains (Loffler et al. 2005) and for mixed dechlorinating communities (Duhamel and Edwards 2007; Vainberg et al. 2009; Ziv-El et al. 2011) to maintain a favorable pH. *Dehalococcoides* optimum pH has been reported to range from 6.9-7.5 (Loffler et al. 2013); yet, to date, there is a lack of systematic studies defining both the pH boundaries for these important organisms, and the effect pH exerts on each step in the TCE reduction pathway. Beyond its function as a buffer, HCO_3^- also serves as an electron acceptor for other microorganisms commonly encountered with

[3] Throughout this work, HCO_3^- is used to denote the buffer HCO_3^-/CO_2. At the pH ranges observed in this study, HCO_3^- accounted for 90% or greater of the two species.

organohalide respirers in the environment and in laboratory-cultured consortia. For example, at neutral pH, hydrogenotrophic methanogens consume HCO_3^- and H_2 to generate methane (Cordruwisch et al. 1988):

Hydrogenotrophic methanogenesis:

$$HCO_3^- + 4\ H_2 + H^+ \rightarrow CH_4 + 3\ H_2O \qquad \text{(Equation 4.1)}$$

The competition for H_2 among organohalide respirers and methanogens has been well documented (Aulenta et al. 2005; Ballapragada et al. 1997; Carr and Hughes 1998; Fennell and Gossett 1998; Fennell et al. 1997; Smatlak et al. 1996; Yang and McCarty 1998). However, none of these studies have addressed how consumption of H_2, whether added as gas or as a result of fermentation, is affected by varying HCO_3^- concentrations.

Homoacetogens are other important microorganisms commonly encountered with organohalide respirers. Homoacetogens produce H_2 from fermentation of complex substrates and/or consume available H_2 (Drake 1994; Rittmann and Herwig 2012). Hydrogenotrophic homoacetogens catalyze the formation of acetate from H_2 and HCO_3^- in their energy metabolism (Drake 1994):

Hydrogenotrophic homoacetogenesis:

$$2\ HCO_3^- + 4\ H_2 + H^+ \rightarrow CH_3COO^- + 4\ H_2O \qquad \text{(Equation 4.2)}$$

They, too, compete for H_2 with organohalide respirers. To date, the limited number of studies that have investigated hydrogenotrophic homoacetogenesis in TCE dechlorinating consortia (Yang and McCarty 1998; Yang and McCarty 2000) has not included HCO_3^- concentration as a variable driving the extent and the rates of reductive dechlorination.

Hydrogenotrophic methanogens and homoacetogens can also affect pH in dechlorinating communities. Methanogens produce methane as the end product (Eq. 4.1)

by expending one proton and one HCO_3^-, while hydrogenotrophic homoacetogens generate acetate (Eq. 4.2) from one proton and two HCO_3^-. Both reactions increase the pH while consuming HCO_3^-, which often is the only buffer in the system. The effect of HCO_3^- concentration in TCE dechlorinating microbial communities has not been studied. Few studies focusing on other dechlorinating systems have recognized its importance and examined the effect of HCO_3^- concentrations on the formation of chlorinated daughter products, thus motivating this work. For example, removal of chlorophenols from simulated wastewater in upflow anaerobic sludge blanket (UASB) reactors revealed significant inhibition on dechlorination at high HCO_3^- (3500 mg L^{-1} as $CaCO_3$) and high pH (Majumder and Gupta 2009). In microcosms showing microbial dechlorination of polychlorinated biphenyls with H_2 gas as electron donor, 100 mg L^{-1} HCO_3^- (1.64 mM) yielded the fastest rates of dechlorination, whereas addition of 1000 mg L^{-1} HCO_3^- (16.4 mM) resulted in the slowest polychlorinated biphenyls dechlorination rates and triggered the most acetate to form (Yan et al. 2006).

In this study, we evaluate the role of HCO_3^- as a buffering agent and as an electron acceptor in TCE reductively dechlorinating mixed communities using a previously described culture, DehaloR^2, as a model consortium (Ziv-El et al. 2011). H_2, and not fermentable substrates, was used as the sole electron donor to directly and accurately measure hydrogenotrophic production of methane and acetate from HCO_3^-. The concentrations of HCO_3^- tested reflect typical groundwater concentrations (2.5-10 mM), as well as commonly reported laboratory concentrations (30 mM).

4.2 Materials and methods

Microbial inoculum and preparation of batch cultures

The sediment-free microbial consortium, DehaloR^2, described by Ziv-El et al. (2011) was used as an inoculum for all experiments. For the experiments in this study, we preconditioned the inoculum culture by growing it in 10 mM HCO_3^- medium, with excess H_2 as electron donor, and two consecutive feedings of 10 µL neat TCE in 120 mL medium.

Reduced anaerobic mineral medium was prepared containing the following reagents per liter: 1 g NaCl, 0.06 g $MgCl_2 \times 6H_2O$, 0.2 g KH_2PO_4, 0.3 g NH_4Cl, 0.3 g KCl, 0.005 g $CaCl_2 \times 2H_2O$, and 1 mL of Trace A and Trace B solutions described elsewhere (Loffler et al. 2005). During medium preparation, nitrogen was the sole gas for boiling and bottling and the reducing agents were 0.2 mM L-cysteine and 0.2 mM $Na_2S \times 9\ H_2O$. No buffer was added to the medium before autoclaving. For bottling, we used 160-mL glass serum bottles containing 90 mL liquid and 70 mL headspace sealed with black butyl rubber stoppers and aluminum crimps.

The concentrations of $NaHCO_3$ tested were 2.5, 5, 10, and 30 mM. In the cultures where both $NaHCO_3$ and HEPES (pK_a = 7.55) were used as buffers, we supplemented 5 mM HEPES in combination with 2.5, 5 and 10 mM HCO_3^-, and 10 mM HEPES in the 30 mM HCO_3^- cultures. $NaHCO_3$ and HEPES were delivered to each bottle from 1 M sterile anaerobic stock solutions. The initial pH was adjusted with sterile 2.25 N HCl or NaOH to 7.5 (± 0.1). At time 0, we added to each culture bottle 0.55 mmol L^{-1} TCE (5 µL neat or 71.3 mg L^{-1}), 1 mL ATCC vitamin mix, 50 µL of 1 g L^{-1} vitamin B_{12} solution, 8.2 mmol L^{-1} H_2 (20 mL H_2 gas), and 10 mL DehaloR^2 microbial culture corresponding

to a 10% inoculum. The working volume was 100 mL of liquid with 60 mL of headspace. The bottles were incubated in the dark at 30°C without shaking. An additional 8.2 mmol L^{-1} H_2 was added on day 12 (all cultures) and on day 26 (only to cultures still undergoing dechlorination). All experimental conditions were tested in triplicates and the experiments were also performed on two separate occasions.

Chemical and pH measurements for the time course experiments

We measured TCE, *cis*-DCE, VC, ethene, and methane using a gas chromatograph (GC) (Shimadzu GC-2010; Columbia, MD) equipped with a flame ionization detector (FID). The compounds were carried by helium gas through an Rt-QS-BOND capillary column (Restek; Bellefonte, PA). The oven temperature was maintained at 110°C for 1 min, followed by a temperature increase of 50°C min^{-1} to 200°C. Then, the temperature ramp was further raised to 240°C with a 15°C min^{-1} gradient and held for 1.5 mins. The temperatures of the FID and the injector were 240°C. Chloroethenes, ethene and methane calibrations in 160-mL bottles with 100 mL liquid volume were performed in a range of 0.05-2.45 mmol L^{-1}. The detection limit for all compounds measured on the GC-FID is $\leq$0.018 mmol L^{-1}.

A GC instrument equipped with a thermal conductivity detector (TCD) was employed to measure H_2 before reinjecting additional H_2 to the cultures on day 12. The instrument settings used were those previously outlined (Parameswaran et al. 2011). The H_2 detection limit for the GC-TCD is 0.8% vol/vol.

We quantified acetate, propionate, and formate from 0.75-mL liquid samples filtered through a 0.2 μm polyvinylidene fluoride membrane syringe filter (Pall Corporation; Ann Arbor, MI) into 2-mL glass vials (VWR; Radnor, PA) via high

performance liquid chromatography (HPLC) using a previously published method (Parameswaran et al. 2011). Five point calibration curves (0.5-10 mM) were generated for acetate, propionate, and formate during every HPLC run. The detection limit for the compounds measured on the HPLC was ≤0.1 mM.

0.29 ± 0.06 mM propionate was carried over from the inoculum culture and the final measured concentration was 0.33 ± 0.04 mM, indicative that propionate did not serve as a significant source of electrons. Formate was sometimes also detected at low concentrations (0.1-0.3 mM), however, we did not identify a clear trend on the formation/consumption of this product. Therefore, propionate and formate were omitted from the electron balances in Figure 4.3.

The pH was measured using an Orion 2-Star pH bench top meter (Thermo Scientific, USA) that was calibrated regularly with 4.01, 7.00, and 10.01 standard solutions from the manufacturer.

All cultures were sampled for gas and liquid analyses until dechlorination of TCE to ethene was complete or until the end of experiments on day 40.

DNA extraction and molecular microbial characterization

Pellets were formed by centrifugation from 2-mL liquid from each culture replicate and they were stored at -20°C until the DNA extraction. Genomic DNA was extracted for two time points for all sets of HCO_3^- & HEPES, and two time points for the set with 30 mM HCO_3^- only. Before DNA extraction, the replicate pellets were thawed, resuspended in the supernatant and combined so that only extraction per set per time point was performed. This was done to increase total biomass and DNA yield. The DNA extraction was performed as previously described (Ziv-El et al. 2011).

We employed quantitative real-time PCR (qPCR) to target the 16S rRNA gene of *Dehalococcoides* and *Archaea* (TaqMan® assays) and the FTHFS gene of homoacetogens (SYBR Green assay). Triplicate reactions were setup for the six point standard curves and the samples in 10 µL total volume using 4 µL of 1/10 diluted DNA as template. We generated standard curves by serially diluting 10 ng μL^{-1} plasmid DNA. The primers and probes, reagents concentrations, and thermocycler (Realplex 4S thermocycler; Eppendorf, USA) conditions were those described for *Dehalococcoides* (Holmes et al. 2006), *Archaea* (Parameswaran et al. 2010; Yu et al. 2005b), and FTHFS (Parameswaran et al. 2010; Xu et al. 2009). Acetoclastic methanogens (the order *Methanosarcinales*) were not assayed because they are absent in the culture employed in this study, which this was confirmed by qPCR previously (Ziv-El et al. 2011).

Time 0 for all qPCR assays was generated by amplifying genomic DNA from the inoculum culture and assigning 10% as the starting concentrations of gene copies per L culture.

Calculations

The distributions of electrons from Figure 4.4 were calculated in units of me^- equivalents for each compound from the equation below:

$$\%\ \text{compound} = \frac{[\text{compound}] \times \frac{\#\text{electrons}}{\text{mol}}}{[H_2] \times \frac{2\ \text{electrons}}{\text{mol}\ H_2}} \times 100$$

The number of me^- equivalents for dechlorination is 2, 4, and 6 for DCE, VC and ethene, respectively, 8 for acetate and methane, and 2 for H_2.

4.3 Results and discussion

Chloroethenes reductive dechlorination at different HCO_3^- concentrations

The time course dechlorination measurements presented in Figure 4.1 show a short lag time for the onset of dechlorination of 0.55 mmol L^{-1} TCE. TCE to *cis*-DCE conversion was the fastest dechlorination step in all cultures, with only VC and ethene detected after day 5, regardless of the concentration of HCO_3^- added. A close monitoring of VC to ethene reduction rates between each GC measurement revealed that after day 5, dechlorination rates had slowed down at all HCO_3^- concentrations, especially in the cultures containing 30 mM (Figure 4.1G-H), suggesting an electron donor limitation. The measured H_2 levels on day 12 were 1.5 mmol L^{-1} at 2.5 mM HCO_3^- and 0.5 mmol L^{-1} at 5 mM HCO_3^-. At 10 and 30 mM HCO_3^-, no H_2 peak was detected on the GC-TCD on day 12. Immediately after injecting an additional 8.2 mmol L^{-1} H_2 on day 12, we observed an increase in the rates of VC consumption and ethene formation, as seen in Figure 4.1A-H.

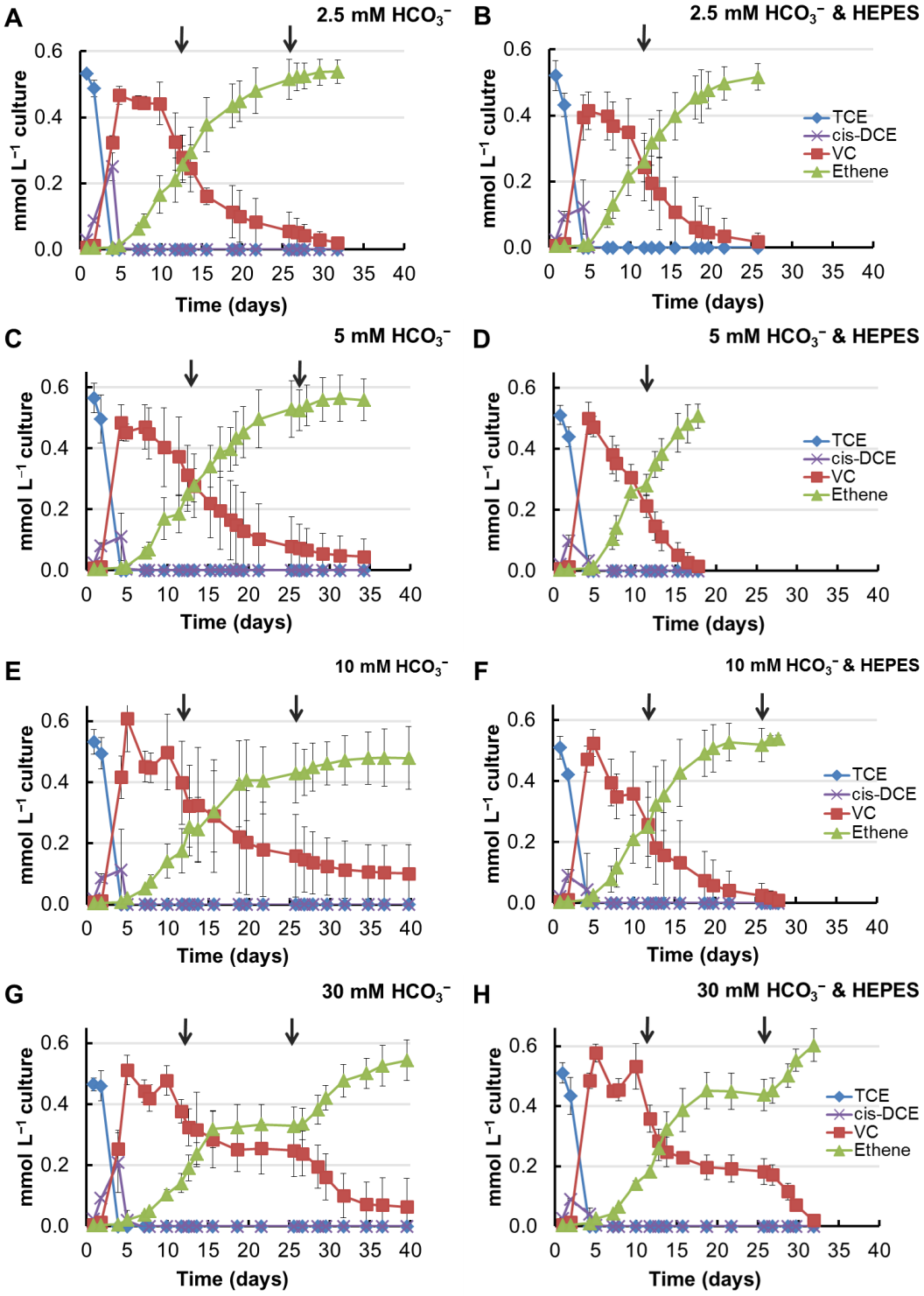


Figure 4.1 Chloroethenes dechlorination at different HCO_3^- concentrations.
Time course of chloroethenes dechlorination to ethene in cultures amended with H_2 as the sole electron donor and with HCO_3^- buffer (graphs A, C, E, and G) and a combination of HCO_3^- and HEPES buffers (graphs B, D, F, and H). The arrows represent the 2nd and 3rd addition of 8.2 mmol L^{-1} H_2. The error bars are standard deviations of triplicate cultures.

Following the second addition of H_2, all cultures reached ≥70% conversion of TCE to ethene. Complete TCE dechlorination (Figure 4.1D) was first observed between days 17 and 18 in cultures containing 5 mM HCO_3^- and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), which was provided as an additional buffer. Complete conversion to ethene was further observed in the cultures with 2.5 mM HCO_3^- & HEPES on day 26. A threefold increase in the 16S rRNA *Dehalococcoides* genes (data not shown) from 1.13×10^{11} ($\pm 4.98 \times 10^9$) copies L^{-1} (time 0) to 3.71×10^{11} ($\pm 2.78 \times 10^{10}$) and 3.67×10^{11} ($\pm 8.04 \times 10^9$) copies L^{-1} was detected after complete dechlorination at 5 mM HCO_3^- & HEPES and 2.5 mM HCO_3^- & HEPES, respectively. Chloroethenes conversion rates in the cultures containing 10 and 30 mM HCO_3^- were the slowest, as seen in Figure 4.1. The *Dehalococcoides* 16S rRNA gene copies per L in the cultures with HCO_3^- and HEPES after complete conversion to ethene were 2.07×10^{11} ($\pm 5.79 \times 10^9$) at 10 mM and 2.03×10^{11} ($\pm 5.59 \times 10^9$) at 30 mM (data not shown). The lower resulting cell density coupled to decreased dechlorination rates indicates that *Dehalococcoides* growth was diminished at the higher HCO_3^- concentrations (Student's *t* test; ≥70% confidence level).

We observed a second H_2 limitation at 10 and 30 mM HCO_3^-, with the complete cessation of VC reduction at 30 mM between days 18 and 26 (Figure 4.1G-H). Consequently, an additional dose of H_2 (8.2 mmol L^{-1}) was injected into all cultures still undergoing dechlorination. With the 3rd addition of electron donor, the 10 and 30 mM HCO_3^- cultures supplemented with HEPES dechlorinated all TCE to ethene by day 28 and 32 (Figure 4.1F and H), respectively. The parallels without HEPES showed incomplete conversion to ethene even by day 40 (Figure 4.1E and G) and VC

dechlorination had stalled once again on day 35, or it was proceeding at much reduced rates.

Methane and acetate production during TCE reductive dechlorination

In Figure 4.1, we show how H_2 was limiting dechlorination rates before the 2nd and 3rd H_2 addition at the different concentrations of HCO_3^- tested. The theoretical H_2 demand for 0.55 mmol TCE L^{-1} TCE is 1.65 mmol H_2 L^{-1}. Considering that the H_2 at time 0 was 8.2 mmol L^{-1}, five times in excess of the theoretical demand for dechlorination, the slower dechlorination rates observed, together with H_2 depletion, indicated that competing microorganisms were consuming H_2 faster than the dechlorinators. An increase in methane of only 0.01 mmol L^{-1} was detected at all HCO_3^- concentrations before day 4 (Figure 4.2A), which coincides with the disappearance of TCE and formation of less chlorinated daughter products (Figure 4.1). The lack of methane production was also confirmed by the qPCR data which show no relative increase in the numbers of *Archaea* gene copies L^{-1} at this time point compared to the 10% inoculum (Figure 4.2B, day 7). The lag time for methane production could have been due to the previously reported longer lag time of the methanogenic microorganisms (Fey and Conrad 2000) and the toxic effect of TCE on methanogens (Yang and McCarty 2000). Additionally, besides *Dehalococcoides*, other dechlorinators can use TCE as electron acceptor and H_2 as electron donor to produce *cis*-DCE. A competitive advantage of *Geobacter* spp., the other identified TCE to *cis*-DCE respirers in the inoculum culture (Ziv-El et al. 2011), over methanogens could have also contributed to a delayed onset of methanogenesis.

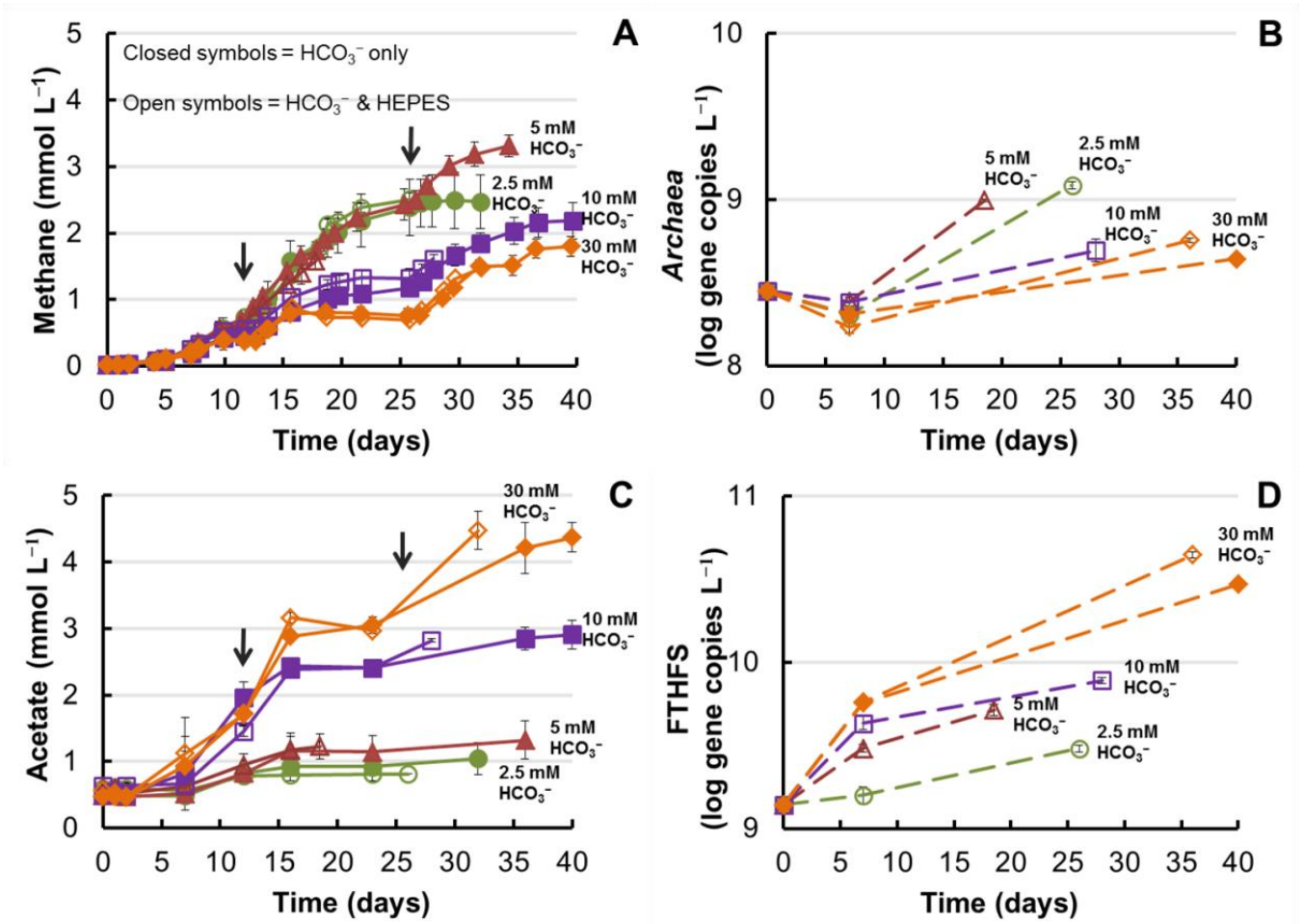


Figure 4.2 Methanogenesis and homoacetogenesis during active evolvement of reductive dechlorination. Methane (A) and acetate (C) production during reductive dechlorination in medium buffered with 2.5 (circles), 5 (triangles), 10 (squares), and 30 (diamonds) mM HCO_3^-. The error bars are standard deviations of triplicate cultures and the arrows represent the 2nd and 3rd addition of 8.2 mmol L^{-1} H_2. Quantification of methanogens, *Archaea* (B), and homoacetogens, FTHFS (D) using qPCR. The error bars are standard deviations of triplicate analytical runs.

Methanogenesis was mostly stimulated at 2.5 mM HCO_3^- and 5 mM HCO_3^-, and it was less active with increasing concentrations of HCO_3^- (Figure 4.2A). The methane production trends observed are supported by a higher increase in *Archaea* numbers at the lower HCO_3^- concentrations (2.5 and 5 mM in Figure 4.2B) compared to 10 and 30 mM (Figure 4.2B). At 30 mM HCO_3^-, we detected no net increase in methane between day 10 and 12, suggesting that methanogens, like dechlorinators, were also experiencing H_2 limitation. Once H_2 became available after the second addition, methane production rates quickly increased in all cultures (Figure 4.2A).

Upon the third addition of H_2 (day 26), methane no longer increased at 2.5 mM HCO_3^- even though H_2 was provided (Figure 4.2A, day 26-32), indicating a HCO_3^-, and not a H_2 limitation. Even though HCO_3^- was not measured due to analytical limitations, we were able to track HCO_3^- consumption via production of methane and acetate, as illustrated in Figure 4.3. The HCO_3^- utilization balance presented in Figure 4.3 shows that production of methane (and to a lesser degree acetate) exhausted all the HCO_3^- in the systems initially supplemented with 2.5 mM.

Homoacetogenesis exhibited the opposite trend to methanogenesis. According to the time course concentrations recorded and shown in Figure 4.2C, more acetate was produced when more HCO_3^- buffer was present. Additionally, among all conditions tested, the greatest increase in copies L^{-1} culture by day 7 of the formyltetrahydrofolate synthase (FTHFS) gene, a functional marker for acetogens, was detected at 30 mM HCO_3^- (Figure 4.2D), and the relative numbers of gene copies were lower with decreasing concentrations of HCO_3^-. Before the second addition of H_2, all cultures showed an increase of 0.3-1.3 mM acetate (Figure 4.2C). However, after injecting the

second dose of H_2, only a small rise in acetate was observed at 2.5 and 5 mM HCO_3^-. In contrast, at 10 and 30 mM HCO_3^-, we detected a boost in homoacetogenesis (Figure 4.2C) and corresponding higher increases in the FTHFS gene (Figure 4.2D).

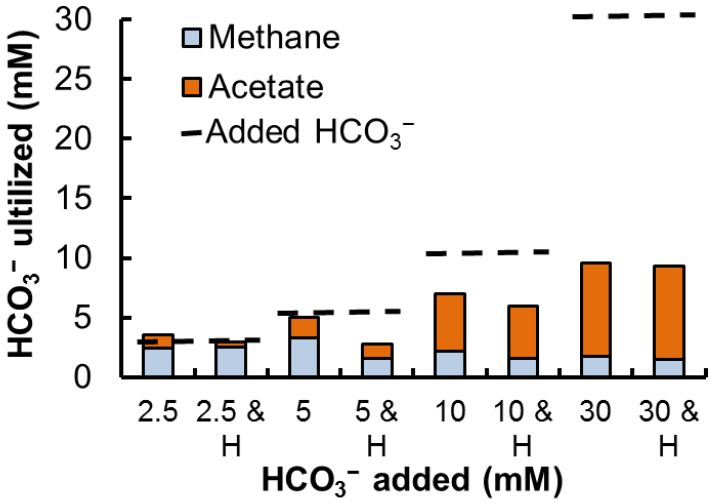


Figure 4.3 Calculated HCO_3^- consumption for methane and acetate production. Calculated HCO_3^- utilization by hydrogenotrophic methanogens and hydrogenotrophic homoacetogens at the end of the experiments in the absence or presence of HEPES (denoted as H on the X-axis). A maximum of 1 mM HCO_3^- was assumed as carryover from the 10% inoculum culture, which was grown in 10 mM HCO_3^- medium. The stoichiometric requirement for methane is one HCO_3^- and for acetate is two HCO_3^-.

The qPCR data for both methanogens and homoacetogens correlate well with our analytical data. The resulting increased levels of homoacetogens coupled to the lowest levels of methanogens at 30 mM HCO_3^- indicate benefits for the first group at the higher HCO_3^- concentrations. Unlike homoacetogens, the resulting methanogenic microorganisms were present at similar levels in cultures initially containing 2.5 and 5 mM HCO_3^- and less plentiful in cultures initially containing 10 and 30 mM HCO_3^- (Figure 4.2B). Overall, our findings are consistent with the lower HCO_3^- requirement for methane production: one mol HCO_3^- consumed for one mol methane (Eq. 4.1) vs. two mol HCO_3^- consumed for one mol acetate (Eq. 4.2). Additionally, these data are in

agreement with the findings of Florencio et al., 1995 (Florencio et al. 1995) on substrate competition between methylotrophic methanogens and methanol-utilizing acetogens in UASB reactors, where acetogenesis was significant and outcompeted methanogenesis only in the presence of exogenously supplemented HCO_3^-.

Distribution of electrons for H_2-consuming processes

The fate of electrons fed as H_2 is depicted in Figure 4.4. By day 12 (after one addition of H_2; Figure 4.4A), 70% or greater of the total added electrons can be accounted for towards the three main energy-deriving reactions, dechlorination, homoacetogenesis and methanogenesis, under all conditions tested. Biomass was not included in these balances, however, a 10-20% fraction of the total electrons can be assumed for cell synthesis (Rittmann and McCarty 2001). 1.65 mmol H_2, the theoretical H_2 requirement for dechlorination of 0.55 mmol TCE, equals to 3.3 me^- equivalents H_2, and each 8.2 mmol L^{-1} H_2 addition represents 16.4 me^- equivalents. Out of the three main processes occurring in our test systems, TCE dechlorination utilized a small fraction of 9.3% out of the total me^- equivalents for the cultures that completed dechlorination with two H_2 additions (Figure 4.1B and D), and 6.7% of the total me^- equivalents for those that received three H_2 additions (Figure 4.1A, C, E, F, G, and H).

From the H_2 me^- equivalents provided at time 0, only 18.3% would have been required to completely reduce TCE to ethene. As seen in Figure 4.1 and 4.4A, none of the cultures, regardless of their H_2 demand, completed dechlorination with the initial H_2. Additionally, the 10 and 30 mM HCO_3^- amendments with or without HEPES received H_2 fifteen times in excess of the theoretical demand for dechlorination, yet only the sets

supplemented with HEPES completed dechlorination, implicating an important pH factor, which is discussed in the next section.

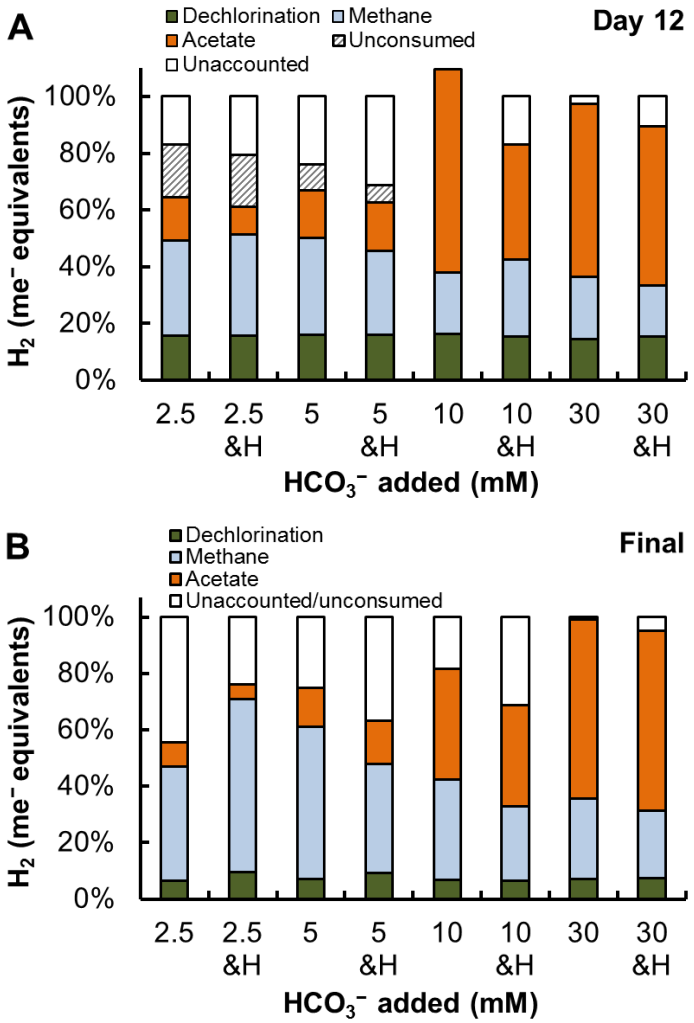


Figure 4.4 Distribution of electrons fed as H_2 towards dechlorination, methanogenesis, and homoacetogenesis at various HCO_3^- concentrations. (A) Average data from triplicate cultures on day 12 after addition of 16.4 me^- equiv. H_2 (8.2 mmol L^{-1}). (B) Average final data from triplicate cultures after addition of 33 me^- equivalents (16.4 mmol L^{-1} H_2 in 2.5 mM HCO_3^- & HEPES and 5 mM HCO_3^- & HEPES) and 49 me^- equivalents (32.8 mmol L^{-1} in all other sets). The presence of the additional buffer, HEPES, is denoted as H on the X-axis.

Overall, the fate of most H_2 me^- equivalents was to HCO_3^--driven reactions towards the production of methane and acetate. Acetate from hydrogenotrophic homoacetogenesis was also found to be the main sink of electrons in a field study that

used H_2 gas for remediation of chlorinated ethenes in groundwater (Edstrom et al. 2005). Moreover, Duhamel and Edwards 2007 (Duhamel and Edwards 2007) investigated the growth and yields of hydrogenotrophic methanogens, acetogens and dechlorinators during the process of dechlorination. The authors found that most of the electrons fed as methanol in 30 mM HCO_3^- buffered medium went towards acetogenesis and that methanogens were outcompeted by acetogens. Our data from 10 and 30 mM HCO_3^- corroborate their findings; however, one important additional finding from our experiments, as seen in Figure 4.2 and 4.4, is that methanogens can outcompete homoacetogens at low HCO_3^- concentrations (2.5 and 5 mM).

The results on TCE dechlorination, methanogenesis and homoacetogenesis from this work at different HCO_3^- concentrations offer some insights into which competing microbial groups will prevail and how HCO_3^- consumption affects rates of dechlorination. Furthermore, our study also alludes to how HCO_3^- drives the H_2 competition between organohalide respirers, methanogens, and homoacetogens. This important aspect has not been determined previously in reductive dechlorination, to our knowledge. In addition, for application purposes, it is important to consider how temperature could affect these findings, as these predictions might be somewhat different at lower temperatures, such as those in groundwater. Our experiments were performed at 30°C, however, temperature studies on organohalide respirers (i.e. *Dehalococcoides*) have documented slower rates of dechlorination at 10-15°C compared to their maximum rates at 30-35°C (Friis et al. 2007). Homoacetogens are even greater H_2 and HCO_3^- consumers than methanogens at lower temperatures (Fey and Conrad 2000; Kotsyurbenko et al. 2001), hence, the predominance of homoacetogens would be greater

in groundwater systems. Furthermore, because many homoacetogens can consume fermentables and/or H_2 to produce acetate (Drake 1994), it is important to consider homoacetogenesis as an electron sink and alkalinity-consuming process in dechlorination at the laboratory and field scale. Although comprehensive models on *in situ* reductive dechlorination have been developed (Clapp et al. 2004; Fennell and Gossett 1998; Robinson and Barry 2009; Robinson et al. 2009), the introduction of hydrogenotrophic homoacetogenesis in these models has not been considered.

Effect of pH on dechlorination in HCO_3^- -amended cultures

We supplemented HEPES to all HCO_3^- concentrations tested to separate between the effect of HCO_3^- as an electron acceptor/sink and the effect of pH changes resulting from microbial processes that use HCO_3^- as an electron acceptor, i.e. methanogenesis and homoacetogenesis. The time course measurements presented in Table 4.1 and the final measurements in Figure 4.5 uncovered a trend when HCO_3^- was the sole buffer: a higher pH increase with increasing HCO_3^- concentrations due to methanogenesis and homoacetogenesis HCO_3^--consuming reactions. This was not the case at 30 mM HCO_3^-, where we recorded a lower final pH than at 10 mM HCO_3^- (Figure 4.5) due to the buffering capacity from the 20 mM unconsumed HCO_3^- (Figure 4.3). However, in a separate experiment where we increased the total concentration of H_2 to 41 2 mmol L^-1 in cultures containing 30 mM HCO_3^-, we recorded a final pH of 9.6 under these conditions (data not shown). These cultures also exhibited slower rates of dechlorination compared to the data from Figure 4.1 and no ethene formed by day 40 of the experiments (data not shown).

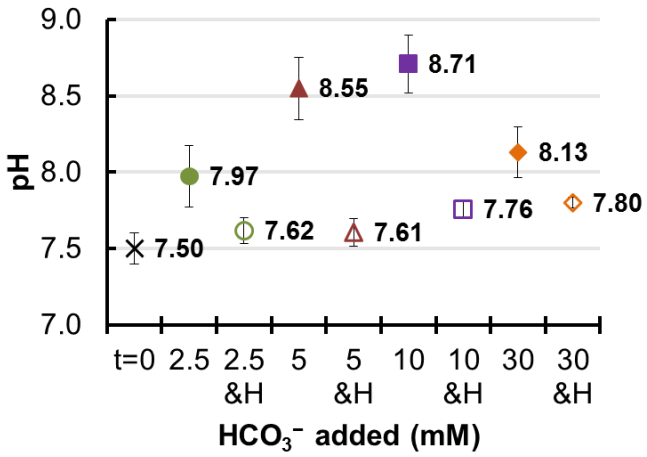


Figure 4.5 pH changes resulting from biological HCO_3^- consumption. Average initial (t=0) and final pH measurements in all HCO_3^- amendments from this study in the absence (closed symbols) or presence (open symbols) of HEPES. The error bars are standard deviations of triplicate cultures. The buffer HEPES is denoted as H on the X-axis.

An increase in pH at all HCO_3^- concentrations tested was also observed when HEPES was present as an additional buffer but the pH increase was within a much narrower range (Figure 4.5). We ran statistical analyses and determined that, because of better pH buffering, the rates of dechlorination were significantly faster (Student *t*-test, P <0.05) in the presence of HEPES, compared to when HCO_3^- was the sole buffer (Figure 4.1). In this study, we show that high pH can also occur in dechlorinating systems, especially in engineered systems fed with H_2, and this pH change can negatively impact chloroethenes reduction. A detrimental effect on TCE dechlorination that resulted in accumulation of mainly *cis*-DCE at pH 8.3 was previously observed in an anaerobic biotrickling filter (Popat and Deshusses 2009). Our results show that high pH is stressful to TCE dechlorinating microorganisms, hence, research on bioremediation of chloroethenes will greatly benefit from comprehensive pH studies.

Table 4.1 Time course pH measurements. Average pH values with standard deviations of triplicate cultures containing 2.5, 5, 10, and 30 mM HCO_3^- as the sole buffer and a combination of HCO_3^- and HEPES. The values in bold are the final pH measurements

	[HCO_3^-] mM							
Day	2.5	2.5 & HEPES	5	5 & HEPES	10	10 & HEPES	30	30 & HEPES
0	7.49 ± 0.06	7.48 ± 0.08	7.47 ± 0.05	7.51 ± 0.07	7.54 ± 0.05	7.46 ± 0.04	7.44 ± 0.03	7.43 ± 0.03
1	7.41 ± 0.07	7.43 ± 0.08	7.45 ± 0.07	7.51 ± 0.04	7.56 ± 0.03	7.45 ± 0.06	7.45 ± 0.09	7.50 ± 0.02
7	7.23 ± 0.05	7.38 ± 0.09	7.37 ± 0.06	7.44 ± 0.09	7.66 ± 0.18	7.47 ± 0.06	7.56 ± 0.07	7.58 ± 0.03
17	7.29 ± 0.03	7.26 ± 0.12	7.36 ± 0.03	7.48 ± 0.12	7.87 ± 0.11	7.53 ± 0.04	7.60 ± 0.11	7.60 ± 0.03
19	–	7.58 ± 0.01	–	**7.61 ± 0.09**	–	–	–	–
20	–	–	–		–	–	–	–
23	7.81 ± 0.06	–	7.92 ± 0.09		8.12 ± 0.11	–	–	–
26	–	**7.62 ± 0.09**	–		–	7.64 ± 0.01	7.68 ± 0.09	7.65 ± 0.01
28	–		–		–	**7.76 ± 0.05**	–	–
32	**7.97** ± 0.20		–		–		–	**7.80 ± 0.04**
35			**8.55 ± 0.20**		8.73 ± 0.10		8.07 ± 0.19	
40					**8.71 ± 0.19**		**8.13 ± 0.17**	

4.4 Conclusions

Despite the fact that HCO_3^- is a common natural buffer and addition of more HCO_3^- can counteract pH deviations from the optimum range for dechlorination, the results of our study point out that 1) high HCO_3^- concentrations increase the H_2 demand, and that 2) consumption of HCO_3^- contributes to pH increases that could adversely affect TCE dechlorination rates or result in accumulation of toxic intermediate by-products (i.e., DCE and VC). Our findings regarding the effect of pH increases from HCO_3^--consuming reactions are relevant for *ex situ* chloroethenes remediation technologies that provide H_2 and for laboratory amendments. When fermentable substrates are used to stimulate reductive dechlorination, or, in the case of groundwater where HCO_3^- is replenished from minerals dissolution or organics oxidation, this increase in pH will likely be offset by the protons produced from fermentation or by the constant supply of buffer.

However, the lessons learned from this study on dechlorination, methanogenesis, and homoacetogenesis highlight that HCO_3^-, especially when abundant, could be an important variable for biologically-driven TCE dechlorination, as it has a prominent role as an electron acceptor by stimulating competing H_2-consuming processes. Our findings also point out that a shift in the main H_2 competitors occurs depending on the HCO_3^- concentration available in the environment, with homoacetogens as the greater electron sink at high HCO_3^-, and methanogens as the main H_2 competitors at low HCO_3^-.

CHAPTER 5

SUCCESSFUL OPERATION OF CONTINUOUS REACTORS AT SHORT RETENTION TIMES RESULTS IN HIGH-DENSITY, FAST-RATE *DEHALOCOCCOIDES* DECHLORINATING CULTURES[4]

5.1 Introduction

In the United States, at least 60% of the National Priorities List Superfund sites and at least 17% of groundwater sources have detectable levels of chlorinated solvents, including trichloroethene (TCE) and perchloroethene (PCE) (ATDSR 2011; Moran et al. 2007). The presence and persistence of these compounds in the environment is a major threat to public health. Biological reduction by members of the bacterial genus *Dehalococcoides* is a common and cost-effective avenue for *in situ* bioremediation of sites contaminated with chlorinated solvents. *Dehalococcoides mccartyi* strains provide a unique solution to remediating chlorinated ethenes as they can reductively dechlorinate PCE and TCE to the non-toxic end product, ethene, with transient production of *cis*-dichloroethene (*cis*-DCE) and vinyl chloride (VC) (Ellis et al. 2000; Loffler et al. 2013; Maymo-Gatell et al. 1997).

The common laboratory cultivation method for TCE- and PCE-dechlorinating cultures containing *D. mccartyi* is in batch reactors under batch-fed conditions. To achieve high concentrations of *Dehalococcoides* (e.g., 10^{11}-10^{12} cells L^{-1}), these cultures must be fed with high concentrations (mM range) of chlorinated ethenes. Batch systems can be cumbersome, as self or competitive inhibition of dechlorination, and toxicity of

[4] This chapter was prepared as a manuscript and has been submitted for publication.

Dehalococcoides and other community members prevents feeding TCE or PCE in high concentrations (Chambon et al. 2013). Therefore, batch cultivation of *Dehalococcoides* entails receiving and reducing (mostly to ethene) several non-inhibitory, successive feeds of electron acceptors.

Laboratory studies, as well as bioaugmentation applications at contaminated sites, often require large volumes of culture containing high-density *Dehalococcoides* cells. Continuous stirred-tank reactors (CSTRs) are well established sources for yielding large volumes of steady-state cells or proteins (Hoskisson and Hobbs 2005). Moreover, a short-hydraulic retention time (HRT) CSTR is an ideal tool for community-based transcriptomics or proteomics studies, which require cells constantly growing and at high densities. Unlike in a batch reactor, theoretically, inhibition or toxicity to microbial community members can be minimized in a CSTR by continuously maintaining low concentrations of TCE or PCE. These low concentrations should enable feeding higher concentrations of chlorinated solvents in the same time interval than in a batch reactor, thus achieving higher *Dehalococcoides* concentrations.

Kinetic parameters suggest the potential for culturing *Dehalococcoides* at a short HRT in a CSTR. Specifically, doubling times of ≤1 d have been reported for some *D. mccartyi* pure cultures (Cheng and He 2009; Maymo-Gatell et al. 1997) and *D. mccartyi* enrichment cultures (Vainberg et al. 2009). Furthermore, the low Monod half-maximum rate concentrations (K_s) for TCE, *cis*-DCE, and VC of *D. mccartyi* (<5 µM) (Popat and Deshusses 2011) indicate that these microbes should perform well in a continuous-flow reactor where the aqueous concentrations of electron acceptors are low. Despite these potential advantages, dechlorination studies using CSTRs are limited (Berggren et al.

2013b; Carr et al. 2000; Drzyzga et al. 2001; Sabalowsky and Semprini 2010; Yang and McCarty 1998; Zheng et al. 2001). In the past two decades since the discovery of *D. mccartyi*, there has been little success in achieving sustainable growth of dechlorinating cultures that are able to reduce chloroethenes to mostly ethene. In fact, Yang and McCarty (1998) and Berggren et al. (2013) are the only two cases reported for the complete conversion of PCE and TCE to ethene in CSTRs (*Dehalococcoides* concentrations were not reported) operated at a 36- and a 50-d HRT, respectively. These HRTs are even longer than those of methanogenic anaerobic digesters (Tchobanaglous et al. 2003), even though the growth rates of *D. mccartyi* are faster than those of acetoclastic methanogens (Tchobanaglous et al. 2003).

A good understanding of the growth requirements and microbial interactions in dechlorinating cultures containing *Dehalococcoides* should allow for cultivation of *Dehalococcoides* in a high-growth rate system, such as a short-HRT CSTR. We hypothesized that culturing *Dehalococcoides* communities in a short-HRT CSTR has, thus far, been impeded for two major reasons. First is inhibition due to toxicity of the chlorinated electron acceptors. For growth of dechlorinating cultures to occur, a high enough concentration of chlorinated solvents must be fed to attain high concentrations of *Dehalococcoides*. Yet, very high removal of TCE or PCE to ethene must occur to avoid inhibition (the effluent concentrations of chlorinated ethenes must be low). Second is the stringent competition between *Dehalococcoides* and other community members for the obligate electron donor, H_2.

We report here the successful cultivation and performance of a *D. mccartyi*-containing culture in a CSTR operated at a 3-d HRT and fed with 1 and 2 mM TCE. To

achieve this successful, proof-of-concept CSTR operation, we built upon data from prior CSTR runs in our laboratory and a systematic study evaluating HCO_3^- as a competing electron acceptor in microbial dechlorination of TCE (Delgado et al. 2012). In the previous CSTR runs, summarized in the Table 5.1, we tested different operating conditions (TCE concentration, electron donor concentration, and HRT) in 30 mM bicarbonate (HCO_3^-)-buffered medium. In the HCO_3^- study (Delgado et al. 2012), we saw that high HCO_3^- levels (i.e., 30 mM) increase the H_2 demand by stimulating homoacetogenesis and methanogenesis, two processes competing for H_2 and, therefore, potentially limiting reductive dechlorination of chloroethenes. Thus, the successful CSTR runs presented here were achieved with an optimized medium composition with a low bicarbonate concentration, thereby managing the microbial communities and achieving low effluent concentrations of chlorinated ethenes.

Table 5.1 Experimental conditions tested for CSTR optimization

Run	HRT (d)	[Substrate]$_{influent}$ TCE (mM)	Lactate (mM)	Methanol (mM)	HCO_3^-/CO_2 buffer (mM)	Notes[a]
1	4	3	10	12	30	Ethene was the most prevalent dechlorination end-product throughout three HRTs; low methanogenesis.
2	2	4	10	12	30	Ethene was the most prevalent dechlorination end-product throughout three HRTs; low methanogenesis.
3	8	8.37	15-20	12	30	Conversion to mostly ethene occurred initially, however TCE accumulated after two HRTs and performance did not recovered; active methanogenesis.
4	4	8.37	20	12	30	Conversion to ethene and VC occurred within the first two HRTs. *cis*-DCE accumulated after four HRTs and performance did not recover; active methanogenesis.
5	3	4	20	12	30	TCE accumulated after four HRTs and performance did not recover.
6	4	4	20	12	30	Conversion to *cis*-DCE and VC occurred operating for six HRTs; active methanogenesis.
7[b]	3	1	7.5	15	5 (+20 mM HEPES)	Conversion to mainly ethene was achieved and was sustained.
8[b]	3	2	10	15	5 (+20 mM HEPES)	Conversion to mainly ethene was achieved and was sustained.

[a] all CSTR conditions were tested for at least three HRTs or until performance of the reactors decreased.
[b] run 7 and 8 were the most succesful and are presented in detail in the Results and Discussion sections.

5.2 Materials and methods

Bioreactor design and operation

A schematic and photograph of the reactor setup used (Bioreactor 1 and 2) are shown in Figure 5.1. Each reactor consisted of a 0.65-L glass bottle sealed with a butyl rubber stopper and a screw cap. The stopper was perforated to fit the influent and effluent lines, and a gas sampling port containing a removable septum (IceBlue® Septa, Restek, USA). The septum was changed several times throughout the runs and some losses of headspace compounds occurred due to brief flushing with ultra-high purity (UHP) N_2. The actual liquid and headspace operating volumes were 0.5 L and 0.1 L, respectively. Each reactor was magnetically stirred at 200 RPM and submerged in a water bath set at 30°C. Influent medium was pumped from 5-L glass bottles containing 4 L of medium with a Minipuls 3 peristaltic pump (Gilson, Inc., USA) to achieve a 3-d HRT. All lines and tubing used were 1/8" diameter stainless steel or Viton material. The liquid sampling port was located before the effluent collection bottle. The effluent culture was collected into 1-L glass bottles equipped with 1-L gas Tedlar bags (SKC Inc., USA) for gas collection.

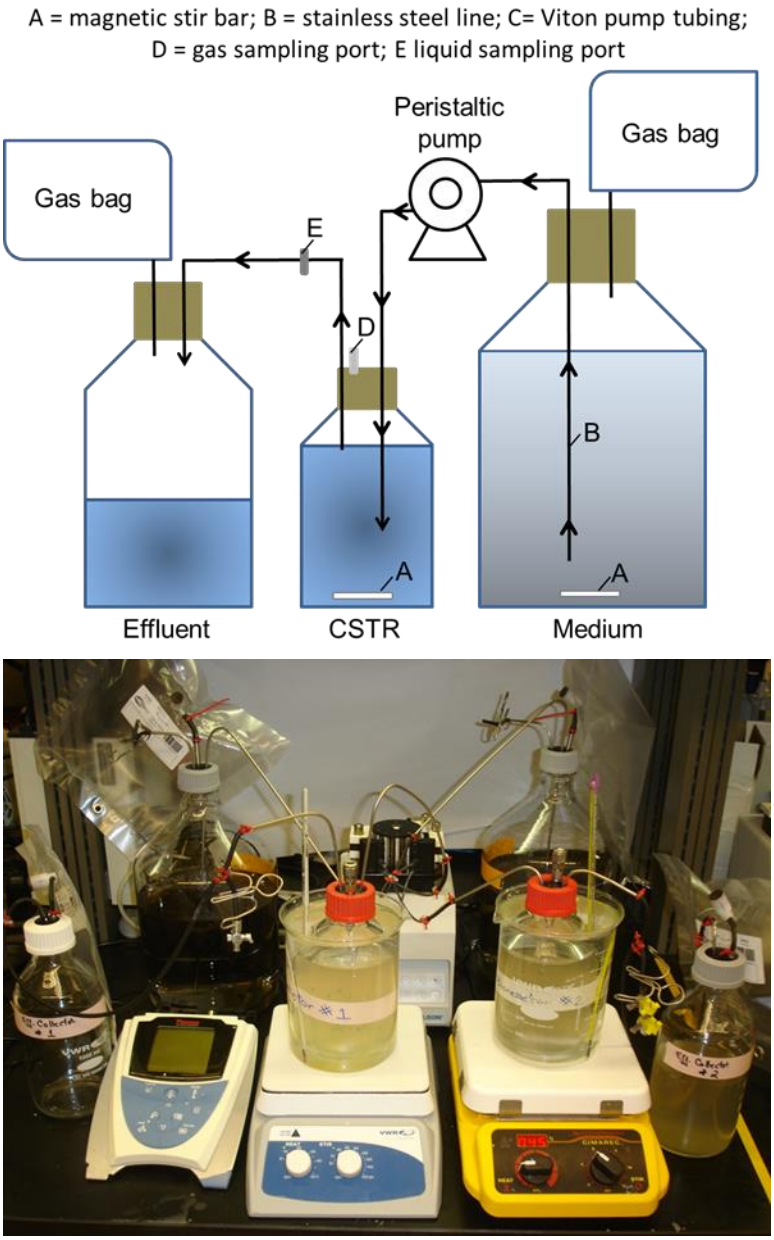


Figure 5.1 Schematic (top panel) and photograph (bottom panel) of the experimental apparatus employed in this study.

Bioreactor 1 and Bioreactor 2 were operated under identical conditions for a total of 120 and 100 days, respectively. During this time, the bioreactors were fed TCE-containing medium continuously at all times, except for the initial four days after inoculation and for seven days in between switching the concentrations of TCE from 1 to

2 mM. Before increasing the TCE concentration in the influent medium, the bioreactors were also flushed with UHP N_2 to remove headspace gases.

Inoculum culture and medium composition

The culture employed for the studies herein was DehaloR^2 (Ziv-El et al. 2011), a TCE to ethene dechlorinating consortium containing *Dehalococcoides* and *Geobacter*. DehaloR^2 was initially grown in a CSTR fed with 3 mM TCE at a 4-d HRT (Table 5.1, Run 1). The culture from this run was collected and stored at 4°C for 15 months prior to inoculating the bioreactors presented herein. 0.5 L DehaloR^2 culture (100% vol/vol) per reactor was inoculated on day 0. Trace concentrations of *cis*-DCE and VC were present in this culture during storage; therefore, we added 2 mM lactate and kept the reactors in batch mode for ~4 days to reduce the chlorinated ethenes to ethene before proceeding to continuous operation.

We prepared reduced anaerobic mineral medium containing 1 mM TCE (aqueous concentration), 7.5 mM sodium DL-lactate, 15 mM methanol, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5 mM $NaHCO_3$, 5 mL L^{-1} ATCC vitamin supplement, 500 μg L^{-1} vitamin B_{12}, 0.25 μg L^{-1} resazurin, 0.2 mM L-cysteine, and 0.2 mM Na_2S x 9 H_2O. The salts and trace nutrients added per liter medium were those described in Delgado et al. (Delgado et al. 2012). In the medium with 2 mM TCE, the lactate and HEPES were increased to 10 mM and 20 mM, respectively, NaCl was decreased to 0.1 g L^{-1}, and methanol was kept at 15 mM. The influent medium pH was adjusted to 7.5-7.8 with 10 N NaOH. The same base medium composition was used for previous CSTR runs presented in Table 5.1, except the noted differences summarized in the table. We first autoclaved the medium, boiled it under a stream of UHP N_2, and

then added the reducing agents. To avoid fluctuations in TCE concentrations in the media bottles from changes in the liquid-headspace ratios during continuous operation, the bottles were fitted with collapsible 3-L gas Tedlar bags filled with UHP N_2. Abiotic degradation or TCE losses did not occur.

Chemical analyses

We sampled gas from the reactors to quantify the concentrations in the headspace of TCE, *cis*-DCE, VC, ethene, methane, and H_2. The methods for the Shimadzu gas chromatography instruments was previously described (Delgado et al. 2012). The concentrations of chlorinated ethenes and ethene in the liquid were calculated using Henry's constants (K_H) for each compound:

$$[Compound]_{liq} = [Compound]_{gas}/K_H \quad \text{(Equation 5.1)}$$

We obtained dimensionless Henry's constants (mM_{gas}/mM_{liq}, T = 30 °C) experimentally for the mineral medium used in this study for TCE (0.49), *cis*-DCE (0.17), VC (1.32), and ethene (9.00). Gas concentrations were used to estimate liquid concentrations based on the above Henry's constant. The flow of chlorinated ethenes and ethene out of the reactors was mainly through the liquid phase, although a small fraction of these compounds was in the gas, as shown in Figure 5.2. Because of the small gas flow rates and the difficulty in separating liquid and gas effluents, we did not measure the total gas production, but estimated it according to the mol balance equation below:

$$[TCE]_{in} \times Q_{liq} = [Ethenes]_{out\ gas} \times Q_{gas} + [Ethenes]_{out\ liq} \times Q_{liq} \quad \text{(Equation 5.2)}$$

in which [TCE] = TCE aqueous concentration (mM), [Ethenes] = cumulative concentration of chlorinated ethenes (TCE, *cis*-DCE, VC), and ethene in the reactor and effluent (mM), and Q = flow rate (mL d^{-1}).

We removed liquid samples to measure lactate, methanol, acetate, and propionate using high purity liquid chromatography (HPLC) (Delgado et al. 2012). We used an Orion pH meter (Thermo Scientific, USA) to monitor the pH, which ranged from 6.3 to 7.5. We performed pH adjustments to ~7 with 10 N NaOH only when the pH inside the reactors dropped to 6.3.

Microbial ecology

We extracted total genomic DNA from pellets made with 1.5 mL liquid samples according to the protocol previously published (Ziv-El et al. 2011). Quantitative real-time PCR (qPCR) assays were performed targeting the 16S rRNA genes of *D. mccartyi*, *Geobacteraceae*, and *Archaea*, and formyltetrahydrofolate synthase (FTHFS) (gene involved in the pathway for acetate production by homoacetogens) as described by Ziv-El et al. (Ziv-El et al. 2012b). We also performed qPCR tracking the reductive dehalogenase genes of *D. mccartyi*, *tceA*, *vcrA*, and *bvcA*, using the qPCR protocol, primers, probes, reagent concentrations, and PCR conditions detailed previously (Ziv-El et al. 2012b), except each reductive dehalogenase gene was assayed separately.

Conversion rates and long-term viability of CSTR-grown culture

Once pseudo steady-state (defined as stable conversion to ethene) was achieved for the 1 mM and 2 mM TCE continuous runs, we determined the maximum rates of conversion, R_{max}, for TCE, *cis*-DCE, and VC. We transferred 100 mL effluent culture to 160-mL serum glass bottles, and flushed for 20 min with UHP N_2 gas to remove any carry-over ethenes. Then, we provided a chlorinated electron acceptor (0.5 mmol $L_{culture}^{-1}$ of either TCE, *cis*-DCE, or VC), 5 mM lactate, 12 mM methanol, and 10 mL H_2 (4.1 mmol $L_{culture}^{-1}$). The bottles were incubated at 30 $^{\circ}$C on an orbital shaker set at 200

RPM. We measured the concentration of dechlorination products formed over short time intervals (five hours or less) in order to minimize increases in dechlorinating populations. qPCR tracking the *D. mccartyi* 16S rRNA gene confirmed that these bacteria had not grown significantly throughout the course of these short tests (data not shown). All R_{max} values were determined from at least triplicate experiments.

The culture produced in the CSTR from Runs 1-2 and 7-8 in Table 5.1 was stored in a 4°C refrigerator and periodically monitored for activity. Viability experiments consisted of transferring 10 mL stored culture to 160-mL serum bottles containing 90 mL anaerobic medium (10% inoculum vol/vol), adding 0.5-1 mmol $L_{culture}^{-1}$ TCE, 5 mM lactate, and 12 mM methanol, and monitoring TCE dechlorination to ethene in time course experiments.

5.2 Results

Dechlorination performance in a 3-d HRT CSTR fed with 1 mM or 2 mM TCE

We initially assessed the dechlorination activity (performance of the culture) in the CSTRs by measuring TCE and its dechlorination products using a GC. Figure 5.2 shows the performance of the replicate CSTRs fed 1 mM TCE at a 3-d HRT. *cis*-DCE and VC initially accumulated in the bioreactors within the first two HRTs; however, by day 11, ethene became the prevalent dechlorination end-product, and >90% conversion of TCE to ethene was observed thereafter. Both bioreactors reached dechlorination pseudo steady-state after ~5.5 HRTs, which was maintained until the end of this continuous run.

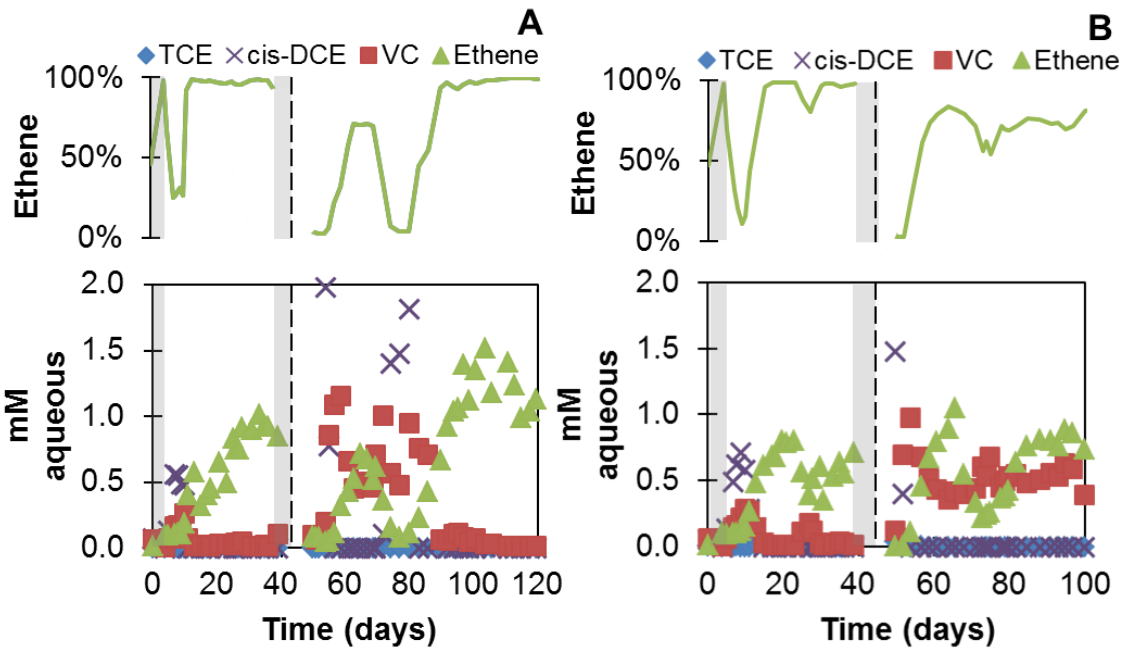


Figure 5.2. Dechlorination of 1 mM TCE and 2 mM TCE influent and the corresponding percent ethene conversion (top line graphs) in CSTRs operated at a 3-d HRT. The light gray shaded areas are periods of batch operation and the dashed line represents the start of the 2 mM TCE continuous feed. Shown are (A) Bioreactor 1 and (B) Bioreactor 2.

When the influent was 2 mM TCE, the bioreactors exhibited the same conversion trends as when initially fed with 1 mM TCE (Figure 5.2). For the first several HRTs, *cis*-DCE and VC were the main dechlorination products. Conversion to mostly ethene was achieved in ~14 days (day 65), but performance declined shortly after (Figure 5.2A). We believe this decline was due to an oxygen leak into the reactor from a damaged influent pump tubing. Once the tubing was replaced, the reactor recovered, and a pseudo steady-state with greater than 93% conversion to ethene was achieved by day 94 with 2 mM TCE influent concentration, and sustained for 9 subsequent HRTs (Figure 5.2A). The duplicate bioreactor presented in Figure 5.2B also reached conversion to mostly ethene, with a pseudo-steady state of ~80% reduction to ethene of 2 mM-fed TCE.

Growth of *Dehalococcoides* and enrichment of efficient dechlorinating microbial communities

The high conversion to ethene was coupled to increases in *Dehalococcoides* densities. We monitored the growth of *D. mccartyi* every HRT until pseudo steady-state was achieved. Figure 5.3A shows the initial concentration of *D. mccartyi* and the average pseudo steady-state abundances of 1.3×10^{12} and 1.6×10^{12} cells $L_{culture}^{-1}$ when continuously feeding 1 or 2 mM TCE, respectively, at a rate of biomass production of 3.3×10^{11} *D. mccartyi* cells $L_{culture}^{-1}$ d^{-1}. In terms of *D. mccartyi* diversity/composition, the CSTR-grown culture contained the three previously identified reductive dehalogenase genes, *tceA* (Magnuson et al. 1998), *vcrA* (Muller et al. 2004), and *bvcA* (Krajmalnik-Brown et al. 2004). Figure 5.3B highlights that concentrations of the three reductive dehalogenase genes increased during operation, reaching their highest levels during the 2 mM-TCE pseudo-steady state, with abundances of 10^{11} copies L^{-1} for *tceA* and *vcrA*, and 10^{8} copies L^{-1} for *bvcA*. Besides *Dehalococcoides*, DehaloR^2 culture contains one other identified dechlorinating genus, *Geobacter* (Ziv-El et al. 2011; Ziv-El et al. 2012c), which only partially reduces TCE to *cis*-DCE (Sung et al. 2006a). *Geobacter*, assayed using the 16S rRNA gene of *Geobacteraceae*, also increased throughout the two operating conditions (Figure 5.3A). The densities obtained for *Geobacteraceae* in our CSTRs were 6.4×10^{10} gene copies $L_{culture}^{-1}$. Data on growth of TCE/PCE-reducing *Geobacter* in CSTRs for pure culture or for mixed communities are absent from the literature; however, the abundances obtained for these microbes in our CSTRs are also on the high end compared to those in batch-fed mixed dechlorinating cultures (Duhamel and Edwards 2007; Ziv-El et al. 2011).

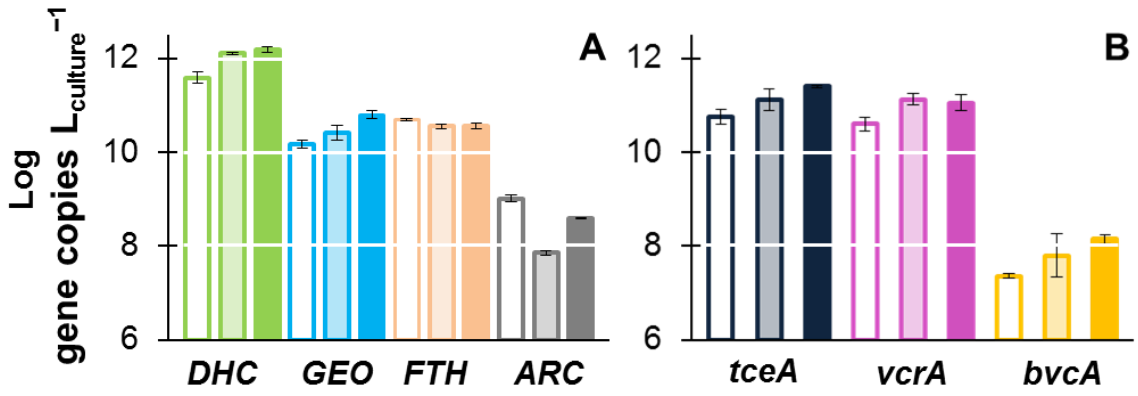


Figure 5.3 Microbial populations abundance in a 3-d HRT CSTR determined by qPCR at time 0 (no-fill bars), 1 mM TCE pseudo steady-state (light-filled bars), and 2 mM TCE pseudo steady-state (dark-filled bars). (A) Log concentrations of *Dehalococcoides mccartyi* (DHC), *Geobacteraceae* (GEO), FTHFS (FTH), and *Archaea* (ARC). (B) Log concentrations of *Dehalococcoides mccartyi* functionally-defined reductive dehalogenase genes, *tceA*, *vcrA*, and *bvcA*. All error bars show standard deviations of replicate samples (*time 0*, n = 2; *1 mM TCE*, n = 4; *2 mM TCE*, n = 3) and analytical qPCR reactions (*time 0*, n = 6; *1 mM TCE*, n = 12; *2 mM TCE*, n = 9).

Fate of electron donors and H_2-induced microbial interactions

Consumption of the provided fermentable substrates is presented in Figure 5.4A and C. Lactate was not detectable in all measurements at both influent TCE concentrations. Approximately half of the 15 mM methanol was consumed for the phase with 1 mM TCE influent, while close to complete methanol consumption was recorded at 2 mM TCE pseudo-steady state (Figure 5.4A and C). The duplicate bioreactors behaved similarly in terms of lactate and methanol consumption in the 1 mM TCE and 2 mM TCE runs, although the bioreactor converting TCE to ~80% ethene (Figure 5.2B) did not completely consume the influent methanol until the last HRT.

As a result of lactate and methanol fermentation, H_2 concentrations in the headspace of the bioreactors were 0.3-0.5 mM for the 1 mM TCE run. However, with 2 mM TCE, H_2 was no longer detected in the headspace (the detection limit for our H_2 measurements was 0.018 mmol L^{-1} gas concentration) despite a higher lactate

concentration feed. One of the driving hypotheses of this study was that growth of *Dehalococcoides* coupling high cell densities to dechlorination of TCE to ethene can occur if competition for H_2 by non-dechlorinating populations is minimized. H_2-oxidizing methanogens, the only type of methanogens in the inoculum culture, were initially present at concentrations of 10^9 gene copies $L_{culture}^{-1}$ and decreased by two orders of magnitude to 10^7 gene copies $L_{culture}^{-1}$ during the 1 mM TCE run (Figure 5.3A). The decrease in gene copies of methanogens was also corroborated by the gradual decrease in methane in the bioreactors, as seen in Figure 5.4B and D. During the 2-mM TCE continuous run, the gene copies of methanogens per liter culture increased to 10^8 (Figure 5.3A) and, hence, methane concentrations in the liquid were up to 1.3 mM (Fig. 5.4B and D). This was likely a consequence of the increase in lactate influent concentration from 7.5 mM to 10 mM, which subsequently yielded additional H_2 and HCO_3^-/CO_2 as growth substrates for hydrogenotrophic methanogens. The FTHFS gene copies of hydrogenotrophic homoacetogens, another competing sink coupling H_2 oxidation to the reduction of HCO_3^-, were 5×10^{10} initially, decreased during the 1-mM run and remained fairly constant at the 2-mM pseudo steady-state (Figure 5.3A).

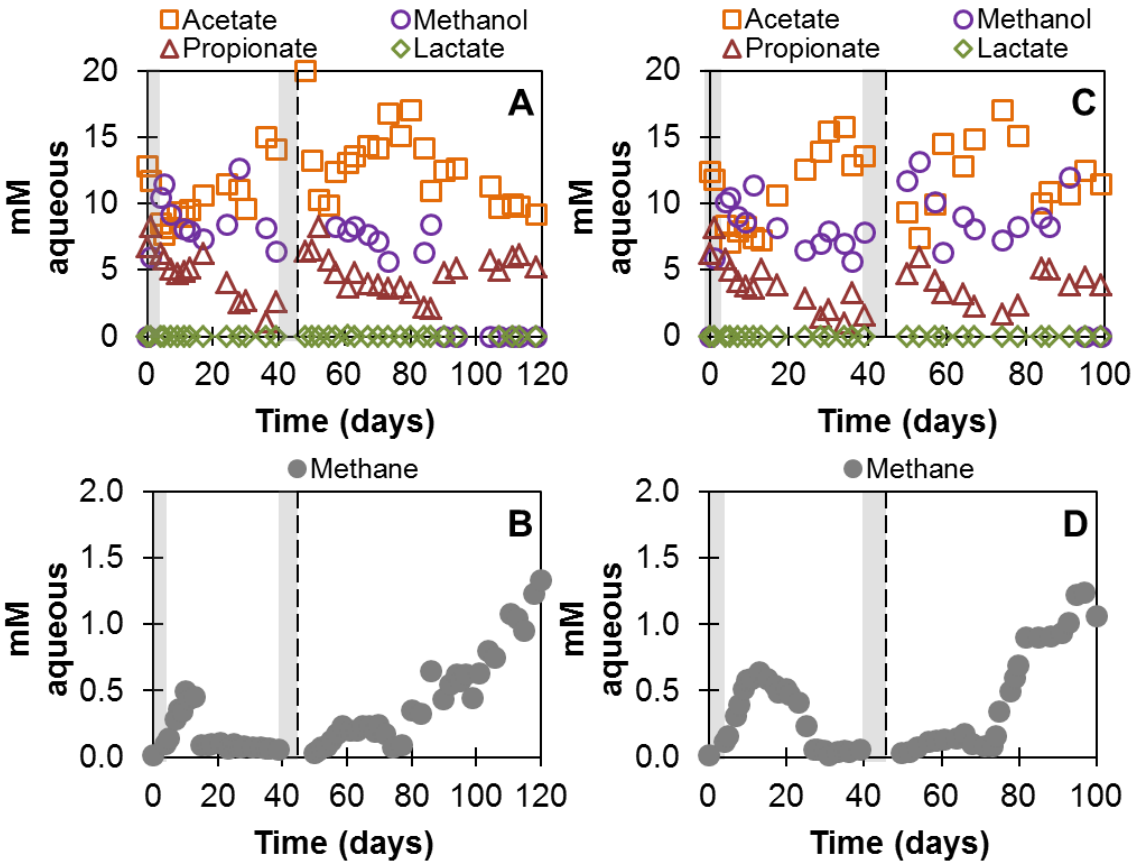


Figure 5.4 Consumption of influent lactate and methanol and production of acetate, propionate, and methane during continuous feed of medium containing 1 mM TCE and 2 mM TCE in a 3-d HRT CSTR. The light gray shaded areas are periods of batch operation and the dashed line represents the start of the 2 mM TCE continuous feed. Shown are (A-B) Bioreactor 1 and (C-D) Bioreactor 2.

Dechlorination kinetics of the CSTR-grown culture

Table 5.2 summarizes the maximum conversion rates, R_{max}, at pseudo-steady state obtained from the culture produced in the CSTR grown with the two concentrations of TCE. Experimental data for the values in Table 5.2 were obtained from separate short-term batch experiments for each individual chlorinated ethene (examples shown in Figure 5.5). These experiments were performed to ensure that the rates of dechlorination were within the same order of magnitude for all chlorinated ethenes. With the culture produced when continuously feeding 2 mM TCE, we obtained an overall rate of

dechlorination of 0.16 (±0.010) mmol Cl^- $L_{culture}^{-1}$ h^{-1}. This rate surpasses the previously reported batch-grown DehaloR^2 maximum rate of 0.04 mmol Cl^- released $L_{culture}^{-1}$ h^{-1} (or 0.92 mmol Cl^- $L_{culture}^{-1}$ d^{-1}) (Ziv-El et al. 2012a), which was obtained by feeding a total of 3 mmol $L_{culture}^{-1}$ TCE in three consecutive additions of 1 mmol L^{-1}.

Table 5.2 Maximum conversion rate (R_{max}) of chloroethenes by DehaloR^2 culture produced in a CSTR fed with 1 mM TCE and 2 mM TCE influent concentrations. The R_{max} values are averages with standard deviations of at least triplicate experiments as those shown in Figure 5.5

	R_{max} (mmol $L_{culture}^{-1}$ h^{-1})		
$[TCE]_{in}$	**TCE**	***cis*-DCE**	**VC**
1 mM	0.044 (±0.004)	0.023 (±0.002)	0.007 (±0.001)
2 mM	0.134 (±0.016)	0.055 (±0.018)	0.017 (±0.007)

The methodologies to determine culture rates vary between research groups, which makes comparisons challenging. Schaefer et al. (Schaefer et al. 2009) employed a similar experimental approach as described in our study to determine maximum rates of conversion. As seen in Table 5.2 at 2 mM TCE influent, R_{max} values for the culture produced in this study are four times greater for TCE to *cis*-DCE and *cis*-DCE to VC than those reported by SDC-9 culture (in Schaefer et al. (2009), 0.04 and 0.02 Cl^- mmol $L_{culture}^{-1}$ h^{-1}, respectively), while VC to ethene rates of DehaloR^2 measured here are lower than those of SDC-9 by a factor of two (in Schaefer et al. (2009), 0.04 mmol Cl^- $L_{culture}^{-1}$ h^{-1}).

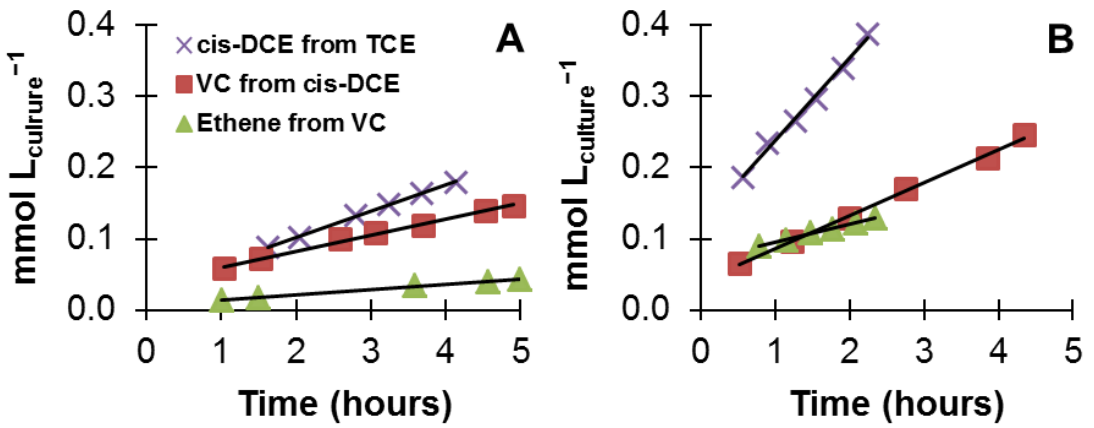


Figure 5.5 Experimental time-course measurements to determine the maximum rate of conversion, R_{max}, for the culture produced in a 3-dHRT CSTR fed with an influent containing (A) 1 mM TCE and (B) 2 mM TCE. 0.5 mmol L^{-1} TCE, *cis*-DCE, or VC was added to the effluent culture in serum batch bottles in separate experiments. The production rate of the lesser chlorinated products, *cis*-DCE, VC, or ethene, was measured over short periods (5 hours or less) to minimize microbial growth. The points are experimental measurements and the lines are linear fits of the experimental data.

We predicted that R_{max} and *D. mccartyi* cell density would roughly double when the influent concentration of TCE was increased from 1 mM to 2 mM. Table 5.2 and Figure 5.5 reveal that the rates of TCE and *cis*-DCE dechlorination were three times greater, while VC dechlorination rates were two times greater at 2 mM TCE influent. Yet, *D. mccartyi* concentrations increased only from 1.3×10^{12} (1-mM TCE run) to 1.6×10^{12} cells $L_{culture}^{-1}$ (2-mM TCE run). A plausible explanation for the apparent discordance between the higher rates of dechlorination and those of *D. mccartyi* cell copies is related to the growth of *Geobacter* dechlorinators in the culture. *Geobacter* increased ~2.5 fold when the CSTR medium contained 2 mM TCE (Figure 5.3A). This suggests that the contribution of *Geobacter* in the reduction of TCE to *cis*-DCE increased significantly at 2 mM TCE feed, when compared to the contribution of *D. mccartyi* for this dechlorination step.

Culture viability after prolonged storage

An advantage in producing dense microbial cultures containing *Dehalococcoides* is that they can be cultured in the laboratory and stored for later usage for prolonged periods without significant loss in activity. The culture initially produced in our CSTR (Run 1, Table 5.1) was stored for extended periods at 4°C. Figure 5.6A shows that complete dechlorination of ~0.7 mmol $L_{culture}^{-1}$ TCE occurred in 6 days after the culture had been stored in a refrigerator for seven months. After 15 months of storage, the same concentration of TCE was reduced to 80% ethene in 15 days (Figure 5.6B), implying that, while some loss of activity will occur (due to cell decay), these cultures maintain good dechlorinating activity profiles when the appropriate conditions are provided for revival and growth.

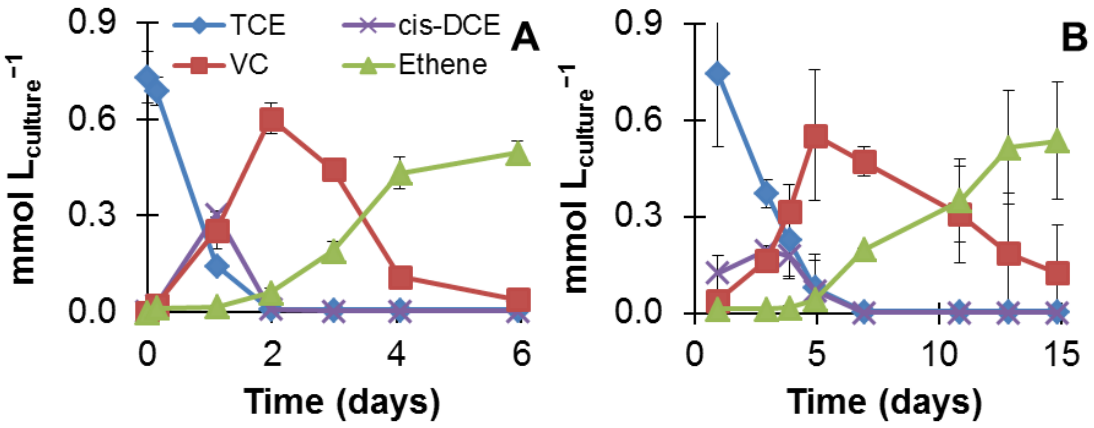


Figure 5.6 Viability and performance of DehaloR^2 culture produced in a CSTR after storage at 4 °C for 7 months (panel A) and 15 months (panel B). Dechlorination of TCE to ethene was assessed by transferring 10 mL refrigerated culture into 90 mL reduced anaerobic mineral medium amended with TCE and electron donors. The error bars are standard deviations of triplicate cultures.

Discussion

In this study, we show that using carefully selected conditions in a CSTR, cultivation of *Dehalococcoides* at short HRTs is feasible, resulting in robust communities capable of fast dechlorination. A compilation of previous CSTR studies on dechlorination of chlorinated ethenes is shown in Table 5.3. As revealed in Table 5.3, in most previous CSTR studies, the main reduced end-product of dechlorination of TCE and PCE was *cis*-DCE. This suggested that *D. mccartyi* respiring *cis*-DCE or VC to ethene were inhibited by high concentrations of chlorinated solvents, washed out, or outcompeted by other microbes. Our study is the first to document conversion to mostly ethene in a CSTR at a 3-d HRT (Table 5.3). We also report for the first time pseudo steady-state densities and production rates for *Dehalococcoides* cultivated in a CSTR. Moreover, the values for *D. mccartyi* presented in Figure 3A are close to the maximum ever reported for these microbes; the only past study to obtain growth to 10^{12} *Dehalococcoides* cells $L_{culture}^{-1}$ was Vainberg et al. (2009).

The community data regarding methanogens and homoacetogens abundances, in conjunction with the CSTR dechlorination performance and the concentrations of methane and acetate, support the fact that competing sinks for H_2 were minimized using our medium composition, thus allowing H_2 to be used optimally for dechlorination. Indeed, *Dehalococcoides* and *Geobacter* growth correlated with good TCE dechlorination performance in these continuous reactors. Moreoever, recently, *Geobacter* was documented to provide *D. mccartyi* with required corrinoids for dechlorinating activity and cellular growth (Yan et al. 2012) and, therefore, may be a desired partner in *Dehalococcoides*-containing cultures.

Table 5.3 Summary of key parameters and microbial inocula employed in chlorinated ethenes CSTR studies

Chlorinated ethene	e^- donor and C source	Undefined nutrients	Buffer	HRT (d)	Major reduced product	Inoculum culture
1 mM TCE	7.5 mM lactate and 15 mM methanol		15 mM HEPES and 5 mM HCO_3^-	3	Ethene	DehaloR^2 dechlorination culture converting TCE to ethene (this study)
2 mM TCE	10 mM lactate and 15 mM methanol		20 mM HEPES and 5 mM HCO_3^-	3	Ethene	
1.12 mM PCE	4.3 mM lactate	0.02 g L^{-1} yeast extract	35 mM Na_2CO_3 and 6 mM K_2HPO_4	50-55	Ethene	Point Mugu (PM) dechlorinating culture converting PCE to ethene (Berggren et al. 2013b)
7.4 mM TCE	25.6 mM lactate		CO_3^{2-}	5.9-25.3	*cis*-DCE	Evanite (EV) subculture converting PCE to *cis*-DCE (Sabalowsky and Semprini 2010)
0.52 mM PCE	52 mM methanol, 20 mM pyruvate or 80% H_2/20% CO_2, and 2 mM acetate	0.2 g L^{-1} yeast extract, 1% spent medium	90 mM HCO_3^-	11 5.8 2.9	VC *cis*-DCE *cis*-DCE	Methanol/PCE enrichment culture converting PCE to VC and ethene (Zheng et al. 2001)
≤ 50 mM PCE (nominal)	45 mM lactate		10 mM $(NH_4)H_2PO_4$ and 20% CO_2	~2	*cis*-DCE	Co-culture of *Desulfitobacterium frappieri* TCE1 and *Desulfovibrio* sp. strain SULF1 (Drzyzga et al. 2001)
0.2 g PCE in hexadecane NAPL	10 mM formate	0.2 g L^{-1} yeast extract	10 mM HCO_3^-	3	*cis*-DCE	Methanol/PCE enrichment culture converting PCE to VC and ethene (Carr et al. 2000)
0.98 mM PCE	1.7 mM benzoate	0.02 g L^{-1} yeast extract	14 mM Na_2CO_3 and 3 mM K_2HPO_4	36	Ethene	Dechlorinating source culture converting PCE to ethene (Yang and McCarty 1998)

The CSTR-produced culture exhibited very rapid rates of dechlorination, as shown in Table 5.2. The lower R_{max} for VC compared to TCE and *cis*-DCE (Table 5.2) implies that the limiting step in the CSTRs was dechlorination of VC. VC to ethene is commonly the slowest dechlorination step (Yu et al. 2005a), which might explain some of the rate differences between VC and TCE and *cis*-DCE dechlorination. Another factor we identified that could have led to lower apparent rates for VC dechlorination is the poorer gas-liquid transfer properties of VC, given its higher Henry's constant. In abiotic batch experiments using our medium composition (data not shown), we determined that 0.5 mmol L^{-1} VC added as gas did not equilibrate between the liquid and gas within the time of the R_{max} experiments (5 hours or less). Therefore, the slower dissolution of VC into the medium might have limited its bioavailability. Hence, the reported values for VC in Table 5.2 are the minimum R_{max} for this electron acceptor, with the possibility that the rates were higher as we did not observe significant VC accumulation during reactor operation (Figure 5.2).

The high abundances of *D. mccartyi* obtained in our CSTRs (10^{12} *Dehalococcoides* cells L^{-1}) clearly support the opportunity for their efficient cultivation in continuous reactors at short HRTs, which brings about numerous advantages when working with dechlorinating cultures. In the laboratory, such a system is ideal to provide a continuous supply of uniform culture for downstream applications requiring large volumes of cultures. These could include studies on microbial interactions, inhibition, transcriptomics and proteomics, experiments testing a large matrix of environmental conditions, or pilot-scale bioaugmentation applications. Moreover, a CSTR can

minimize reactor size requirements and/or time of operation to achieve high-density *Dehalococcoides* cultures.

For field applications, a short-HRT CSTR would be ideal for production of robust cultures capable of fast-rate of dechlorination containing high-cell density *Dehalococcoides*. Depending on the site to be remediated, bioaugmentation can require hundreds to thousands liters of bioaugmenting culture (Aziz et al. 2012). Fast dechlorination rates linked to high concentrations of *D. mccartyi* have been demonstrated in few well-characterized, batch grown bioaugmentation cultures, including the commercially produced culture, SDC-9, where PCE is constantly supplied to batch fermenters and 10^{11}-10^{12} *Dehalococcoides* cells are produced (Vainberg et al. 2009). This study demonstrates that a similar outcome in terms of *Dehalococcoides* densities and rates of dechlorination can also be achieved using a continuous-flow bioreactor, and provides the first scientific platform for a potential future implementation of such system at a larger scale.

CHAPTER 6

EFFECT OF HIGH AMMONIA ON MICROBIAL COMMUNITIES DRIVING CHLORINATED ETHENES REDUCTIVE DECHLORINATION[5]

6.1 Introduction

Decades of improper chemical disposal, careless handling, and accidental spills, along with the continuous generation of waste by all communities, industries, technologies and military, have led to extensive contamination of soil and groundwater. To date, ~1300 Superfund sites and hundreds of thousand sites are polluted with organic and inorganic compounds requiring decontamination (US EPA 2012). One of the most common organic pollutants of soil and groundwater is the industrial solvent trichloroethene (TCE) (ATDSR 2011). TCE is a toxic, carcinogenic compound (ATDSR 2011). Because of its frequency, of toxicity, and potential for human exposure, TCE has been placed in the most recent CERCLA Priority List of Hazardous Substances at number 16 out of 275 substances (ATDSR 2011).

TCE can be biologically detoxified to ethene *via* reductive dechlorination by *Dehalococcoides mccartyi* species (Maymo-Gatell et al. 1997). These hydrogen-oxidizing anaerobes utilize TCE as an electron acceptor for energy metabolism (Cupples et al. 2003; He et al. 2003b; Maymo-Gatell et al. 1997; Sung et al. 2006b) and acetate as carbon source for cell synthesis (Tang et al. 2009). Reductive dechlorination of TCE occurs in a step-wise manner with transient production of *cis*-dichloroethene (*cis*-DCE) and vinyl chloride (VC). Because of their fruitful ability to transform chlorinated ethenes

[5] This chapter was prepared as a manuscript and will be submitted for publication.

to a harmless end-product, *Dehalococcoides* have been extensively and successfully employed for *in situ* bioremediation scenarios at contaminated sites (Ellis et al. 2000; Lendvay et al. 2003; Major et al. 2002; Schaefer et al. 2010).

A large number of environments containing TCE, including Superfund sites, U.S. National Priorities List (NPL) hazardous waste sites, and groundwater sources, are also polluted by multiple contaminants (Moran et al. 2007; US EPA 2012). These include other industrial solvents (e.g. perchloroethene, trichloroethane, and dichloromethane), metals (e.g. lead, arsenic, copper, and cadmium), petroleum hydrocarbons (benzene, toluene, ethylbenzene, and xylene), and nitrogen-containing compounds (e.g. nitrate and ammonia). Ammonia is another priority pollutant in the 2011 CERCLA list and was detected at 135 NPL hazardous waste sites (ATDSR 2011). Contamination with high ammonia has been reported in urban and rural areas from sewage and mains leakage, septic tanks, industrial spillages, river or channel infiltration, fertilizers, house building, storm water and direct recharge, contaminated land, and landfills (Bohlke et al. 2006; Wakida and Lerner 2005).

Past studies on TCE *in situ* bioremediation in the presence of ammonia have exclusively focused on cometabolism with aerobic conditions (Arciero et al. 1989; Ely et al. 1997; Rasche et al. 1991; Yang et al. 1999). Aerobically, TCE can be biodegraded by ammonia-oxidizing bacteria using ammonia as a growth substrate (Arciero et al. 1989; Ely et al. 1997; Rasche et al. 1991; Yang et al. 1999). In the past two decades, however, anaerobic reductive has emerged as the most feasible avenue for bioremediation of TCE (Stroo 2012). Yet, investigations on the effect of ammonia on anaerobic TCE reductive dechlorination have not been carried out. Ammonia is the preferred nitrogen source for

synthesis of cellular components in *Dehalococcoides* (He et al. 2007) and in a multitude of bacterial or archaeal microbial groups. However, when present at high concentrations, ammonia exerts inhibition via disturbance of cellular homeostasis. Specifically, NH_3 readily diffuses into the cell, where it gets protonated to NH_4^+ (Kadam and Boone 1996). Consequently, high ammonia can increase the intracellular pH and alter the cell redox potential (Sprott and Patel 1986). Depletion of H^+ from conversion of NH_3 to NH_4^+ also disrupts the proton motive force and energy acquisition required for growth (Hajarnis and Ranade 1994; Kadam and Boone 1996; Sprott and Patel 1986).

Tolerance to high ammonia concentrations is microorganism-dependent (Lay et al. 1998; Muller et al. 2006) and tolerance to ammonia by *Dehalococcoides* is not known. The knowledge gap on the effect of high ammonia in TCE- reductively dechlorinating microbial communities containing *Dehalococcoides* is significant. For field applications, this knowledge limitation prevents us from assessing or predicting the outcome of anaerobic *in situ* bioremediation where TCE and ammonia are co-contaminants. In the present study, we assessed the effect of high total ammonia[6] nitrogen (TAN) in defined medium and in landfill leachate. We evaluated a range of concentrations (up to 2 g L^{-1} NH_4^+-N) and quantitatively tracked reductive dechlorination, fermentation, homoacetogenesis and methanogenesis in *Dehalococcoides*-containing enrichment cultures fed with TCE and fermentable substrates.

[6] Comprises both unprotonated (NH_3) and protonated (NH_4^+) forms. Where appropriate, NH_3 and NH_4^+ chemical formulas are used to denote the species discussed.

6.2 Materials and methods

NH_4Cl batch experiments

Microbial inocula, composition, and growth. Two mixed microbial cultures, ZARA-10 (Chapter 3) and DehaloR^2 (Ziv-El et al. 2011), dechlorinating TCE to ethene, were used in this study. For the experiments herein, ZARA-10 culture was cultivated in serum batch bottles, while DehaloR^2 inoculum was cultivated in a chemostat as described in Chapter 5. Both cultures were grown on TCE as the chlorinated electron acceptor, and fermentable substrates, lactate and methanol, were the electron donors and carbon sources. The microbial composition of these cultures was previously determined using 454 pyrosequencing and quantitative real-time PCR (qPCR) in Ziv-El et al. (2011) and in Chapter 3. Both cultures are enriched in *Dehalococcoides* and fermenting and homoacetogenic bacteria (predominated by the genera *Acetobacterium* and *Clostridium*). ZARA-10 is more active in methanogenesis than DehaloR^2; however, both contain only hydrogenotrophic methanogens (Ziv-El et a. 2011 and Chapter 3).

Medium and concentrations of TAN tested. We prepared reduced anaerobic mineral medium buffered with 30 mM HCO_3^- as previously described (Ziv-El et al. 2011) in 160-mL glass serum bottles containing 90 mL medium. TAN was added in the form of NH_4Cl powder before sealing the bottles with butyl rubber stoppers and crimping with aluminum crimps. NH_4Cl was supplemented to obtain the following final concentrations in g L^{-1} NH_4^+-N: 0.5, 1, and 2. The concentration of NH_4^+-N in the controls was 0.08 g L^{-1} (6 mM NH_4Cl). Ammonia measurements at time 0 and at the end of the expriments confirmed that no ammonia losses occurred. We setup triplicate bottles for each condition. At the beginning of the experiments, each bottle received 5 μL neat TCE (0.5

mmol L^{-1}), 5 mM sodium DL-lactate, 50 µL neat methanol (12 mM), 1 mL ATCC vitamin supplement, 50 µg mL^{-1} vitamin B_{12} and 10 mL inoculum culture (10% vol/vol).

Landfill leachate batch experiments

Landfill leachate was obtained from the Northwest Regional Landfill, Surprise, AZ in the summer of 2011 (sample L11) and summer of 2012 (sample L12). Immediately after being brought to the laboratory, we characterized several parameters, which are summarized in Table 6.1. The leachate experiments were performed in triplicates in the same size serum bottles as stated in section NH_4Cl batch experiments except the medium was replaced with landfill leachate (90 mL) buffered with 5 mM HCO_3^-. We performed pH adjustments to near neutral using 2.25 N HCl. We tested the following combinations of experimental conditions using landfill leachate: TCE (no culture), TCE + DehaloR^2 but no additional fermentable substrates, TCE + DehaloR^2 + fermentable substrates (5 mM lactate and 12 mM methanol), and TCE + ZARA-10 + fermentable substrates.

Table 6. 1 A Northwest Regional Landfill leachate characterization from Surprise, AZ

	Landfill leachate	
Sample name	L11	L12
Total Ammonia (g NH_3-N L^{-1})	0.5 ± 0.01	0.6 ± 0.01
Total Nitrogen (TN) (g N L^{-1})	0.6 ± 0.01	0.9 ± 0.02
COD (mg L^{-1})	1980 ± 220	4300 ± 30
Alkalinity (mg $CaCO_3$ L^{-1})	1900 ± 20	4400 ± 110
pH	7.7	8.2

Gas and liquid chemical analyses

We extracted 200 µL of headspace gas from each bottle to measure TCE, *cis*-DCE, VC, ethene, and methane using a gas chromatograph (GC) instrument (Shimadzu GC-2010; Columbia, MD) equipped with a flame ionization detector (FID) and a Rt-QS-BOND capillary column (Restek; Bellefonte, PA). The GC settings were those previously published (Delgado et al. 2012). We performed calibration curves within a range of 0.05-2.45 mmol L^{-1} for all chlorinated ethenes, ethene and methane in the same size serum bottles as those used for the experiments. To measure H_2, we employed a GC equipped with a thermal conductivity detector (TCD) with the instrument settings were outlined elsewhere (Parameswaran et al. 2011). Even though it measured, H_2 never accumulated to detectable levels in the headspace of the bottles. The detection limit for H_2 is 0.8% vol/vol.

We quantified lactate, methanol, acetate, and propionate via high performance liquid chromatography (HPLC) using a previously published method (Parameswaran et al. 2011) from 1-mL liquid samples filtered through a 0.2 µm polyvinylidene fluoride membrane syringe filter (Pall Corporation; Ann Arbor, MI). Five point calibration curves were generated for each acid and alcohol during every HPLC run.

Total Chemical Oxygen Demand (COD) was determined using the HACH COD kits, and Total Nitrogen with HACH TNT 828 (Hach Company Loveland, CO).

The pH was measured using an Orion 2-Star pH bench top meter (Thermo Scientific, USA) calibrated with the standard solutions purchased from the manufacturer.

Quantitative real-time PCR (qPCR)

We extracted DNA from 0.5 mL pellets formed by centrifugation and stored at -20°C until the DNA extraction. The detailed DNA extraction protocol was previously described (Ziv-El et al. 2011). We then employed qPCR for the following targets: *Dehalococcoides* 16S rRNA gene, *Geobacteraceae* 16S rRNA gene, *Archaea* 16S rRNA gene (methanogens), and the formyltetrahydrofolate (FTHFS) gene (homoacetogens). We setup triplicate reactions for the six point standard curves and the samples in 10 µL total volume using 4 µL of 1/10 diluted DNA as template. The standard curves were produced by serially diluting 10 ng μL^{-1} plasmid DNA. The primers and probes, reagents concentrations, and thermocycler (Realplex 4S thermocycler; Eppendorf, USA) conditions were those previously published (Ziv-El et al. 2012b).

6.3 Results and discussion

We supplemented increasing concentrations of NH_4Cl (0.5, 1, and 2 g L^{-1} NH_4^+-N) to ZARA-10, a representative TCE-dechlorinating culture containing *Dehalococcoides* (the main TCE respirers), fermenters, hydrogenotrophic homoacetogens, and hydrogenotrophic methanogens. Figure 6.1 presents the effect of increasing TAN on dechlorination performance under all conditions tested in time-course experiments. TAN concentrations induced differences in the rates of reductive dechlorination. The rates of dechlorination decreased with increasing concentration of ammonia (Figure 6.1B-D), but, dechlorination to ethene occurred at all conditions tested (Figure 6.1A-D). As expected, dechlorination rates with the ZARA-10 inoculum were

fastest in the Controls (0.08 g L^{-1} NH_4^+-N), where TAN was supplemented at non-toxix concentrations as an essential growth nutrient (Figure 6.1A).

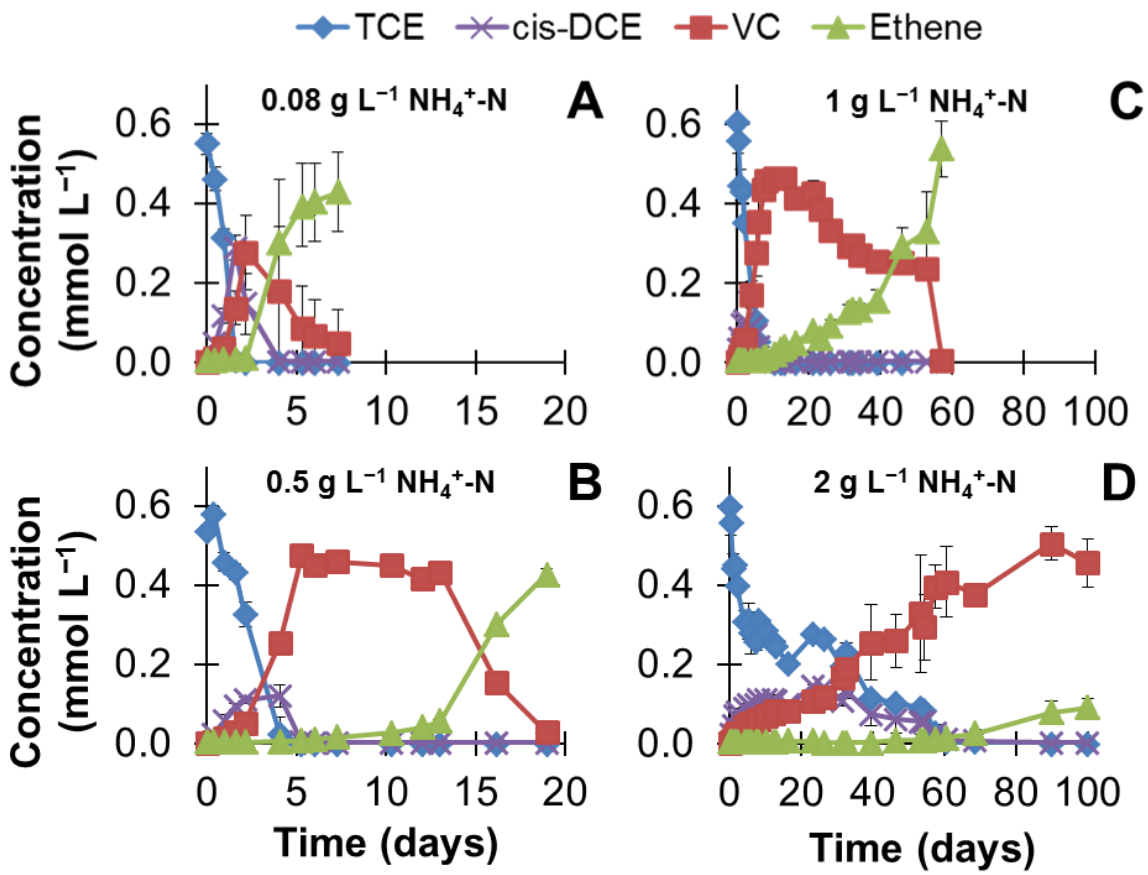


Figure 6.1 Dechlorination of TCE to ethene by ZARA-10 culture in bottles containing (A) 0.08 (Control), (B) 0.5, (C) 1 and (D) 2 g L^{-1} NH_4^+-N. All cultures initially received 5 mM lactate and 12 mM methanol as electron donors and carbon sources. The cultures in panel C and D received an additional 5 mM lactate on day 46. The error bars are standard deviations of triplicate cultures are not shown when smaller than the symbols.

Because the greatest effect of TAN on biological reductive dechlorination was observed at 2 g L^{-1} NH_4^+-N, we tested it with an additional dechlorinating culture, DehaloR^2, to strengthen our observations. As shown in Table 6.2, the end-reduced products of reductive dechlorination with DehaloR^2 inocula were also VC and ethene at 2 g L^{-1} NH_4^+-N. Both cultures contain multiple strains of *Dehalococcoides* involved in the different steps of reductive dechlorination of TCE to ethene (Ziv-El et al. 2011). The

findings with two separate dechlorination cultures from our study are strongly indicative that *Dehalococcoides* populations carrying out dechlorination of chlorinated ethenes are tolerant to concentrations as high as 2 g L^{-1} NH_4^+-N. This is an important finding for bioremediation, as the function (ability to reduce TCE to ethene) of either ZARA-10 or DehaloR^2 was slowed, but not lost when these microorganisms were exposed to TAN ranges from 0.5-2 L^{-1} NH_4^+-N.

Table 6.2 Extent of TCE reductive dechlorination observed at the ammonia concentrations tested in this study. N/D = not determined

	TAN (g NH_4^+-N L^{-1})		
Inoculum culture	**0.5**	**1**	**2**
ZARA-10	Ethene	Ethene	VC and ethene
DehaloR^2	N/D	N/D	VC and ethene

An investigation into the fate of the electrons provided as fermentable substrates, lactate and methanol, in ZARA-10 cultures is shown in Figure 6.2. The measurements for lactate and methanol also reveal an influence of ammonia on the consumption of these compounds and the subsequent production of fermentation products. Specifically, the rates of fermentation at the different ammonia concentrations correlated negatively with increasing NH_4^+-N concentrations as follows (in descending order): 0.08 g L^{-1} NH_4^+-N (Control) > 0.5 g L^{-1} NH_4^+-N > 1 g L^{-1} NH_4^+-N > 2 g L^{-1} NH_4^+-N (Figure 6.2A-B). The cultures mostly affected by this lag time in fermentation were those containing 2 g L^{-1} NH_4^+-N, with the complete depletion of the methanol provided at time 0 only after ~16

days (Figure 6.2A-B), compared to the controls, which depleted the methanol after 4 days. Previous work on glucose fermentation to H_2 and volatile acids in batch experiments containing TAN at 0.5 and 2 g L^{-1} N also showed differences in the metabolic rates of fermentation (slower rates and longer lag time at higher ammonia concentrations) (Salerno et al. 2006), consistent with our findings for lactate and methanol.

The differences in lactate and methanol fermentation rates were also accompanied by variances in the concentrations of acetate and propionate formed at high TAN (0.5-2 g L^{-1} NH_4^+-N) when compared to Controls (Figure 6.2C-D). With 5 mM lactate and 12 mM methanol initially added, acetate production was positively correlated to increasing TAN concentrations (Figure 6.2C). The cultures containing 2 g L^{-1} NH_4^+-N accumulated ~15 mM acetate, while the Controls accumulated 8 mM acetate (Figure 6.2C). Significant differences were not observed for propionate formation at 0.5 and 1 g L^{-1} NH_4^+-N (3.8 mM under both conditions). On the other hand, at 2 g L^{-1} NH_4^+-N, production of propionate was drastically diminished (Figure 6.2D).

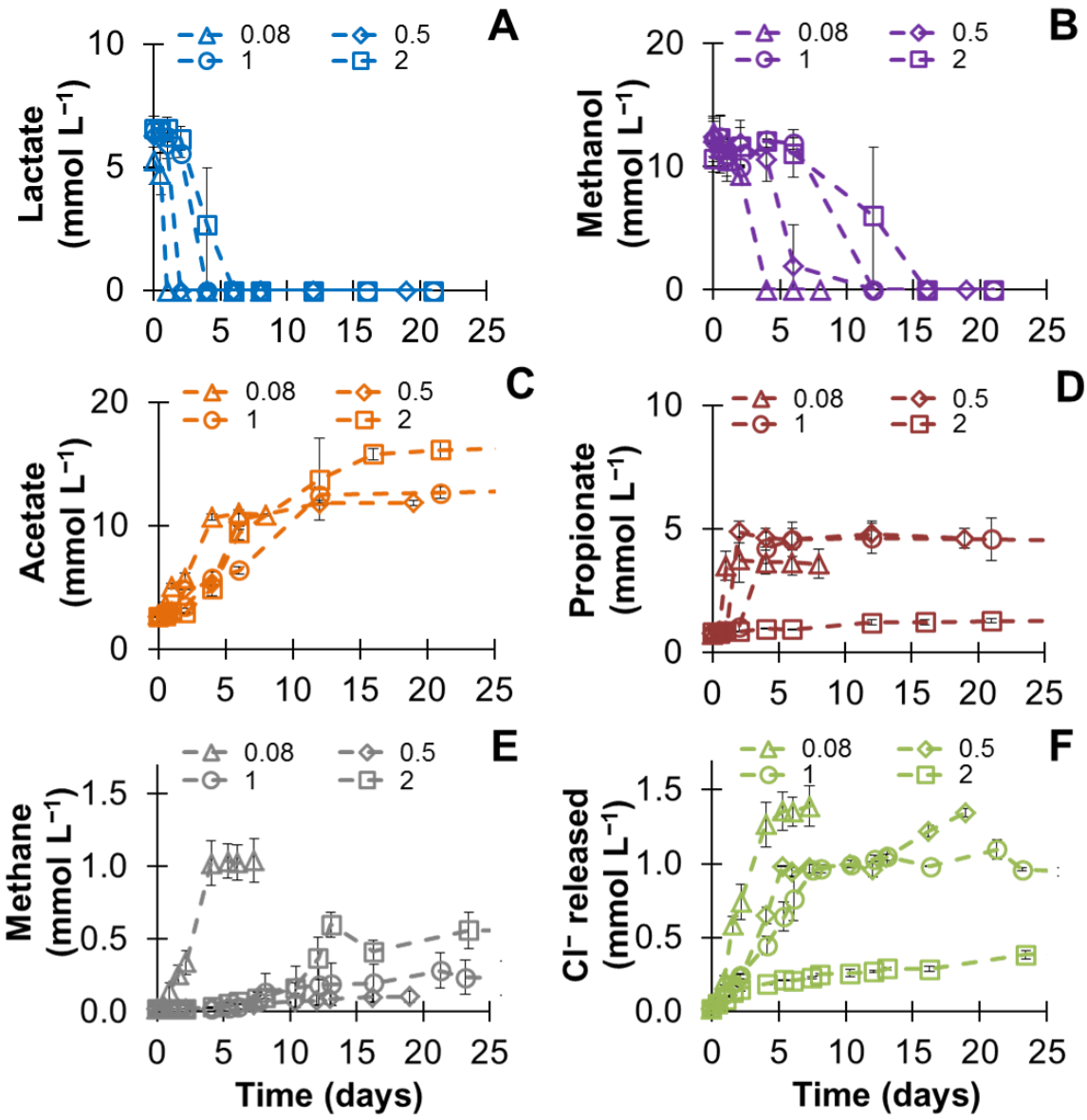


Figure 6.2 Effect of ammonia on fermentation (A-D), methanogenesis (E) and reductive dechlorination (F) for ZARA-10 culture. Symbols: triangles, 0.08 g L^{-1} NH_4^+-N; diamonds, 0.5 g L^{-1} NH_4^+-N; circles, 1 g L^{-1} NH_4^+-N; squares, 2 g L^{-1} NH_4^+-N. The error bars are standard deviations of triplicate cultures.

Besides dechlorination, acetogenesis, and fermentation, of particular interest to reductive dechlorination is methanogenesis. As discussed in Chapter 4, hydrogenotrophic methanogens compete for H_2 with *Dehalococcoides*, and, therefore, their excessive proliferation must be avoided. In animal waste streams containing

concentrations of 1 g L^{-1} N or higher, TAN decreased the specific methanogenic activity by up to 50% (Braun et al. 1981; Hansen et al. 1998; Sossa et al. 2004). Consisten with observations in other systems, methanogenesis was slowed in our system by the presence of high TAN, compared to Controls (Figure 6.2E). The inhibitory effect of high TAN was distinguishable between cultures in terms of lag time and total methane produced. Explicitly, the methanogenic lag times were positively correlated with increasing TAN concentrations. Methanogenic lag times in descending order were: 2 g L^{-1} NH_4^+-N > 1 g L^{-1} NH_4^+-N > 0.5 g L^{-1} NH_4^+-N. From the lactate and methanol initially added as H_2 precursors at time 0, the total methane concentrations produced in the presence of excess TAN in ascending order were 2 g L^{-1} NH_4^+-N > 1 g L^{-1} NH_4^+-N > 0.5 g L^{-1} NH_4^+-N (Figure 6.2E). On the second addition of lactate on day 46 at 1 and 2 g L^{-1} NH_4^+-N, the methane concentration also increased and reached similar levels to Controls at 2 g L^{-1} NH_4^+-N (data not shown in Figure 6.2E).

To correlate the chemical data shown in Figure 6.2 with microbial growth, we employed qPCR targeting select groups of interest in the representative culture, ZARA-10. Figure 6.3 shows the qPCR data enumerating *Dehalococcoides*, *Geobacteraceae*, FTHFS (homoacetogens), and *Archaea* (methanogens) in the ZARA-10 inoculum and at the end of the experiments for Controls and for the cultures supplemented with 0.5-2 g L^{-1} NH_4^+-N. Figure 6.3 and Table 6.3 highlight the effect of TAN on growth when ammonia was present in excess than the amount required as a nitrogen source for growth. Interestingly, out of all the microbial groups assayed, *Dehalococcoides* was least affected by increasing TAN (Figure 6.3 and Table 6.3). At the opposite end, *Geobacteraceae*, the other bacterial group involved in partial reduction of TCE to *cis*-DCE in ZARA-10

cultures was significantly impacted by high TAN. *Geobacteraceae* showed no growth relative to the inoculum at 1 and 2 g L^{-1} NH_4^+-N, respectively, and lower concentrations than the initial ones were detected (Figure 6.3 and Table 6.3). These findings imply that batch concentrations ≥ 1 g L^{-1} NH_4^+-N completely inhibit growth of *Geobacter*-type dechlorinators.

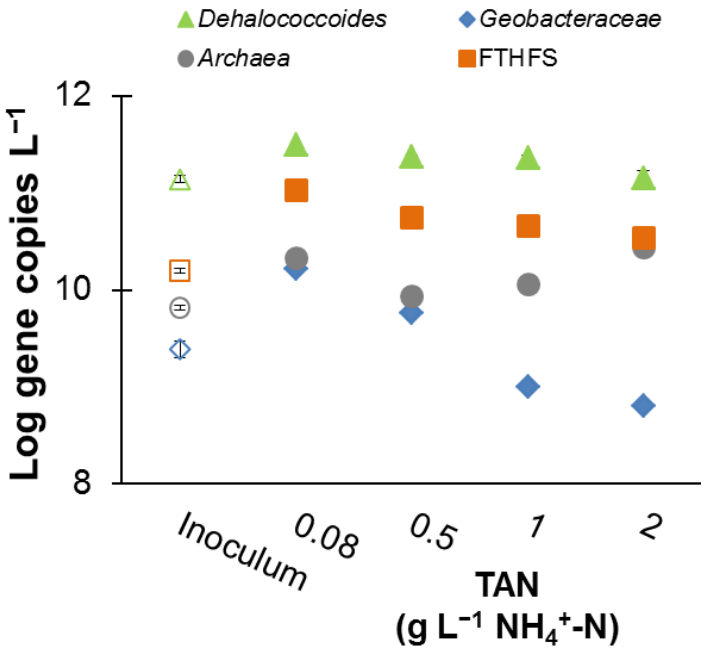


Figure 6.3 Quantitative PCR (qPCR) enumerating the 16S rRNA gene copies of *Dehalococcoides*, *Geobacteraceae*, and *Archaea*, and FTHFS gene copies for each ammonia concentration tested at time 0 (10% inoculum) and at the end of the experiments (Controls (0.08 g L^{-1} NH_4^+-N), day 8; 0.5 g L^{-1} NH_4^+-N, day 19; 1 g L^{-1} NH_4^+-N, day 60; 2 g L^{-1} NH_4^+-N, day 100). The data are averages of triplicate qPCR reactions. The error bars are standard deviations of the mean and are not shown when smaller than the symbols.

Homoacetogens were assayed using the FTHFS gene, which is a highly conserved gene in the acetyl-CoA pathway (Figure 6.3). The homoacetogens present in the inoculum culture are able to grow on the fermentable substrates and/or on the H_2 produced from fermentation and HCO_3^-/CO_2. The FTHFS genes copies decreased as a function of increasing TAN (Figure 6.3), indicating a negative impact on cell synthesis.

The qPCR trends for methanogens were opposite to those for homoacetogens at 0.5-2 g L^{-1} NH_4^+-N, potentially implying an increased tolerance towards ammonia in methanogens. The increases in the gene copies of all microbial groups tracked from inoculum to end points were highest in the Controls, which concur with uninhibiting conditions provided in these cultures.

Table 6.3 Comparison of microbial growth as determined by qPCR

Dehalococcoides (16S rRNA gene copies L^{-1})	**NH_4^+-N** ($g\ L^{-1}$)	**Inoculum[a]**	**Final[b]**	**Fold increase**
	0.08	1.39E+11	3.15E+11	2.3
	0.5	1.39E+11	2.37E+11	1.7
	1	1.39E+11	2.33E+11	1.7
	2	1.39E+11	1.44E+11	1.0
Geobacteraceae (16S rRNA gene copies L^{-1})	0.08	2.48E+09	1.67E+10	6.8
	0.5	2.48E+09	5.80E+09	2.3
	1	2.48E+09	1.01E+09	-0.4
	2	2.48E+09	6.32E+08	-0.3
FTHFS (gene copies L^{-1})	0.08	1.58E+10	1.08E+11	6.8
	0.5	1.58E+10	5.69E+10	3.6
	1	1.58E+10	4.66E+10	3.0
	2	1.58E+10	3.46E+10	2.2
Archaea (16S rRNA gene copies L^{-1})	0.08	6.58E+09	2.18E+10	3.3
	0.5	6.58E+09	8.81E+09	1.3
	1	6.58E+09	1.16E+10	1.8
	2	6.58E+09	2.80E+10	4.3

[a]The inoculum culture was assayed for each qPCR target and 10% was assigned to each set of cultures as the starting microbial concentration on day 0.
[b]Final time points (Controls (0.08 g L^{-1} NH_4^+-N), day 8; 0.5 g L^{-1} NH_4^+-N, day 19; 1 g L^{-1} NH_4^+-N, day 60; 2 g L^{-1} NH_4^+-N, day 100).

Fermentation of lactate and methanol provides *Dehalococcoides* with H_2, their electron donor (He et al. 2003b; Maymo-Gatell et al. 1999), acetate, their carbon source (Tang et al. 2009) and with other micronutrients (i.e. specific amino acids (Zhuang et al.

2011) and vitamin B_{12}, a co-factor cobalamin required for their reductive dehalogenase enzymes) (He et al. 2007). Therefore, a plausible explanation for the decreased rates in dechlorination at increasing TAN concentrations includes beside the direct, toxic effect of ammonia on *Dehalococcoides*, a H_2 limitation, and potentially a nutrient limitation. Moreover, in a previous work (Yan et al. 2012), it was demonstrated that *Geobacter lovleyi* also provide *Dehalococcoides* with vitamin B_{12}. The complete inhibition of this bacterium at 1 and 2 g L^{-1} NH_4^+-N might have also contributed to slower TCE to ethene reduction rates.

We sought to expand our findings on the effect of TAN in defined medium with experiments using leachate collected from a landfill in Arizona. Landfills are prime examples of environments containing high TAN (Arigala et al. 1995; Christensen et al. 2001; Kjeldsen et al. 2002). Landfills are also a noteworthy source of a variety of polluting substances, including TCE or other chlorinated ethenes (Chang et al. 2003; Kjeldsen et al. 2002). Moreover, a large number of cases of groundwater contamination with landfill leachate have been documented (Christensen et al. 2001), and leachate sometimes contains TAN concentrations as high as 1 g L^{-1} NH_3-N (Wakida and Lerner 2005).

A time-course experiment testing the effect of landfill leachate on TCE biological reduction is presented in Figure 6.4. Reduction of TCE was not observed in abiotic controls or in unbuffered landfill leachate with a starting pH of 8.2. Figure 6.4A demonstrates that remediation of TCE to ethene in landfill leachate containing 0.6 g L^{-1} N (Sample L11, Table 6.1) occurs, even without external electron donors. Landfill leachate typically contains abundant sources of organics (Kjeldsen et al. 2002) which can

be fermented to H_2. However, TCE detoxification occurred at faster rates when we supplemented lactate and methanol (Figure 6.4B).

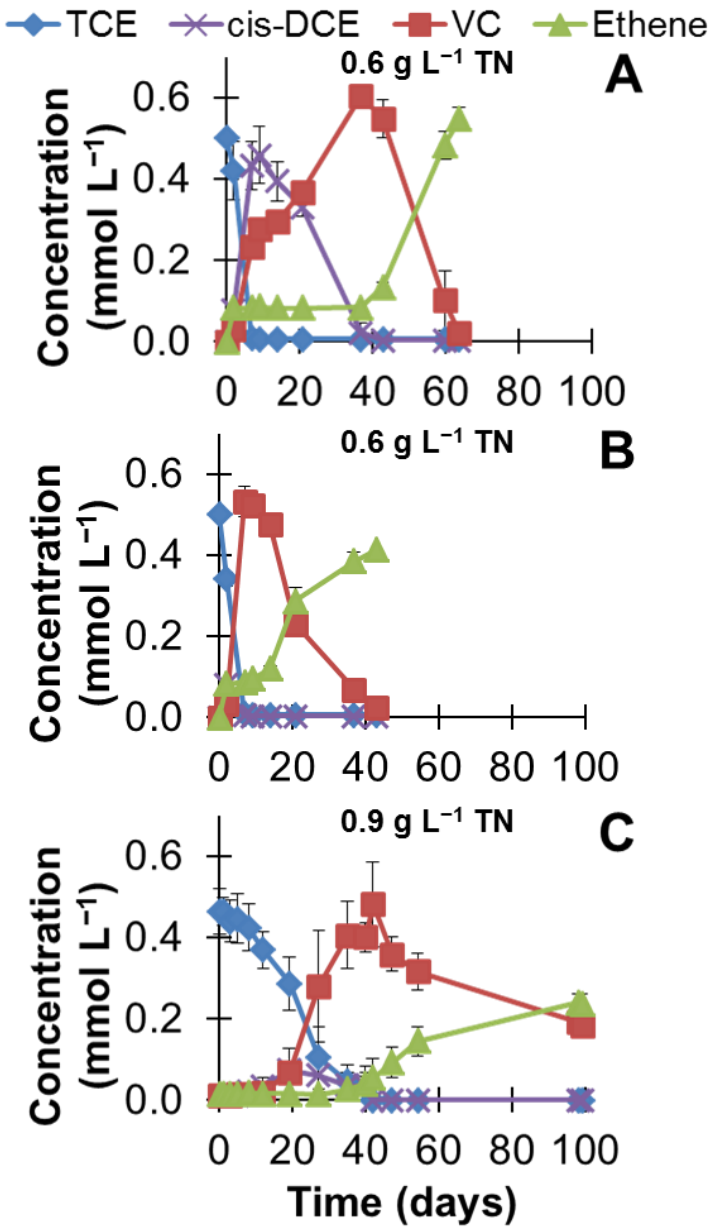


Figure 6.4 Assessment of dechlorination of TCE in bottles containing landfill leachate and bioaugmented with a dechlorinating culture (10% inoculum) (A) without provided electron donors or (B-C) with additional fermentable substrates as sources of electrons. The error bars are standard deviations of triplicate cultures are not shown when smaller than the symbols.

As expected, when we employed a higher strength leachate containing 0.9 g L^{-1} N (Sample L12, Table 6.1), the rates of TCE reductive dechlorination were slower (Figure 6.4C), supportive of the results obtained in defined medium. Nonetheless, after 100 days of incubation, ethene was the predominant metabolic product from reductive dechlorination even at this higher TAN concentration from landfill leachate.

This work provides the first and systematic insights into the effects of high ammonia on bioremediation of chlorinated ethenes in environments polluted with both contaminants, such as groundwater and landfills. Our data on reductive dechlorination by *Dehalococcoides*-containing cultures reveals relative ammonia tolerance for *Dehalococcoides* of up to 2 g L^{-1} NH_4^+-N. Despite the fact that reductive dechlorination rates and the growth of *Dehalococcoides* as well as the rates of fermentation were affected by high TAN, dechlorination of TCE proceeded all the way to ethene. This demonstrates that detoxification of chlorinated ethenes is feasible even at the high ammonia concentrations tested in this study.

CHAPTER 7

KEY FINDINGS AND RECOMMENDATIONS FOR FUTURE WORK

7.1 Key findings

In situ bioremediation using *Dehalococcoides* is a promising, environmentally friendly solution for restoring soil and groundwater contaminated by chlorinated ethenes. My research advances the knowledge on reductive dechlorination and the involved microbial communities. This work details novel or optimized approaches to improve reductive dechlorination and growth of *Dehalococcoides* in mixed communities and provides insights into some of the challenges and factors potentially limiting biostimulation or bioaugmentation.

In Chapter 3, I provide a different explanation on the inability to consistently promote reductive dechlorination of PCE/TCE beyond DCE in microcosms or at contaminated sites, even when the presence of *Dehalococcoides* communities is confirmed. Previously, this inability was explained by the presence of inhibiting conditions or by the absence of *Dehalococcoides* capable of complete dechlorination. My results from Cuzdrioara and Carolina biostimulated and later bioaugmented microcosms cannot be explained by either of those two hypotheses. I instead propose that the stalling at DCE was due to an electron donor competition in which components of the soil or sediment served as electron acceptor for competing microorganisms. Furthermore, I provide evidence on the importance of specific enrichment techniques and growth protocols. Using the three soil/sediment-free enrichment cultures described in Chapter 3 (originating from geographically distinct regions), I demonstrate that

Dehalococcoides can become significant competitors for electron donor, and I show how to obtain fast rates and high concentrations of *Dehalococcoides* in the laboratory by diminishing competitors for H_2.

As a result of the study in Chapter 3, I achieved a heightened awareness of the fierce competition for electron donor at sites contaminated with chlorinated solvents or in laboratory cultures. In Chapter 4, I then identified HCO_3^-, the natural buffer in groundwater and the buffer of choice in the laboratory and at contaminated sites undergoing biological treatment, as a potential H_2 sink for reductive dechlorination. HCO_3^- is the electron acceptor for hydrogenotrophic methanogens and hydrogenotrophic homoacetogens, two microbial groups competing for H_2 with *Dehalococcoides*. Indeed, data from Chapter 4 clearly shows that high HCO_3^- concentrations increase the H_2 demand and negatively affect the rates and extent of dechlorination. The lessons learned from that study on competition among dechlorination, methanogenesis, and homoacetogenesis highlight that HCO_3^-, especially when abundant, could be an important, but not previously considered, variable for biologically-driven TCE dechlorination.

By applying the knowledge gained from microbial community management from Chapters 2 (DehaloR^2 work) and Chapter 3 and 4, I achieved the first ever successful high growth-rate continuous reactor (3-d HRT CSTR) for cultivation of dechlorinating cultures containing *Dehalococcoides*. Critical for the successful operation of this CSTR (Chapter 5) was maximizing certain interaction while minimizing others. I show that by using carefully selected conditions in a CSTR, including minimizing HCO_3^-, I could prevent the excessive growth of methanogens and homoacetogens, making cultivation of

Dehalococcoides-containing cultures at short HRTs feasible. This resulted in 10^{12} *Dehalococcoides* cells L^{-1} and robust cultures capable of fast dechlorination. The evidence presented in Chapter 5 and in Chapter 3, consistently exemplifies that *Dehalococcoides* are not slow-growing anaerobes. To the contrary, they perform reduction of TCE to ethene at fast rates when present in well managed mixed communities.

One of the challenging aspects of bioremediation of chlorinated solvents is their co-occurrence with other contaminants. In Chapter 6, I provide a first and systematic insight into the effect of high ammonia on bioremediation of chlorinated ethenes in environments impacted by both contaminants, such as contaminated groundwater or landfills. Specifically, I examined the effect of total ammonia nitrogen on mixed microbial communities driving the reductive dechlorination of TCE and the possibility of using leachate as electron donor for dechlorination or ammonia-nitrogen as specific inhibitor of the microbial community members. Fermentation rates were impacted by high ammonia concentrations while *Geobacter* bacteria were completely inhibited at the higher ammonia concentrations tested. However, I present a previously undocumented increased tolerance of *Dehalococcoides* to high ammonia concentrations without loss of the ability to dechlorinate TCE to ethene. This demonstrates that detoxification of chlorinated ethenes is feasible even at 2 g L^{-1} NH_4^+-N.

Next, I succinctly describe several research works which I believe merit to be pursued. These studies are recommended because they are either a logical next step from my dissertation work or because they lie within my research interests.

7.2 Recommendations for future work

Constructing and deconstructing reductively dechlorinating communities

After the first isolation of *Dehalococcoides mccartyi*, it was hypothesized that *Dehalococcoides* have undefined nutritional requirements, as, when grown in isolation, strain 195 required filtered supernatant from an anaerobic digestor enrichment culture (Maymo-Gatell et al. 1997). The isolation of *Dehalococcoides mccartyi* strain BAV1 occurred in completely defined basal salts medium, which demonstrated that no unknown nutrients are necessary for growth of this microbe coupled to reductive dechlorination (He et al. 2003b). However, all isolates described in Table 2.2 grow poorly and perform reduction of chlorinated ethenes at drastically lower rates when compared to mixed enrichment cultures containing *Dehalococcoides*. And so, in recent years, a line of research has emerged into unveiling the required syntrophic interactions that enhance reductive dechlorination by *Dehalococcoides* in mixed communities.

The Krajmalnik-Brown Laboratory has also partaken into investigating syntrophic interactions and ideal reductively-dechlorinating microbial communities through the work of Ziv-El et al. (Ziv-El et al. 2011; Ziv-El et al. 2012c) and the work in Chapters 3 and 5. Ideal, robust communities, showing major improvements over previously described cultures in the literature have been enriched in the Krajmalnik-Brown Lab on multiple occasions. Yet, our work has not elucidated whether certain specific microbes or microbial groups are either required or 'better" partners than others to obtain fast rates of reductive dechlorination. The literature also presents somewhat unclear evidence towards this scientific inquiry in tri- and co-cultures containing *D. mccartyi* strains and

pure strains of either a fermenter, methanogen, or another TCE-respirer (Men et al. 2012; Yan et al. 2013; Yan et al. 2012).

In light of the multiple changes I made to the medium composition used for growth of dechlorinating cultures and the strictly anaerobic cultivation techniques I practiced (Chapter 3,4, and 6), it would be worthwhile to first test whether growth of pure cultures *D. mccartyi* can be improved. For this, I propose to grow *D. mccartyi* isolates using the optimized medium from Chapter 5, except lactate and methanol should be replaced with H_2 and acetate. These pure cultures should be grown as described in Chapter 3 by feeding multiple consecutive additions of chlorinated ethenes, H_2 and acetate. The purpose of this study would be to compare the rates of reductive dechlorination, yields, and maximum cell concentrations achieved with published values from the literature.

Regardless of the outcome from the experiments with *Dehalococcoides* isolates in terms of better or similar growth compared to the literature, I propose to further test *D. mccartyi* strains in defined cultures with other microbes. These microbes should be chosen based on the knowledge gained on the microbial composition of the four enrichment cultures, DehaloR^2 (Ziv-El et al. 2011), ZARA-10, LINA-09, and ISLA-08 (Chapter 3). Both common genera enriched in each culture, as well as unique genera should be tested. Potential candidates for these experiments would be strains of *Acetobacterium* and *Clostridium* (fermeters and acetogens), *Methanobacterium* (hydrogenotrophic methanogen), *Geobacter* and *Desulfitobacterium* (TCE to DCE respirers). These experiments could potentially elucidate whether an ideal community can be constructed and/or whether fast rates of dechlorination and enhanced growth of

Dehalococcoides is linked to a specific microbe (certain genera) or a specific microbial function (i.e. fermenter, corrinoid-producing methanogen).

"Natural' electron acceptors in the metabolism of Dehalococcoides

The role and lifestyle of *Dehalococcoides* in natural environments remains, to this day, an enigma. In the laboratory, these bacteria have been grown exclusively on anthropogenic halogenated substances and the majority of *Dehalococcoides* studies have been in conjunction with contaminated sites. But beyond contaminated sites, *Dehalococcoides* have also been detected in an array of pristine environments, including freshwater river sediments (He et al. 2005), freshwater lake sediments (Krzmarzick et al. 2013), forest and state park soils (Krzmarzick et al. 2012), estuarine sediments (Kittelmann and Friedrich 2008b), and marine subsurface sediments (Futagami et al. 2009). Yet, our understanding of *Dehalococcoides* in these environments is limited. This lack of knowledge is partially due to the unknown identity of the compounds serving as electron acceptors in these natural habitats (Krzmarzick et al. 2012), and the inherent challenges associated with studying microbial functions in complex systems such as soils or sediments (Zhou et al. 2002).

A first elegant effort in exploring the lifestyle of *Dehalococcoides*-like *Chloroflexi* in natural habitats was recently published by Krzmarzick et al. (2012). In that study, the authors established growth of *Dehalococcoide*s in soil slurries and showed dechlorination of enzymatically chlorinated organic matter (Krzmarzick et al. 2012). Thus, Krzmarzick et al. (2012) hypothesized that *Dehalococcoides* may play a role in the cycling of halogens in nature (chlorine and bromine), which may explain their ubiquity in

natural habitats. In Chapter 3, I employed two samples containing *Dehalococcoides* (garden soil from Romania and mangrove sediment from Puerto Rico) originating from environments with no history of contamination. Thus, Chapter 3 expands the limited number of *Dehalococcoides* studies from uncontaminated sites. Moreover, Chapter 3, together with the other works on *Dehalococcoides* in natural environments, concurs with their yet unexplored natural metabolic capabilities and ecological interactions. Therefore, I recommend a study to search for "natural" electron acceptors for *Dehalococcoides*.

For this search, I propose to do a thorough chemical characterization on pristine soil and sediment employed in Chapter 3. In nature, thousands of halogenated compounds are produced from volcanic activity, forest fires, geothermal processes and from micro- and macroorganisms (Griebler et al. 2004; Haggblom and Bossert 2003). Therefore, the chemical characterization of the soil and sediment should comprise of at least the composition of ions, including F^-, Br^-, Cl^-, and I^-, total organic carbon, and humic acids. Next, I will generate hypotheses on the nature of the compound/s potentially utilized by *Dehalococcoides*. These hypotheses will be generated taking into account i) the chemical characterization of the soil and sediment, ii) the type of biota (microorganisms, macroorganisms, and plats) found in these environments, iii) reported halogenated compounds produced in environments similar to those where the soil and sediment were collected, and iv) compounds that can be halogenated by microorganisms. In support of this recommended work is preliminary evidence from experiments performed by Kylie Kegerreis advised by Dr. Rosa Krajmalnik-Brown that debromination of 2,6-dibromophenol, produced in nature by marine hemichordates

(Steward et al. 1995), is debrominated to phenol by DehaloR^2 and ZARA-10 enrichment cultures.

Once a library of electron acceptor candidates is identified, I propose to first detect them in the soil and sediment using mass spectroscopy. Then, I will test these compounds in microcosms containing the soil and sediment from the uncontaminated sites and in the enrichment cultures generated in Chapter 3. If the compounds are respired, the respiration should be coupled to the growth of *Dehalococcoides*. Therefore, I will monitor the biotrasformation of the compounds tested and the growth of *Dehalococcoides* and *Eubacteria* using quantitative PCR. The discovery of a 'natural" electron acceptor utilized by *Dehalococcoides* will not only help us understand their role in nature, but will also help us improve their growth in the laboratory. The "natural" electron acceptors could be used to grow *Dehalococcoides* for bioaugmentation application. Additionally, these acceptors could potentially be supplemented at sites undergoing bioremediation where the contaminants (e.g. PCE or TCE) are present in very low concentrations, and therefore do not support significant growth.

Modeling reductive dechlorination and growth of Dehalococcoides in a CSTR

In Chapter 6, I operated a CSTR at a 3-d HRT for the cultivation of *Dehalococcoides*-containing cultures. I now propose to model and bring further optimizations to the CSTR concept with the main goal to increase the production rate of high-cell density *Dehalococcoides* bioaugmentation cultures. I propose to develop a mathematical model using MATLAB software. The model will be an expansion of the CSTR equations for bioreactors presented in biotechnology textbooks, particularly in

Rittmann and McCarty (2001) (Rittmann and McCarty 2001). The following equations exemplify the substrate, S and the mass of a microorganism, X_a in a typical steady-state CSTR:

$$S = K\frac{1+b\theta}{(Yq_{max}\theta)-(1+b\theta)} \quad \text{(Equation 7.1)}$$

$$X_a = Y(S^0 - S)\frac{1}{1+b\theta} \quad \text{(Equation 7.2)}$$

where S = effluent substrate concentration (mmol L^{-1}), K = concentration giving one-half the maximum rate (mmol L^{-1}), b = endogenous decay coefficient (d^{-1}), and θ = HRT (d), Y = yield for cell synthesis (mmol L^{-1} $cell^{-1}$), q_{max} = maximum specific rate of substrate utilization (mmol L^{-1} d^{-1}), X_a = concentration of active biomass (cells L^{-1}), and S^0 = influent substrate concentration (mmol L^{-1}).

The development of the model will occur in two phases. In the first phase of the model, I will focus on reductive dechlorination and growth of *Dehalococcoides*, assuming excess H_2, which will be provided from fermentation of lactate and methanol. In the case of a TCE- or PCE-reducing CSTR, Equation 7.1 and 7.2 do not suffice to explain reductive dechlorination due to the unique multiple steps: PCE→TCE→*cis*-DCE→VC→ethene, and the involvement of different bacteria in each step. Specifically, reductive dechlorination of PCE and TCE to *cis*-DCE only can be carried out by non-*Dehalococcoides* organohalide respirers (Smidt and de Vos 2004; Tas et al. 2010). Moreover, PCE and TCE strongly inhibit *cis*-DCE and VC reduction, *cis*- DCE strongly inhibits VC reduction, and VC moderately inhibits *cis*-DCE reduction (Chambon et al. 2012). Hence, the model will be expanded to include multiple electron-acceptor steps (PCE, TCE, *cis*-DCE, VC) and multiple dechlorinating genera, competitive inhibition,

and toxicity of the chlorinated ethenes. These modifications are crucial to predict the process of reductive dechlorination.

In order to develop a model that predicts the CSTR performance and limitations, the following parameters are needed: K, μ_{max}, Y, and b. While some of these parameters can be obtained from batch studies in the literature, they should also be determined these parameters experimentally because the proposed work models a CSTR system not previously described. I will carry out experiments to obtain K for each individual chlorinated electron acceptor as described previously (Popat and Deshusses 2011) using effluent culture grown in a CSTR. To determine μ_{max} (maximum specific growth rate (d^{-1})), I will perform growth experiments using a small starting concentration of *Dehalococcoides* (10^7 cell L^{-1}), TCE, and H_2 as electron donor. Growth rates will be obtained from time-course TCE dechlorination data coupled to increases in cells of *Dehalococcoides* and other organohalide respirers over time. To calculate b, I propose to use a methodology not previously employed for consortia containing *Dehalococcoides*: viable PCR. This method uses nucleic acid intercalating dyes (e.g. ethidium monoazide) to discriminate between viable and non-viable cells (Chen and Chang 2010; Shi et al. 2011). Y can be calculated by dividing the concentrations of *Dehalococcoides* cells produced in the CSTR by the concentration of TCE consumed. For all experiments stated here, dechlorination will be measured using gas chromatography and microbial cell densities will be obtained from qPCR assays. The model will be able to predict the optimum S^0 and HRT in order to maximize X_a and the highest rate of *Dehalococcoides* production in TCE bioaugmentation cultures. I will test the model by running CSTR

experiments under the optimum conditions dictated by the model with different consortia to ensure reproducibility across cultures with diverse microbial compositions.

Expanding the CSTR model to include competing biological processes and simulating groundwater conditions

For on-site applications of bioaugmentation, a cost-effective approach would be to grow these bioaugmentation cultures at the contaminated site using the contaminated groundwater as medium (augmented with electron donors and nutrients). Commonly employed electron donors for bioaugmentation include lactate, methanol, emulsified vegetable oil, and hydrogen-releasing compounds (HRC®). For this reason, I will expand the CSTR model to include a multispecies microbial system that includes the process of fermentation of substrates, homoacetogenesis, and methanogenesis, along with the process of reductive dechlorination. Depending on the site chemistry, other microbial process could be included, such as iron, sulfate or nitrate reduction. The parameters required for each of these non-dechlorinating microbial processes are well-known and reported in literature (Loffler et al. 1999; Parameswaran et al. 2011; Rittmann and McCarty 2001; Rittmann and Herwig 2012; Ziv-El et al. 2012b; Ziv-El et al. 2012c). By including the complex microbial community, the model can estimate the production and consumption of H_2, and the possible limitations encountered by *Dehalococcoides* under site-specific conditions. Along with the model development, I will run CSTR experiments with real groundwater and augmented with TCE (to simulate contamination) and with various fermentable substrates utilized for mass-production of bioaugmentation cultures.

Growth of organohalide respiring communities at large-scale and in other bioreactors

Depending on the site to be remediated, bioaugmentation can require hundreds to thousands liters of bioaugmenting culture (Aziz et al. 2012). Batch fermentor production of cultures containing 10^{12} *Dehalococcoides* cells per liter requires 35 days (Vainberg et al. 2009) or longer. The CSTR growth method presented in Chapter 6 requires only three days to produce 10^{12} *Dehalococcoides* cells L^{-1}, thus improving the rate of culture production at least ten times. The work in Chapter 6 provides the first scientific platform for a potential future implementation of such system at larger scale.

Towards this goal, I recommend increasing the size of the reactors to 5-10 L and running CSTR experiments under the optimal conditions previously identified. For application purposes, I also suggest expanding the range of electron donors, electron acceptors, and buffers for the growth of dechlorinating cultures in CSTRs. All enrichment cultures described in this dissertation were grown using lactate and methanol, however other less expensive substrates, such as ethanol, glycerol, emulsified vegetable oil or molasses and buffers, such as phosphate, should be tested. As discussed in Chapter 5, PCE and TCE often occur with other chlorinated solvents at contaminated sites. Therefore, I suggest initially growing the enrichment cultures with PCE and TCE and low levels of other electron acceptors (e.g. trichloroethane, carbon tetrachloride, dichloroethane, trifluoroethane) to precondition the cultures to these alternate electron acceptors. If the cultures are unable to degrade these compounds, I suggest enriching for the appropriate microbes in microcosms and then culturing the enrichment in CSTRs.

The results presented in this dissertation provide compelling evidence that *D. mccartyi* is a fast-growing anaerobic microorganism. The development of a continuous

TCE-dechlorinating bioreactor, along with a successful operation of the membrane biofilm reactor (MBfR) for *ex situ* TCE remediation in our Center (Ziv-El et al. 2012b), proves that dechlorinating consortia can be grown at rates similar to those for fermenting microorganisms. Therefore, I propose expanding the studies of bioreactors in which dechlorinating consortia are grown, including commonly used designs, such as a packed-bed reactor and an upflow anaerobic sludge blanket (UASB).

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