Linking Structure and Function to Manage Microbial Communities Carrying Out Chlorinated Ethene Reductive Dechlorination

by

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ABSTRACT

Contamination by chlorinated ethenes is widespread in groundwater aquifers, sediment, and soils worldwide. The overarching objectives of my research were to understand how the bacterial genus *Dehalococcoides* function optimally to carry out reductive dechlorination of chlorinated ethenes in a mixed microbial community and then apply this knowledge to manage dechlorinating communities in the H_2-based membrane biofilm reactor (MBfR). The MBfR is used for the biological reduction of oxidized contaminants in water using H_2 supplied as the electron donor by diffusion through gas-transfer fibers.

First, I characterized a new anaerobic dechlorinating community developed in our laboratory, named DehaloR^2, in terms of chlorinated ethene turnover rates and assessed its microbial community composition. I then carried out an experiment to correlate performance and community structure for trichloroethene (TCE)-fed microbial consortia. Fill-and-draw reactors inoculated with DehaloR^2 demonstrated a direct correlation between microbial community function and structure as the TCE-pulsing rate was increased. An electron-balance analysis predicted the community structure based on measured concentrations of products and constant net yields for each microorganism. The predictions corresponded to trends in the community structure based on pyrosequencing and quantitative PCR up to the highest TCE pulsing rate, where deviations to the trend resulted from stress by the chlorinated ethenes.

Next, I optimized a method for simultaneous detection of chlorinated ethenes and ethene at or below the Environmental Protection Agency maximum

contaminant levels for groundwater using solid phase microextraction in a gas chromatograph with a flame ionization detector. This method is ideal for monitoring biological reductive dechlorination in groundwater, where ethene is the ultimate end product. The major advantage of this method is that it uses a small sample volume of 1 mL, making it ideally suited for bench-scale feasibility studies, such as the MBfR.

Last, I developed a reliable start-up and operation strategy for TCE reduction in the MBfR. Successful operation relied on controlling the pH-increase effects of methanogenesis and homoacetogenesis, along with creating H_2 limitation during start-up to allow dechlorinators to compete against other microorgansims. Methanogens were additionally minimized during continuous flow operation by a limitation in bicarbonate resulting from strong homoacetogenic activity.

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Chapter 1

INTRODUCTION

Chlorinated ethene contamination.

Contamination by chlorinated ethenes is widespread in groundwater aquifers, sediment, and soils worldwide (Abelson, 1990; McCarty, 1997, Jennings, 2011; U.S. EPA, 2011). A schematic of anthropogenic and naturally produced chlorinated ethenes and ethene in the environment is in Figure 1. The contamination primarily stems from improper disposal of tetrachloroethene (PCE) and trichloroethene (TCE), commonly used solvents in the dry cleaning industry and as metal degreasing agents. PCE and TCE also are converted through biological reductive dechlorination to dichloroethenes (DCE) and then to vinyl chloride (VC). The major anthropogenic release of ethene into the environment is from biomass and fossil fuel combustion; most of the ethene undergoes rapid reactions producing ozone and photochemical smog, leaving a net atmospheric ethene concentration in the ng/µL level (Mattes et al. 2010). In contrast, stricter manufacturing regulations have dramatically decreased contamination of chlorinated ethenes and ethene from manufacturing of plastics, chemicals, anesthetics, and refrigerants (Mattes et al. 2010). VC is naturally produced in abiotic reactions in marshes and forest soils, but only at ng/µL concentrations, PCE and TCE from volcanic eruptions, and ethene from plants and from pathogenic microorganisms (Stern, 2006).

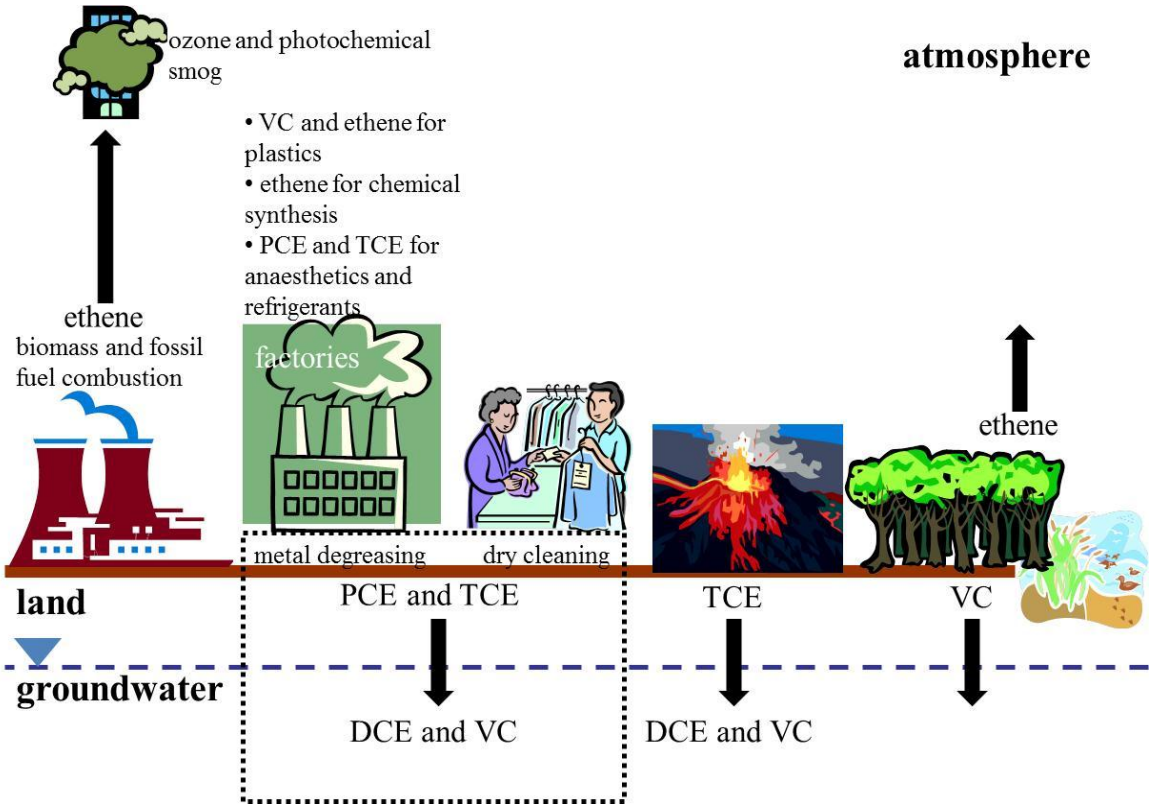


Figure 1.1 Natural and anthropogenic sources of chlorinated ethenes and ethene in the environment based on the review by Mattes et al. (2010). In the dotted black box are the most significant sources of contamination in groundwater. Strict regulations have mostly eliminated new contamination from manufacturing today, but legacy contamination is a serious problem.

PCE, TCE, and DCE cause developmental, neurological, and reproductive disorders (National Research Council 2006), PCE and DCE are probable human carcinogens (National Research Council 2006, U.S. EPA 2011a), and TCE and vinyl chloride (VC) are known human carcinogens (National Research Council 2006, U.S. EPA 2011a). The major concern for human exposure to chlorinated ethenes is from groundwater. Within the U.S., chlorinated ethenes are found as co-contaminants in sediment across 48 states or territories; of the 1646 hazardous waste sites on the National Priority List of the federal government's Superfund program, 80 are contaminated with PCE, 350 with TCE, 334 with DCE, and 447 with VC (U.S. EPA 2011b). The Environmental Protection Agency set maximum contaminant levels in drinking water of 5 µg/L for PCE and TCE, 70 µg/L for the isomers *cis*-1,2-DCE and 1,1-DCE, 7 µg/L for the isomer *trans*-DCE, and 2 µg/L for VC.

A list and brief description of the remediation technologies or strategies for sediment and groundwater remediation are in Table 1. Detailed descriptions of each, schematics, and monitoring requirements are in a review article by Lai et al. (2007). All of these strategies have been employed for chlorinated-ethene remediation, and four can potentially involve microbial bioremediation: pump and treat (P&T), soil vapor extraction in conjunction with air sparging (only aerobic bioremediation), *in-situ* bioremediation, and monitored natural attenuation.

Table 1.1

Technologies or strategies for remediation of contaminants in groundwater, soil, and sediment. A more detailed description with schematics and monitoring methods is in Lai et al. (2007). All of these treatment types have been used for chlorinated ethene remediation. The remediation types that involve or can be combined with microbial bioremediation are shaded in grey.

Remediation Type	Description
***In-situ* Bioremediation**	Biological remediation with microorganisms (bacteria, Archaea, or fungi), through aerobic or anaerobic processes. Bioaugmentation involves addition of organisms and nutrients, and biostimulation involves only nutrient addition. Remediation generally involves a combination of biotic and natural abiotic processes.
***In-situ* Thermal Remediation**	NAPL contaminants(s) are mobilized or vaporized through heating.
***In-Situ* Chemical Oxidation (ISCO)**	Oxidants (e.g. permanganate, sodium persulfate, hydrogen peroxide, or ozone) react with contaminants to produce CO_2, water, inorganic Cl^-, etc.
Monitored Natural Attenuation (MNA)	No active remediation employed, but monitor naturally occurring remediation by physical, chemical, and biological processes.
Permeable Reactive Barriers (PRB)	Contaminants treated when in contact with reactive material on the barrier.
Phytoremediation	Using plants for physical removal or biological degradation of contaminants.
Pump and Treat (P&T)	Groundwater pumped to the surface and treated in a reactor (e.g., physical, chemical, or biological treatment). The effluent is discharged back into the sediment or into a nearby reservoir. May include a containment barrier.
Removal of Contaminated Sediment	Physical removal of sediment. Only applicable for shallow contamination.
Soil Vapor Extraction (SVE)	Applicable for volatile compounds. Sediment flushed with gas and contaminant(s) in sediment and groundwater partition into the gas. Vapors treated in an *ex-situ* reactor before releasing into the atmosphere. Often employed with air sparging to create *in-situ* conditions for aerobic biodegradation.
Surfactant/co-solvent flushing	Increases the solubility or mobility of non-aqueous phase liquid (NAPL) contaminants(s).

Chlorinated ethene biodegradation.

The chemical reactions in microbial biodegradation processes of chlorinated ethenes are summarized in a schematic in Figure 2; these processes can be anaerobic or aerobic, and metabolic (the organisms gain energy from the reactions) or co-metabolic (the reactions occur fortuitously without the organisms gaining energy). Detailed reviews on these processes and the related microbial metabolisms are presented elsewhere (Mattes et al. 2010, Tiehm and Schmidt 2011, Taş et al. 2009), and a comprehensive phylogenetic tree of all involved microorganisms in in Mattes et al. (2010).

Here, I provide a brief overview of aerobic oxidation of chlorinated ethenes by bacteria. Co-metabolic aerobic oxidation has been used *in-situ* with a variety of electron donors including methane, toluene, propane, phenol, ethene, and ethane (Semprini 1997). All of these reactions are mediated by mono- and dioxygenases and produce chlorinated ethene epoxides that have several potential impacts to the host bacteria. PCE and TCE epoxides inactivate the bacteria by spontaneous formation of toxic metabolites such as acetylene and carbon monoxide (Semprini 1997), and *cis-DCE* and VC epoxides inactivate by DNA mutations (Mattes et al. 2010). Another possibility for the latter two is conjugation of the epoxides to co-enzyme-M by ethene-assimilating bacteria who mistake the compounds due to their similar structure to ethene, neutralizing the epoxides and allowing the bacteria to continue to function (Mattes et al. 2010). Aerobic co-metabolic bioremediation can be a valuable remediation strategy in

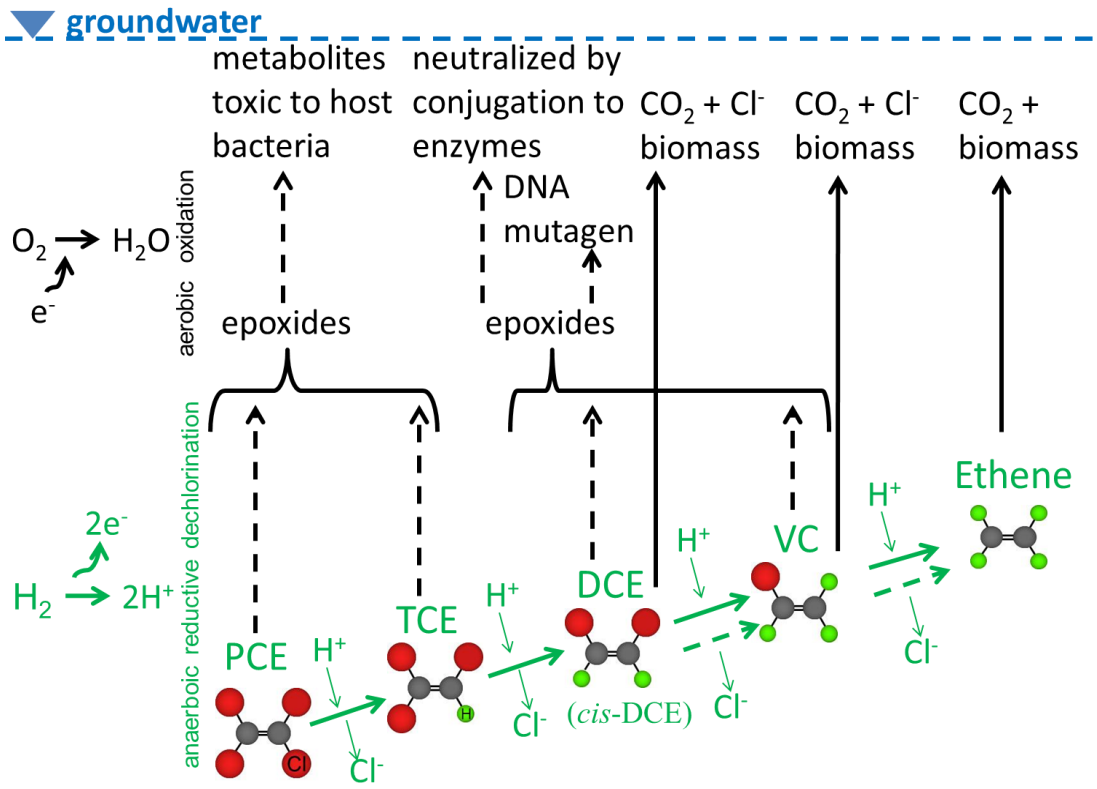


Figure 1.2. Aerobic and anaerobic biodegradation in the subsurface. Metabolic processes are signified with solid arrows and co-metabolic processes with dotted arrows. A comprehensive phylogenetic tree with all bacteria involved in these processes is in Mattes et al. (2010) Figure 2.

select settings, but a significant disadvantage is the difficulty in tracking the remediation progression, since the chlorinated ethenes are destroyed and the bacteria do not grow.

The focus of my research is anaerobic reductive dechlorination, a hydrogenolysis reaction in which each of the Cl^- atoms is replaced in a step-wise manner with a hydrogen atom (Figure 2). This process is much simpler to track than aerobic processes, since mass balances can be carried out on the chlorinated ethenes and ethene. Additionally, the organisms involved in these processes are already comparatively well understood, and abundant molecular techniques are available. A summary of the available eco-genomic methods are in a review article by Maphosa et al. (2010). Certain strains within the genera *Dehalococcoides* (of the *Chloroflexi* phylum), *Desulfitobacterium* and *Dehalobacter* (of the *Firmicutes* phylum), *Geobacter* and *Desulfuromonas* (of the *Delta-Proteobacterial* class), and *Dehalobacter* and *Sulfurosprillum* (of the *Epsilon-Proteobacterial* class) metabolically carry out anaerobic reductive dechlorination to *cis*-DCE (Mattes et al. 2010). Except for *Dehalococcoides*, these genera also can metabolize non-halogenated electron acceptors and can use organic electron donors (Maphosa et al. 2010). Only certain strains within the *Dehalococcoides* genus are capable of carrying out reductive dechlorination of *cis-DCE* to ethene, and, depending on the strain, they do these reactions either metabolically or co-metabolically (Taş et al. 2009, Mattes et al. 2010).

In practice, *in-situ* bioremediation probably involves a combination of several of the above microbial processes in addition to abiotic processes (Hägbolom and Bossert 2003, Dong et al. 2009).

Dehalococcoides, the essential microorganism for chlorinated ethene reduction (described above), have strict growth requirements, summarized in Figure 3. Their electron acceptors are chlorinated organic compounds, the electron donor is H_2, the carbon source is acetate and possibly CO_2 (Islam et al. 2010), the N source is ammonium (He et al. 2007), they are obligate anaerobes (Maymó-Gatell et al. 1995, Amos et. al. (2008)), they grow optimally at a near-neutral pH ((Middeldorp et al. 1999, Robinson et al. 2009), and corrinoids including vitamin B_{12} are essential cofactors for their reductive dehalogenase enzymes carrying out the reductive dechlorination (DiStefano et al. 1992; Maymó-Gatell et al. 1995; He et al. 2007; Amos et al., 2008; Johnson et. al. 2009). Although *Dehalococcoides* can be maintained in isolation with defined media (Löffler et al. 2005), the reductive dechlorination rates of the chlorinated compounds decrease significantly, suggesting that they require yet unidentified growth factors supplied by other members in the community, likely fermenters including homoacetogens (DiStefano et al. 1992, Aulenta et al. 2007, He et al. 2007). In addition, reductive dechlorination may benefit from direct, interspecies H_2 transfer from fermenters (Islam et al. 2010).

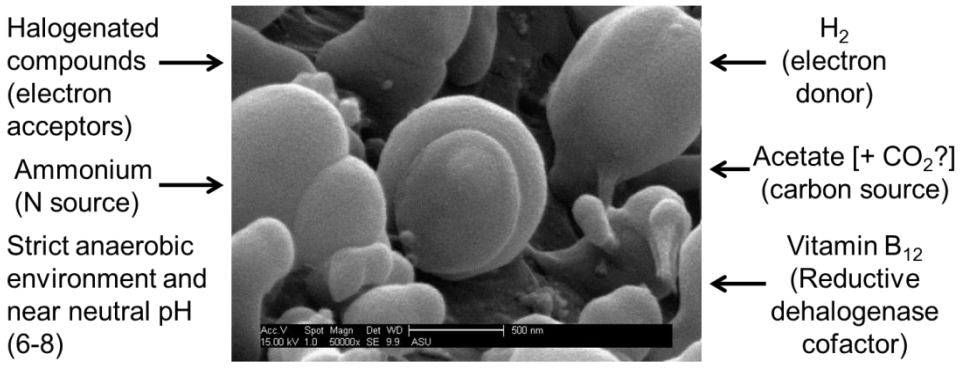


Figure 1.3. Dehalococcoides containing biofilm with known growth requirements.

Anaerobic reductive dechlorination in microbial consortia.

My research focus is the microbial ecology of consortia carrying out anaerobic reductive dechlorination in *ex-situ* treatment; thus, I provide a more in depth review of this process.

Various sediment-free, chlorinated ethene-respiring communities have been developed and characterized for application in bioaugmentation (Duhamel & Edwards, 2006; Macbeth et al., 2004; Richardson et al., 2002; Schaefer et al., 2009). These cultures have several similar features, shown in Figure 4, with fermentable substrates provided as the source of electrons. Most importantly, they all contain at least one strain of *Dehalococcoides* and most contain an additional member of three genera capable of TCE dechlorination to *cis*-DCE: e.g., *Geobacter*, *Dehalobacter*, and *Desulfuromonas*. These previously described microbial consortia are also dominated by bacteria capable of converting

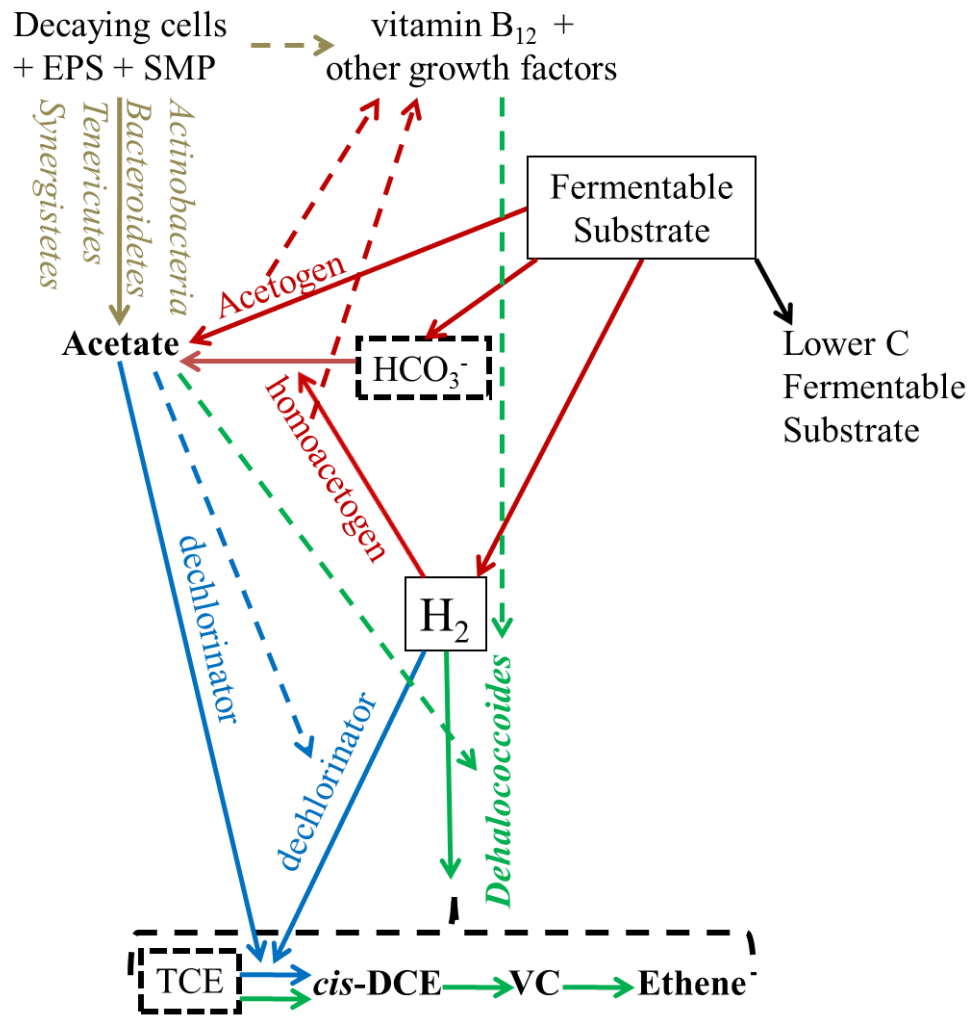


Figure 1.4. Major microbial groups involved in TCE to ethene reductive dechlorination.

fermentable substrates to H_2 and acetate, two metabolites representing, respectively, the required electron donor and carbon source for *Dehalococcoides*. The bacteria responsible for fermentation include at least one species of homoacetogens of the genera *Sporomusa*, *Spirochaetes*, or *Acetobacterium*, believed to provide *Dehalococcoides* with growth factors, including vitamin B_{12} and similar corrinoids, that serve as cofactors for their reductive dehalogenase

enzymes (Johnson et al., 2009). The specific organisms active in consortia are a function of the inoculating sediment and the employed enrichment methods.

One approach to elucidate the interactions within the microbial community, beyond chemical analyses alone, is kinetics-based modeling. Fennel and Gossett (1997) were the first to model the competition for H_2 between methanogens and dechlorinators. Rölling et al. (2007) used a flux-based model to show that H_2 production through fermentation (i.e., H_2 availability) is directly correlated with rates of *in-situ* reductive dechlorination. Another strategy is to use a suite of molecular techniques, including those described in Maphosa et al. (2010). In my research, I used molecular methods combined with analytical detection of chlorinated ethenes, volatile fatty acids, biomass, pH, and scanning electron microscopy (SEM).

Use of molecular methods to investigate chlorinated ethene microbial consortia. In Figure 5 is a dendrogram showing the relative abundance of the major microbial groups described above for various dechlorinating enrichment consortia in the literature. This genomic data were from clone libraries that targeted the 16S rRNA gene of bacteria. Key properties of the enrichment methods for these consortia are in Table 2. The consortia are listed based on the ratio of provided electron donor to acceptor in electron equivalent units. Lactate was 12 e- eq./mol, methanol was 6 e- eq./mol, and TCE was 6 e- eq./mol. While all the expected microbial groups (Figure 4) were present, trends regarding the relative abundance of these different groups are not apparent. The only group with a potential trend is the *Chloroflexi* phylum (contains *Dehalococcoides*); the

dendrogram suggests the requirement of a ratio greater than 1.0 in order to detect this phylum. One cause for the lack of trends may be a result of the employed genomic molecular technique, clone libraries, which provided a very limited number of sequences.

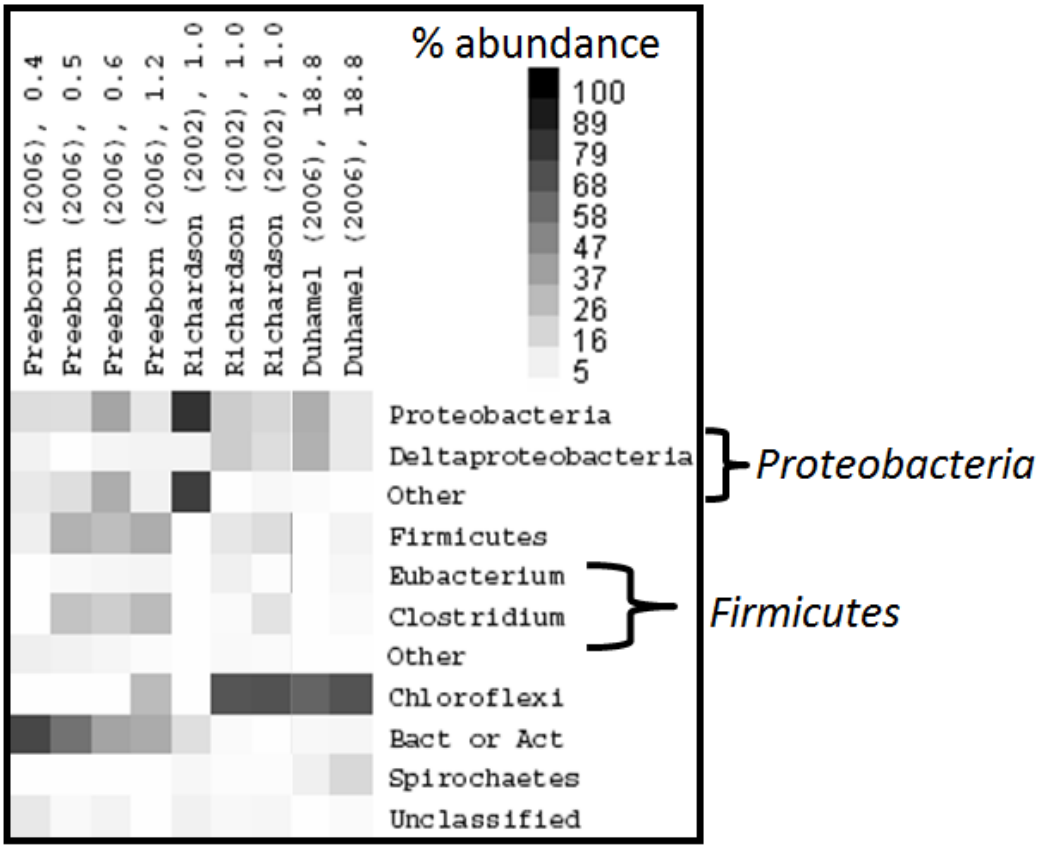


Figure 1.5. Dendrogram comparing the relative abundance of the major microbial groups in various dechlorinating enrichment consortia from the literature.

Table 1.2

Properties of the microbial consortia compared in Figure 4. TCE was the electron donor in all the presented studies. Question marks for various SRT values indicate that these values were not clearly described.

Reference	Freeborn (2006)	Freeborn (2006)	Freeborn (2006)	Freeborn (2006)	Richardson (2002)	Richardson (2002)	Richardson (2002)	Duhamel (2006)	Duhamel (2006)
e- donor	butyrate	propionate	lactate	methanol	lactate	lactate	lactate	methanol	methanol
End product	ethene	ethene	ethene	ethene	VC	ethene	ethene	ethene	ethene
Donor [me- eq./L]	200	140	120	60	120-240	120-240	120-240	5.5-8.5	5.5-8.5
TCE [me- eq./L]	0.72	0.72	0.72	0.72	1.2-2.4	1.2-2.4	1.2-2.4	1.2-1.6	1.2-1.6
Sulfate [me- eq./L]	6.48	6.48	6.48	6.48	6.48	6.48	6.48	4.06	4.06
Ratio of donor e- eq.:TCE e- eq.	0.4	0.5	0.6	1.2	1.0	1.0	1.0	18.8	18.8
SRT (days)	22-28	22-28	22-28	22-28	20-25?	21-35	21-35	>30?	>30?
Microbial consortium	ANAS	ANAS	ANAS	ANAS	ANAS	ANAS	ANAS	KB1	KB1

I relied heavily in my research on pyrosequencing and quantitative polymerase chain reactions (qPCR); using these techniques in combination for environmental samples have been described in Chapter 8 of Kwon and Ricke (2011). I describe the advantages and limitations of these analysis methods next.

Pyrosequencing is a high-throughput sequencing technology that allows for simultaneous sequencing of several hundred thousand sequences. By attaching a barcode to each sample, it is possible to mix samples together and analyze them simultaneously. Similar to clone libraries, pyrosequencing is used to assess the relative abundance of the dominant sequences in a sample, but thousands of sequences are obtained for the same cost as only one hundred from a clone library. Thus, the output from pyrosequencing should be more representative of the microbial diversity in the sample. A shortfall of pyrosequencing is the read length; currently the maximum is around 400 bp, but efforts are underway to increase the read length to at least 1000 bp and match the capabilities of clone libraries.

qPCR is a quantitative technology for targeting specific genes within a sample. It relies on fluorescent emission during each PCR extension step from either: (1) A dye that intercolates non-specifically into any double stranded DNA, known as a SYBR Green based detection, or (2) A fluorophore released from the end of a short sequence probe annealed to the target amplicon, known as a TaqMan assay. qPCR is a valuable complementary technique to pyrosequencing, since the latter provides only an indication of the relative abundance and not of the amount. The major limitation of this technique is the small number of

simultaneous targets; only one target is possible in a SYBR Green assay and typically no more than three in a TaqMan assay.

***Visualizing Dehalococcoides in a community and in biofilms*.** A limited amount of research has been carried out to visualize *Dehalococcoides*. I will describe two significant studies here. Rowe et al. (2008) investigated the planktonic state versus the biofloc of a dechlorinating microbial culture. This work illuminates the spatial positioning of *Dehalococcoides* in communities. They used FISH probes with laser scanning confocal microscopy to look at *D. ethenogenes* and *Archaea*. Imaging showed that, while the methanogens formed long strands predominantly on the outer portions of the bioflocs, and were in low concentrations in the plankton, the *Dehalococcoides* cells were distributed throughout the planktonic phase and in all regions of the biofloc.

Aulenta et al. (2008) studied TCE reduction with a bio-electrochemical reactor. They used two reactors: one with a pure strain of *Geobacter lovleyi*, which was also proven to be active in a biocathode with TCE, and the second was a mixed culture enriched from Venice Lagoon, Italy (Aulenta et al. 2002). SEM imaging showed a clear distinction between colonization of the microorganisms on the electrodes of the two reactors. The mixed culture formed seemingly random aggregates, in contrast to the *Geobacter* pure strain which directly attached to the electrode.

Factors that provide selective pressure for reductive dechlorination in a microbial community.

Table 2 is a summary of the factors that can potentially affect the performance of anaerobic reductive dechlorination in *ex-situ* treatment reactors and *in-situ* treatment. These are: (1) bicarbonate and other electron acceptors, (2) chlorinated ethene concentration, and (3) H_2 delivery strategy. I elaborate here on each of these factors. I do not consider salinity and temperature, since I did not study these parameters for my research and they cannot be readily controlled in practice. Another critical factor for *in-situ* bioremediation is bioavailability of donor and acceptor substrates, meaning partitioning between the solid, liquid, and vapor phases and partitioning between organic and inorganic sediment. I do not consider bioavailability, since I did not work with sediments in my research. An introduction to the importance of salinity, temperature, and bioavailability on reductive dechlorination is in Häggblom and Bossert (2003).

***Bicarbonate and other electron acceptors*.** A slew of oxidized compounds are found in groundwater from the naturally present soil mineralogy, microbial activity, and anthropogenic contaminants. The list includes bicarbonate, nitrate, sulfate, iron, manganese, selenite, chromium, uranium, and halogenated compounds other than chlorinated ethenes. The microorganisms that reduce these contaminants are significant in the context of reductive dechlorination for a number of reasons.

First, they compete with the dechlorinators for H_2, acetate, fermentable substrates, or trace growth factors (Häggblom and Bossert 2003, Maphosa et al.

2010). This can cause inefficient electron utilization and may result in incomplete reductive dechlorination if excess donor substrates are not provided.

Second, the reduced end products of the other oxidized compounds may be undesired. For example, bicarbonate can be reduced to methane, a potent greenhouse gas, or to acetate, that may linger in the water and downstream become biochemical oxygen demand (BOD) (ref). Partial reduction of sulfate to sulfite is undesired as it can be an inhibitor to some dechlorinators and has a distinct, undesirable odor (Häggblom and Bossert 2003).

Third, the oxidized compounds can alter the pH, perhaps putting it outside of the optimum range for *Dehalococcoides*. Bicarbonate is the primary buffer in groundwater and is the central electron acceptor in hydrogenotrophic homoacetogenenis and methanogenesis. This may cause depletion of inorganic carbon and a large increase in pH. Many other electron-accepting processes either consume bicarbonate as a C source or produce bicarbonate from oxidation of an organic donor. These reactions affect the alkalinity and, in turn, the pH of the system. Having the pH go out of the optimum range can be an additional cause of incomplete dechlorination.

An advantage of the presence of electron acceptors other than chlorinated ethenes is that they may provide growth requirements to *Dehalococcoides* (above). Namely acetate and vitamin B_{12} from bicarbonate reduction and

Table 1.3

Selective pressures to the microbial community carrying out reductive dechlorination.

Selective pressure	Effect on bacteria and methanogens
Bicarbonate and other electron acceptors	• Allow for growth of bacteria that compete with dechlorinators for H_2, C, and other growth factors. • Reduced compounds may inhibit or be toxic to reductive dechlorination. • Reduction of bicarbonate or other compounds may alter the pH; bicarbonate is the primary pH buffer and can be consumed directly or indirectly from H^+ consumption or production. • Promotes growth of bacteria and EPS that, when decay may provide beneficial growth factors to dechlorinators • Promotes growth of dechlorinators that can metabolize other electron acceptors. • Energetically unfavorable fermentation processes can proceed when fermenters partner with organisms reducing other oxidized contaminants.
Chlorinated ethene concentration	• Stress to dechlorinating organisms carrying out reductive dechlorination (always disadvantageous). • Stress to organisms that compete with dechlorinators for growth factors; potentially disadvantageous if these organisms provide growth requirement to the dechlorinators. • Reductive dechlorination produces H^+, potentially decreasing the pH if the system is not sufficiently buffered.

H_2 delivery: direct addition or through fermentation	• Provision of excess hydrogen may divert electron away from reductive dechlorination and instead to:
	  ○ Other oxidized compounds
	  ○ Biomass
	  ○ Production of dead-end products that become BOD.
	• Fermentation processes may produce bicarbonate (thereby increasing the buffer capacity of the water) and H^+ (potentially decreasing the pH if water is not sufficiently buffered).

from decaying organisms that grew from reduction of other oxidized compounds. Dechlorinators may be more prevalent if they are able to reduce other oxidized compounds present in the water, e.g. dechlorinating strains of *Geobacter* and *Desulforomonas* also can reduce sulfate and may be more active in the presence of sulfate (Zhang et al. (2010)). An additional potential advantage is discussed next.

***Chlorinated ethene concentration*.** Chlorinated ethenes can induce stress either directly to the dechlorinators or indirectly to other members in the microbial community. Yu and Semprini (2004) demonstrated that sufficiently high concentrations of chlorinated ethenes can cause self-inhibition, also termed Haldane inhibition. Additionally, Yu and Semprini (2005) noted that chlorinated ethenes featuring a larger number of chlorine atoms can inhibit the reduction reactions of those with fewer chlorine atoms, termed competitive inhibition, e.g., PCE on conversion of TCE to DCE and DCE to VC. They indicated that chlorinated ethenes with fewer chlorine atoms also inhibit the dechlorination of compounds with higher numbers of chlorine atoms, but to a much lesser extent. These stressors may be due to thermodynamic limitations or to enzymatic inhibition, but both result in decreased kinetic activity. Haest et al. (2010) documented that, besides the concentration of the chlorinated ethenes, the density of the dechlorinating bacteria controlled the extent of inhibition. Recently, Sabalowsky and Semprini (2010a,b) demonstrated that high concentrations of chlorinated ethenes, >1 mM TCE and >3mM *cis*-DCE, as present in dense non-aqueous phase liquid (DNAPL) plumes, can cause a decrease in the net yield of

Dehalococcoides. They modeled this as an increase in the decay rate due to solubilization of the cell membrane, but they noted that it is also possible that a decreased biomass yield could have resulted from decoupling of biosynthesis from the electron transport chain.

Stress induced by chlorinated ethenes can affect the non-dechlorinating microorganisms in ways that benefit reductive dechlorination by reducing competition for electrons. For example, Yang and McCarty (2000) reported that inhibition to hydrogenotrophic methanogenesis and homoacetogenesis increased electron availability to dechlorination and resulted in higher dechlorination activity; the efficiency of H_2 utilization increased from 10% to 67% when the PCE concentration was increased from 0.1 mM to 1 mM. I hypothesize that suppressing the competing processes also has the potential to be detrimental to reductive dechlorination if the involved organisms provide growth factors or cofactors to the dechlorinators. For example, suppression of homoacetogens could decrease the acetate C-source and the vitamin B_{12} cofactor for *Dehalococcoides*, potentially leading to decreased kinetic activity of reductive dechlorination or to decoupling.

Aside from inhibition, a high concentration of chlorinated ethenes can have a profound impact on pH. Each step in chlorinated ethene reduction produces one mole of protons per mole of chlorinated ethene (Figure 2); so, reductive dechlorination can decreases the pH if the system is not sufficiently buffered.

H_2 delivery strategy. The consensus understanding for *in-situ* bioremediation is that the H_2 needed by *Dehalococcoides* ought to be supplied by fermentation of slowly released organic compounds (Smatlak et al. 1996, Fennell and Gossett 1997). The basic understanding is that excess delivery of H_2 may promote undesired proliferation of other electron accepting processes (above).

Smatlak et al. (1996) provided the first evidence that slow-releasing hydrogen compounds were most effective in promoting efficient reductive dechlorination in the context of competition for H_2 between dechlorinators and methanogens. They experimentally determined the half-maximum-rate concentration of H_2 for these two organisms, finding that the value for methanogenesis was an order of magnitude greater than for reductive dechlorination (960 ± 180 nM and 100 ± 50 nM, respectively). Thus, methanogen competition for H_2 could be reduced by maintaining a sufficiently low bulk H_2 concentration. A low concentration is more readily obtained when H_2 is formed slowly through fermentation. A chlorinated ethene concentration that is inhibitory to methanogens or other electron accepting processes precludes this argument (above, Fennell and Gossett 1998).

An overview of the fermentation of organic compounds is in Figure 6. Fermentation normally begins with relatively large (high-C) compounds that are converted to smaller, simpler molecules. In many cases, the fermentable substrates are made up of relatively reduced C, which increases the reducing potential, and by extension, the H_2 delivery per C. Vegetable oil ($C_{55}H_{98}O_6$), whose oxidation state is -1.56, is often used for *in-situ* bioremediation for this

reason; in theory it has 5.56 e^- eq./C eq. and can thus provide 153 moles of H_2 per mole of oil, according to:

$$C_{55}H_{98}O_6 + 104H_2O \rightarrow 55CO_2 + 153H_2 \quad (1)$$

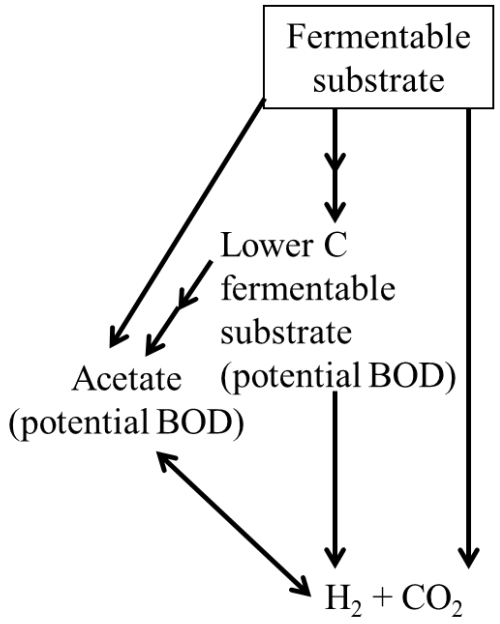


Figure 6. Fermentation reactions that can provide electrons and carbon for reductive dechlorination. Double arrows signify the potential for more than one step in the fermentation process.

In contrast, the fermentation products are lower-C compounds – including propionate, lactate, methanol, butyrate, and ethanol – that also have higher C oxidation states and therefore lower reducing potential per C. For example, acetate has an oxidation state of 0, which means that each C equivalent contains 4 e^- eq. In summary, in order to maximize H_2 availability it is advantageous to supply organic compounds with a high reducing potential.

Vegetable oil is also a superior electron donor compared to other organic compounds because of its physical properties as a large molecule. It is hydrophobic, forming non-aqueous phases, and is thus a slow H_2-releasing compound. The supplied electron donor can last for several years, in contrast to the low-C compounds, which require re-amendment, sometimes monthly. The re-amendments can promote excess bacterial growth around the injection well, leading to clogging, causing the treated plume to migrate around the injection well, and thus the well becomes unusable. Furthermore, the vegetable oil is generally supplied as an emulsion; this reduces its surface charge and therefore its hydrophobicity, allowing for increased spreading and therefore requires fewer holes to deliver the electron donor.

The factors just described may render slow-release H_2 compounds the only viable option for *in-situ* treatment, but various other factors should also be considered. These factors can be generalized to the fate of the electrons supplied and the fate of the protons and other acidic products produced.

The fate of the electrons can be further broken down into three affects to the microbial community. First is the loss of electrons to the biomass metabolism. In each fermentation processes, a fraction of the electrons are used for biomass synthesis, f_s, usually ~0.18 (Rittmann and McCarty 2001). Second, is that some of the electrons are lost to dead-end organic products that downstream become biochemical oxygen demand (BOD). Third is the potential decrease in electron-utilization efficiency for dechlorination due to competition from other oxidized compounds, unless they are inhibited by the chlorinated ethenes (above).

In some cases, these competing electron acceptors can provide energetically favorable conditions for fermentation of the otherwise dead-end organics, thus increasing the H_2 availability for dechlorination. In order for these reactions to proceed, the Gibbs Free Energy, ΔG, must be a negative value:

$$\Delta G = \Delta G^{0'} + RTlnQ \qquad (2)$$

where ΔG are for standard temperature and pressure and Q is the reaction quotient.

When a fermenter partners with a hydrogen scavenger, the fermenter's Q can be reduced, thus making its Gibbs Free Energy negative. For example, methanol fermentation to H_2 has:

$$CH_3OH + 2H_2O \rightarrow 3H_2 + HCO_3^- + H^+ \qquad (3)$$

$\Delta G^{0'}$ = 23.5 kJ/mole CH_3OH.

The standard free energy is positive. However, Cord-Ruwisch and Olliver (1986) found that this reaction proceeded when the methanol fermenter *Sporomusa acidovorans* partnered with the sulfate-reducing bacterium *Desulfovibrio vulgaris* G6 or with the hydrogenotrophic methanogens *Methanobacterium formicicum* or *Methanospirillum hungatei*. The partners consumed hydrogen enough that the actual free energy became negative for *Sporomus acidovorans*. A similar conclusion was found for acetate fermentation:

$$CH_3COO^- + H^+ + 2H_2O \rightarrow 2CO_2 + 4H_2 \qquad (3)$$

$\Delta G^{0'}$ = 95 kJ/mole CH_3OO^-.

Here, Cord-Ruwisch et al. (1998) coupled acetate oxidation by *Geobacter sulfurreducens* with nitrate reduction by *Wolinella succinogenes* or *Desulfovibrio desulfuricans* to give a negative actual free energy.

Another factor to consider is how fermentation reactions can alter the pH of the water. Fermentation produces weak organic acids that act as a pH buffer and dissociate into H^+ ions and a base, e.g., acetic acid dissociates into H^+ and acetate. Fermentation also produces inorganic acid, namely H_2CO_3, that primarily serves to increase the alkalinity and act as a pH buffer. This and the naturally present alkalinity may not be enough to offset the organic acid addition, thereby resulting in changes to the pH. A detailed discussion on the role of electron donor, alkalinity, and pH is in McCarty et al. (2007).

One alternative to fermentation as a source of H_2 is to add formate, which is enzymatically converted to bicarbonate and H_2 (McCarty et al. 2007); this direct conversion means that electrons are not wasted due to production of biomass or fermentation product. The major concern remains competition for H_2 by other electron acceptors (McCarty et al. 2007).

Direct addition of H_2 should be advantageous for the same reasons as for formate, but is not considered for subsurface bioremediation because its low solubility in water makes it difficult to deliver (Häggblom and Bossert 2003, Aulenta et al. 2006). However, H_2 may be a viable option for *ex-situ* treatment of chlorinated ethenes.

The H_2-based Membrane Biofilm Reactor.

The Membrane Biofilm Reactor (MBfR) is used for the biological reduction of oxidized contaminants in water using H_2 supplied through pressurized fibers as the electron donor. Details of the reactor design are provided elsewhere (Rittmann 2007), but I provide here a brief description. At the bench-scale, a typical MBfR consists of two glass modules connected with a peristaltic pump. A bundle of hollow fibers (Mitsubishi Rayon Model MHF 200TL for my research) is potted in the main module, while the second module may hold several fibers used for biofilm sampling. Hydrogen gas diffuses through the fibers' bubbleless gas-transfer membrane to a biofilm growing on the surface of the fibers. Water is pumped into and recirculated at a high flow rate through the modules. The oxidized compounds in the bulk liquid diffuse through the biofilm in the opposite direction from the H_2, and the microorganisms in the biofilm respire the compounds, reducing them to harmless products (e.g. TCE to ethene, nitrate to nitrogen gas and perchlorate to chlorine), which leave the reactor with the effluent water.

The MBfR was used for reduction of TCE in combination with nitrate in Chung et al. (2008a) and in combination with nitrate, sulfate, trichloroethane, and chloroform in Chung et al. (2008b). Bicarbonate was not provided in either study, and CO_2 was only available from the atmosphere, which precluded significant competition from methanogens and homoacetogens. Despite success in the previous studies by Chung et al. (2008a,b), many of my attempts to achieve reductive dechlorination of TCE were completely unsuccessful or resulted in

stalled reduction at *cis*-DCE. Thus, I focused much of my research with the MBfR on understanding the conditions needed to achieve TCE reductive dechlorination with direct delivery of H_2 in the MBfR. This focus builds on my past experience with the MBfR for other oxidized contaminants.

My previous experience with the MBfR for removal of nitrate and perchlorate. In my previous research with the MBfR, I studied the biological reduction of the oxidized contaminants nitrate and perchlorate using groundwater from the City of Pasadena, CA. The water also contained the oxidized compounds sulfate and dissolved oxygen. Nitrate is present in groundwater from fertilizers and can cause so called "blue-baby syndrome," and perchlorate is from rocket fuel and causes thyroid problems (U.S. EPA, 2002).

The primary goal of the project was to study the MBfR performance surface. I described MBfR performance with a relationship among the electron donor availability (by varying the H_2 pressure) and the surface loading of the oxidized compounds on the biofilm (either by varying the flow rate and by extension the hydraulic retention time (HRT) or by spiking the influent with the oxidized contaminants). The data set and key conclusions are presented in Ziv-El and Rittmann (2009a). I also assessed the effluent water quality in a companion paper (Ziv-El and Rittmann, 2009b).

My earlier MBfR work led to four significant conclusions. (1) The biofilm was able to grow using only the groundwater as the inoculum. The groundwater was recirculated in the reactors for one day and then switched to a continuous feed. (2) When H_2 delivery was limited, the acceptors had a clear

hierarchy for bioreduction: oxygen, nitrate, nitrite, chlorate, perchlorate, and then sulfate. This ordering of the acceptors has practical implications. First, in order to have good perchlorate reduction, nitrate and nitrite had to be almost completely reduced. Second, sulfate reduction and the formation of hydrogen sulfide (an undesired byproduct) occurred only when all other acceptors were reduced and hydrogen was still in excess. Third, since nitrite and hydrogen sulfide are easy to measure in the field, this reduction order allows a convenient means for on-site monitoring of the appropriate hydrogen pressure. (3) The maximum hydrogen flux for these fibers could be related almost linearly to the H_2 pressure, indicating that hydrogen was almost always the limiting substrate despite the broad operating conditions tested. (4) The MBfRs used in this research did not generate water-quality problems, such as significant increases in dissolved or biodegradable carbon, acetate, methane, or odor. Also, advective H_2 loss in the effluent was negligible.

Context and objectives of dissertation.

In a review article on *Dehalococcoides*, Taş et al. (2009) stressed the need to move away from isolation and "reductionist" approaches to studying anaerobic reductive dechlorination; instead, they advocated for understanding these organisms in their natural environment, which is as members of mixed microbial consortia. Studies that have relied on molecular techniques to look at the microbial community have focused on who was there and what they could be doing, but most of the studies have not been designed in such a way to provide a

meaningful connection between activity and the relative abundance of the microbial groups.

The overarching objectives of my dissertation are to understand how *Dehalococcoides* function optimally to carry out reductive dechlorination of chlorinated ethenes in a mixed microbial community and then to apply this knowledge to manage dechlorinating communities in the H_2-based membrane biofilm reactor. For all of the experiments, I used the mixed microbial consortium DehaloR^2, developed in our laboratory. The outline and specific objectives for each chapter follow.

Chapter 2. Development of the novel microbial consortium, named DehaloR^2, that dechlorinates chlorinated ethenes was described in the MS thesis of Yao (2009). A crude assessment of the rates of reductive dechlorination (Yao 2009) suggested that this consortium was able to dechlorinate more rapidly than other consortia described in the literature. In Chapter 2, I quantitatively assess the TCE turnover rates in DehaloR^2 in batch serum bottles and compare them to dechlorinating consortia described in the literature. I then provide the first characterization of the microbial community structure of DehaloR^2 assessed with pyrosequencing, qPCR, and a clone library. This chapter was published in an altered format in *Applied Microbiology and Biotechnology* (Ziv-El et al. 2011).

Chapter 3. I was interested in assessing the microbial community structure of a trichloroethene (TCE)-reducing consortium over a range of TCE concentrations using two complementary approaches. The first approach involved predicting trends in the community structure based on an electron

balance analysis, as illustrated in the figure below. The second approach was experimentally assessing the community structure via the molecular-based methods of quantitative PCR and pyrosequencing. My underlying interest was to highlight the potential value in using electron balances in combination with molecular analyses to understand microbial consortia used in bioremediation systems such as *in-situ* treatment or the MBfR. I designed an experiment to track the flow of electrons in DehaloR^2 grown at pseudo steady state in fill-and-draw reactors and assessed the resulting microbial community. I provided TCE at concentrations not previously shown to stress dechlorinating organisms. My hypothesis was that the electron balance analysis would predict the experimental trend as long as stress from the chlorinated ethenes was exhibited by decreased conversion of the chlorinated ethenes; a deviation from the trend would indicate stress to the biomass yields. This chapter was submitted in an altered format to *Biotechnology and Bioengineering*.

Chapter 4. The studies in chapters 2 and 3 analyzed for chlorinated ethenes and ethene by measuring products in the headspace. Many bench-scale reactors do not contain a headspace and additionally have small volumes. It would be advantageous to be able to analyze the chlorinated ethenes with a small volume and for the detection limit to be at or below the maximum contaminant levels (MCLs). In Chapter 4, I describe the method that I optimized to use a 1-mL liquid sample to analyze very close to the MCLs for simultaneous detection of all chlorinated ethenes and ethene using solid phase microextraction with a gas

chromatograph and a flame ionization detector. This chapter has been submitted in an altered format to the *Journal of Chromatograpy A*.

Chapter 5. In this chapter, I combined my knowledge of the DehaloR^2 microbial community from Chapters 2 and 3 and used the analysis method from Chapter 4 to study reductive dechlorination in the H_2-based MBfR. The goal in this study was to develop a reliable start-up strategy for TCE reduction in the MBfR and to then assess the resulting microbial community with molecular techniques and imaging. My hypothesis was that, if methanogens and homoacetogens could be controlled during start-up, this would allow dechlorinators to reduce TCE to ethene and they would continue to be active during continuous flow. The results in Chapter 4 confirmed my hypothesis. This was submitted in an altered format to *Biotechnology and Bioengineering*.

Chapter 6. I summarize in Chapter 6 conclusions from the studies presented in Chapters 2 through 5. I also suggest several studies that expand on the MBfR research for TCE reduction presented in Chapter 5.

Chapter 2

DEVELOPMENT AND CHARACTERIZATION OF DEHALOR^2, A NOVEL ANAEROBIC MICROBIAL CONSORTIUM PERFORMING RAPID DECHLORINATION OF TCE TO ETHENE

This chapter was published in an altered format in *Applied Microbiology and Biotechnology* (Ziv-El et al., 2011).

Introduction

While TCE it is naturally produced by micro- and macro-organisms in marine environments (Abrahamsson et al., 1995; Kittelmann & Friedrich, 2008a), reductively dechlorinating microorganisms from these water sources have not been extensively studied, but could potentially provide a wealth of novel chlorinated ethene-respiring bacteria. Previously reported chlorinated ethene-respiring microbial consortia studied in marine sediment microcosms were distinct from those obtained from soil and groundwater (Griffin et al., 2004; Kittelmann & Friedrich, 2008a; Miller et al., 2005). One of the most striking differences was the transformation of perchloroethene (PCE) and TCE to greater amounts of the intermediary *trans*-dicloroethylene (*trans*-DCE), whereas consortia from soil and groundwater microcosms primarily generate *cis*-DCE as an intermediate or end product (Griffin et al., 2004; Kittelmann & Friedrich, 2008a; Miller et al., 2005). Furthermore, in most microcosms showing an elevated ratio of *trans*- to *cis*-DCE, both DCE congeners accumulated over time (Griffin et al., 2004; Kittelman & Friedrich, 2008b; Miller et al., 2005), indicating

either the absence or inhibition of bacteria able to perform complete dechlorination to ethene.

In addition to the microbial ecology, a significant question for bioremediation of chlorinated solvents is how to compare the kinetics of various chlorinated ethene cultures and how to ascertain the cause of any given differences in chlorinated ethene transformation rates.

The MS thesis of Ying Yao (Yao, 2009) described the development of a sediment-free, anaerobic microbial consortium, designated DehaloR^2. This culture has been maintained stably in our laboratory for over three years and performs reductive dechlorination of TCE to ethene at high rates compared to many similar cultures in the literature. The originating inoculum for this novel consortium was sediment from a brackish tributary of the Chesapeake Bay (CB) near Baltimore, Maryland, where dechlorination products of the antimicrobial compound triclocarban (TCC) previously suggested the presence of dehalorespiring microbiota (Miller et al., 2008). I summarize in this chapter the development of this culture (Yao, 2009) and then describe my contribution in characterizing DehaloR^2.

Using pyrosequencing and phylogenetic analyses, I compared the microbial community structure of CB sediment to DehaloR^2. I also led the effort to characterize DehaloR^2 in terms of various kinetic parameters and investigated the microbial community with a clone library and quantitative PCR (qPCR).

Materials and Methods.

Development and maintenance of a sediment-free, TCE to ethene dechlorinating culture. The sediment was obtained from the Back River, a tributary of the Chesapeake Bay (CB) located near Baltimore, Maryland. This tidal body of water receives effluent from a wastewater treatment plant. Previous work showed possible evidence of microbial reductive dechlorination activity towards polychlorinated aromatics in sediment from the sampling location utilized for this study (Miller et al., 2008; Heidler et al., 2006). Upon arrival at the laboratory, CB sediment samples from different depths of the core were mixed homogeneously in an anaerobic glove chamber (Coy laboratory products Inc. Grass Lake, MI) in an atmosphere of 3.5% H_2 and 96.5% N_2 and stored in sterile Mason jars at 4^{o}C.

Microcosms were set up in the anaerobic glove chamber by transferring 10 g of sediment into 160 mL glass serum bottles containing 90 mL of sterile anaerobic medium as described by Löffler et al. (2005), 1 mL ATCC vitamin supplement, 50 µg/mL vitamin B_{12}, 2 mM lactate, and 450 µM TCE. Microcosms were sealed with butyl rubber stoppers and incubated statically in the dark at 30^{o}C. Two microcosms were set up with autoclaved sediment as abiotic controls.

To generate sediment-free cultures, we transferred 10 mL liquid suspension to triplicate serum bottles (100 mL total liquid volume) amended with 5 mM lactate, 11.1 mM methanol, and 320 µM TCE. We named this culture DehaloR^2 (available through the Arizona State University Arizona Technology Enterprises). When the chlorinated electron acceptors are consumed, the cultures

are re-amended with TCE and electron donors, and are transferred into fresh media regularly with a 10% inoculum.

***Chemical Analyses*.** Concentrations of TCE, 1,1-DCE, *cis*-DCE, *trans*-DCE, vinyl chloride (VC), ethene, and methane were quantified by injecting 200 µL headspace samples with 500 µL gas-tight syringes (Hamilton Company, Reno, NV) into a Shimadzu gas chromatograph (GC-2010, Columbia, MD) equipped with a flame ionization detector. We used an Rt™-QSPLOT capillary column (30 m × 0.32 mm × 10 μm, Restek, Bellefonte, PA) and helium as the carrier gas. The initial oven temperature was 110°C held for 1 min, and then raised with a gradient of 50°C/min to 200°C. A second gradient at 20°C/min raised the temperature to 200°C, followed by a third gradient at 15°C/min to 220°C and held for 2.5 min. The temperature of the FID and injector were 240°C.

Concentrations of lactate, acetate, propionate, and methanol were measured using a Shimadzu high performance liquid chromatography (HPLC, LC-20AT) equipped with an Aminex HPX-87H (Bio-Rad) column for separation of simple acids and solvents. The eluent was 2.5 mM sulfuric acid fed at a flow rate of 0.6 mL/min. We detected chromatographic peaks using a photodiode-array detector at 210 nm and a refractive index detector. The total elution time was 60 min and the oven temperature was constant at 45°C.

***DNA Extractions*.** For the pyrosequencing analysis of the sediment, DNA was extracted from 0.4 g of wet sediment. The extraction was carried out with a FastID (Genetic ID NA, Inc., Fairfield, IA) kit, MoBio bead tubes (MoBio Laboratories, Carlsbad, CA), and 10% SDS solution to increase cell lysis and

improve DNA yields. For all other DehaloR^2 DNA extractions, pellets were formed from 1.5 mL liquid centrifuged for 15 minutes and stored at -20°C. Before proceeding with the DNA extraction protocol for Gram-positive bacteria (QIAGEN DNeasy® Blood and Tissue Kit), we suspended the pellets in 180 µL lysis buffer containing 20 mM Tris-HCl, 2 mM EDTA, 250 µg/mL achromopeptidase, and 20 mg/mL lysozyme, incubated for 60 minutes at 37°C in a Thermomixer® R (Eppendorf), and treated the suspension with SDS (1.2% w/v) before a further incubation for 10 min at 56°C. All extracted DNA was quantified with a Nanodrop-1000 instrument (Nano-Drop Techonologies, Inc.).

***Genomic-based Methods for Microbial Ecology Analysis*.** We amplified genomic DNA with 16S rRNA gene bacteria primers (Zhou et al., 1997) and used the product as a template for nested PCR reactions targeting the 16S rRNA gene of *Dehalobacter* (Schlötelburg et al., 2002) and *Desufuromonas* (Löffler et al., 2000) primers to verify the presence or absence of these organisms. qPCR targeting the 16S rRNA gene was used to quantify Bacteria, *Dehalococcoides,* and *Geobacteraceae*. Since this region is almost identical across *Dehalococcoides,* we quantified the reductive dehalogenase genes *tceA, bvcA,* and *vcrA* to better assess presence of characterized and unique strains (Holmes et al., 2006). We performed TaqMan® triplicate assays and seven-point standard curves in an Eppendorf Realplex 4S realcycler with 4 µL of DNA in 10 µL total reaction volume. The primers, probe, reagent concentrations, and thermocycler conditions for Bacteria were described in Ritalahti et al. (2006), in Cummings et al. (2003) for *Geobacteraceae,* and in Holmes et al. (2006) for *Dehalococcoides*. For the

reductive dehalogenase genes we used a triplicate assay described in Lee et al. (2008).

A Bacteria clone library was constructed following a protocol outlined by Torres et al. (2009). We trimmed the vector sequences with SeqMan Pro software (DNASTAR, Madison WI) and achieved 73 partial sequences. Out of 73 clones, we selected 9 clones as major phylotypes and completed nearly full-length sequences (> 1480 bp, except for clone DhR^2/LM-G05 at 840 bp) for further analysis. We assessed the quality of the sequences by using chimera check programs Bellerophon (Huber et al., 2004) and Mallard (Ashelford et al., 2006) and compared them to previously published sequences using BLAST search tool. We initially aligned 16S rRNA gene sequences using the Nearest Alignment Space Termination (NAST) program (DeSantis et al., 2006), and improved the alignment by manual correction with the editor ARB_EDIT4 that is based on 16S rRNA gene secondary structure (Ludwig et al., 2004). We constructed a neighbor-joining tree by using the ARB software package (Ludwig et al., 2004) with the Jukes-Cantor corrected distance matrix and the LanemaskPH filter that assists in preventing overestimation of branch lengths (Zhang et al., 2009). Our nine major phylotypes included in the phylogenetic tree were deposited in GenBank under accession numbers HQ012835-HQ012843.

Pyrosequence analyses targeting the V4 region of the 16S rRNA gene were performed on the sediment sample. The combined V2 and V3 regions were the target of duplicate DehaloR^2 samples from the next study, after the fourth reduction of TCE to ethene. For the sediment sample, we used 454 GS-FLX

protocols as described by Zhang et al. (2010), and for the DehaloR^2 samples, the bacterial tag-encoded FLX-Titanium amplicon pyrosequencing (bTEFAP) was performed as described by Wolcott et al. (2009). We conducted sequence alignment, clustering, and classfication by following procedures explained by Zhang et al. (2010). We used the program "mother" to calculate rarefaction, Shannon Index and Chao1 estimator (Schloss et al., 2009).

Experiment for Determining Maximum TCE Turnover Rates. We carried out the experiment in triplicate batch reactors consisting of 160 mL glass serum bottles (100 mL liquid, 60 mL headspace). Initially, 10% DehaloR^2 inoculum from a highly enriched culture with complete conversion to ethene was transferred to each bottle, along with 5 mM lactate and 11.1 mM methanol. We added 10 to 15 µL of neat TCE. The initial pH was between 7.2 and 7.5 and the initial bicarbonate concentration was 30 mM. After complete dechlorination to ethene, we removed 1.5 mL of culture for DNA extraction and 0.5 mL for a protein assay to determine biomass concentration (Bicinchoninic Acid Kit, Sigma-Aldrich, Milwaukee, WI). Before the next addition of lactate, methanol, and TCE, the bottles were flushed with N_2 to remove headspace gases and were amended with 10 mM bicarbonate. TCE and electron donors were added five consecutive times, until the rates of reductive dechlorination, the concentration of biomass, and the cell copies measured with qPCR stabilized and then started to decrease, indicating the onset of biomass decay.

Results.

Sediment Microcosm and Culture Development. Figure 1 shows that the sediment microcosms had an 18-day lag time before the onset of dechlorination. TCE was predominantly converted to *trans*-DCE and *cis*-DCE between days 18 and 40, after which reductive dechlorination stalled at a *trans*-to-*cis*-DCE mole ratio of 1.67 ± 0.15, calculated from nine data points over 85 days. The small amount of VC (2.6 µM) and ethene (0.9 µM) indicated only a small proportion of DCE was reductively dechlorinated in the sediment. Concomitant measurements of the headspace by GC-FID showed production of a significant amount of methane in the initial microcosms (data not shown). In the abiotic controls, no TCE reductive dechlorination byproducts were detected over the course of the experiment. In contrast to the microcosms, the sediment-free DehaloR^2 consortium rapidly dechlorinated 320 µM TCE to ethene within 10 days with formation of *cis*-DCE as the primary DCE intermediate (from day 160 in Figure 1).

DehaloR^2 Community Structure. The structures of the microbial community in the sediment used to set up the microcosms and in duplicate enrichment cultures of DehaloR^2 are shown at phyla and genera levels in Figure 2. The numbers of trimmed sequence tags were 6,792 for the sediment 3,323 and

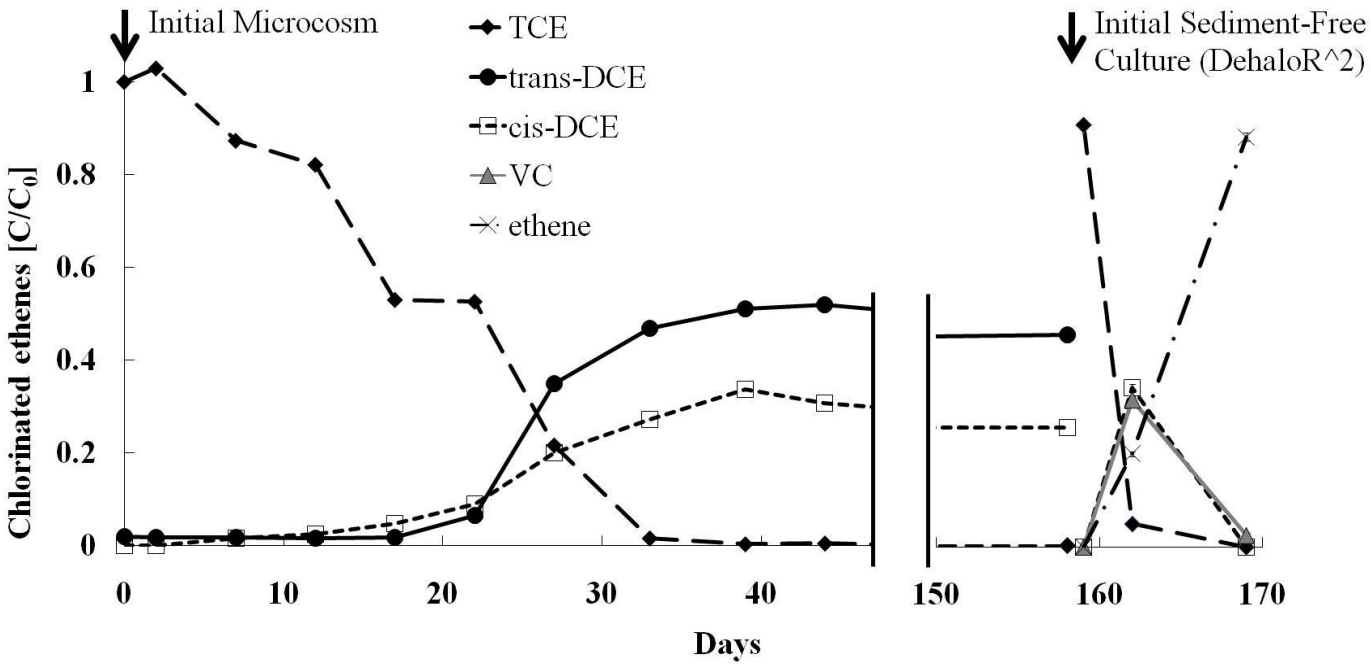
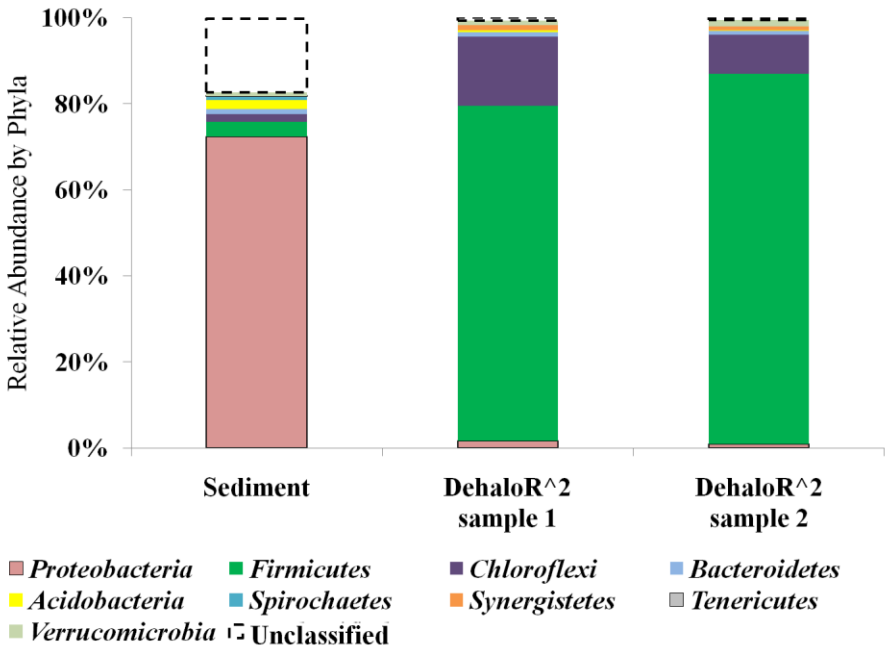
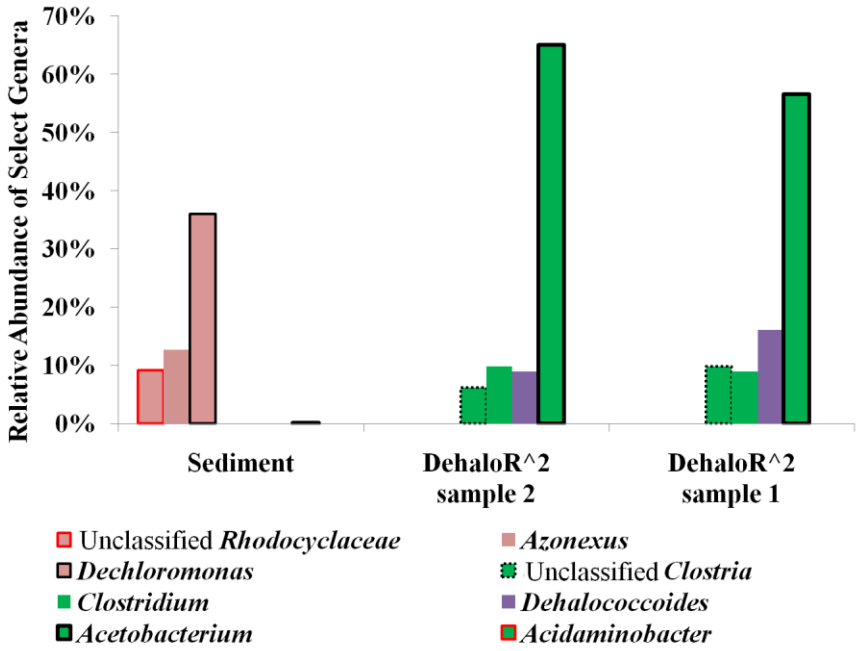


Figure 2.1. Chemical conditions in culture vessels showed a shift from incomplete reductive dechlorination of TCE to DCE (*trans*-to-*cis* isomer ratio of 1.67 ± 0.15) in the initial sediment microcosm to complete and much more rapid dechlorination to ethene with negligible accumulation of *trans*-DCE 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.



(A)



(B)

Figure 2.2. Comparison of the bacterial communities by (A) phyla and (B) select genera 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.

3,215 for the enrichment cultures. These were grouped, respectively, into 508, 169, and 144 unique operational taxonomic units (OTUs) based on 95% similarity; this information and statistical analyses are summarized in Table 1. The actual numbers of OTUs, sample richness, estimated by the Chao1 statistical parameter, were 776, 262, and 212. *Proteobacteria* was the dominant phylum in the sediment sequences, with 72% of all sequences, and it decreased to 0.8-1.7% after enrichment. *Firmicutes* became the major phylum in DehaloR^2 sequences, at 78-86% in comparison to 1.6% in the sediment sequences. Since most *Firmicutes* are fermenters, enrichment on excess lactate and methanol (6.7 and 6 me- eq. per TCE addition, respectively) channeled ~80% of the electron equivalents in approximately equal ratios to acetate (~6.5 mM) and propionate (~3.5 mM), thereby biasing the microbial community towards this phylum. The genus *Dehalococcoides* and its corresponding phylum, *Chloroflexi*, increased from non-detectable to 9-16% in DehaloR^2.

While the sediment sequences at the genus level were dominated by the Proteobacteria *Dechloromonas* (36%) and *Azonexus* (12.7%), in addition to unclassified Rhodocyclaceae (9.1%), three genera from the Firmicutes phylum were highly enriched in the second transfer of DehaloR^2. *Clostridia* and the homoacetogen *Acetobacterium*, both negligible in the sediment, increased to 8.9-9.9% and 56-65%, respectively. Unclassified Clostridium accounted for 6.2-9.8% of the sequence tags. Additionally, *Geobacter*, a dechlorinating organism capable of transforming TCE to *cis*-DCE, stayed relatively constant at 0.5-1.5%.

Table 2.1

Sequence processing data of 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.

Sample	Trimmed Tags	Observed Species (OTUs at 0.05 difference)	Chao1 estimator of richness at 0.05 difference (95% CI)	Shannon Index H' estimator of diversity at 0.05 difference (varH)	Evenness at 0.05 difference
Sediment	6792	508	776 (695,891)	4.015 (0.00061)	0.644
DehaloR^2 sample 1	3323	169	262 (220,337)	2.861 (0.00091)	0.558
DehaloR^2 Sample 2	3215	144	212 (179,276)	2.581(0.00107)	0.519

In order to identify the bacterial species in DehaloR^2, we constructed the clone library depicted in the phylogenetic tree shown in Figure 3. Of the 73 sequenced clones, 73% were fermenters, with homoacetogens constituting the largest fraction (48%), of which 31 were *Acetobacterium* and four were *Spirochaetes*. *Clostridium ganghwense*, a bacterium that may ferment lactate to propionate, as suggested by Zhao et al. (2008), was the closest phylogenetic match (97%) to 14 clones (19.2%). Nineteen clones (26.0%) were *Dehalococcoides* sp., which appear to be represented by several strains, some of which may be novel, as suggested by our sequencing data.

While the relative abundance of fermenters, including *Acetobacterium*, agreed well with the pyrosequencing analysis, we used qPCR to verify the abundance of *Dehalococcoides* compared to Bacteria and found $1.54 \pm 0.27 \times 10^{11}$ and $1.25\ 0.50 \times 10^{12}$ gene copies L^{-1}, respectively. We also quantified the cell density of *Geobacteraceae*, $2.67 \pm 0.38 \times 10^{10}$ genes copies L^{-1}, the family containing the genus *Geobacter*. *Desulfuromonas* and *Dehalobacter,* two other common TCE to *cis*-DCE dechlorinators, were not detected, even when using specific primers and nested PCR (data not shown), thereby indicating absence of these bacteria. Given our current understanding of reductively dechlorinating anaerobes, the reductive dechlorination activity displayed by DehaloR^2 appears to be linked to *Dehalococcoides* and *Geobacteraceae*. Results from qPCR targeting previously characterized *Dehalococcoides* reductive dehalogenase genes revealed that *vcrA, tceA* and *bvcA* were present in high abundance and were larger

than the total amount of *Dehalococcoides* : $1.56 \pm 0.40 \times 10^{11}$, $9.96 \pm 0.41 \times 10^{11}$, and $5.09 \pm 0.78 \times 10^{9}$ gene copies L^{-1}, respectively.

Firmicutes, *Spirochaetes*, and *Synergistes* phyla based on nearly full-length sequences (>1480bp), except for clone DhR^2/LM-G05 (840bp). *Thermotoga thermarum* was used as the outgroup sequence. (B) An expanded branch illustrating the *Dehalococcoides* sp. group. Bootstraps were calculated based on 1000 replicates and values greater than 500 are indicated with filled circles. Sequences from this study are in bold, brackets indicate the number of clones that belong to each phylotype, and accession numbers are in parentheses. The scale bars indicate the number of nucleotide substitutions per site.

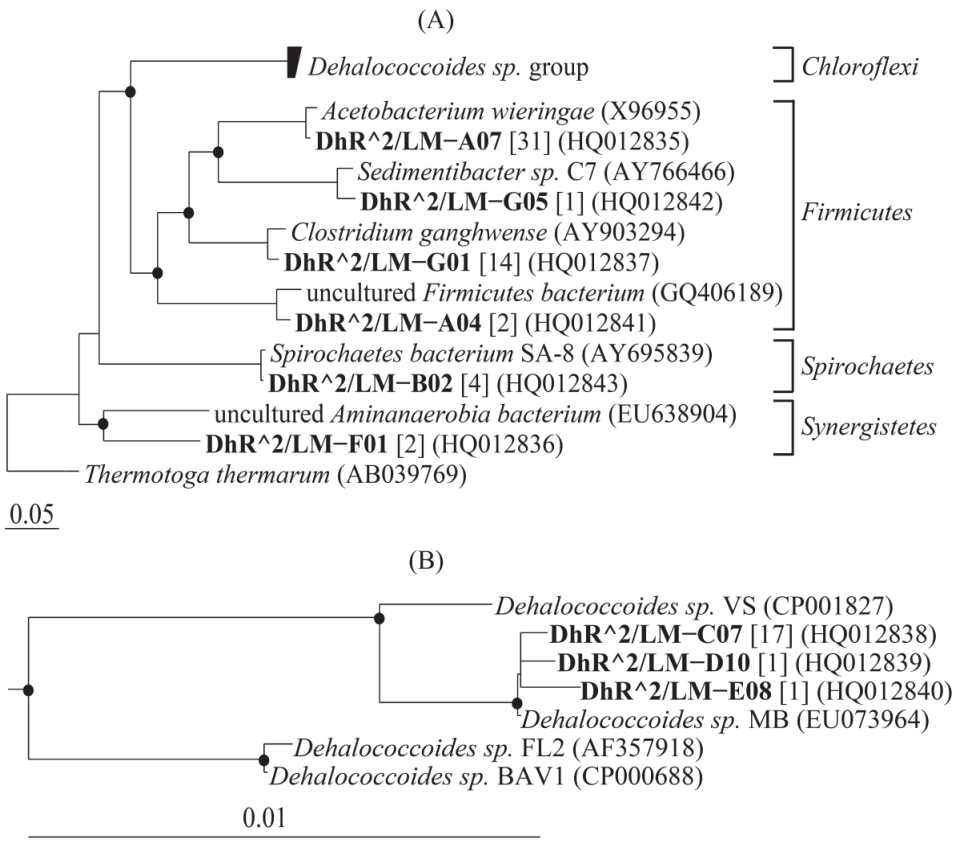


Figure 2.3. Neighbor-joining phylogenetic trees of 16S rRNA gene sequences from a clone library of DehaloR^2 with 73 clones. (A) A subset of *Chloroflexi*, *Firmicutes*, *Spirochaetes*, and *Synergistes* phyla based on nearly full-length sequences (>1480bp), except for clone DhR^2/LM-G05 (840bp). *Thermotoga thermarum* was used as the outgroup sequence. (B) An expanded branch illustrating the *Dehalococcoides* sp. group. Bootstraps were calculated based on 1000 replicates and values greater than 500 are indicated with filled circles. Sequences from this study are in bold, brackets indicate the number of clones that belong to each phylotype, and accession numbers are in parentheses. The scale bars indicate the number of nucleotide substitutions per site.

Maximum TCE Turnover in DehaloR^2. Comparing the maximum rate of reductive dechlorination can be of practical value when selecting potential cultures for bioaugmentation. The results of an enrichment culture time-course experiment are presented in Figure 4. The rate of TCE reductive dechlorination to ethene ($\Delta C\ \Delta t^{-1}$) was calculated for each addition of TCE, and the maximum rate, 2.83 mM $Cl^-\ d^{-1}$, was reached in the third addition when the concentration of *Dehalococcoides* had also reached a maximum (Figure 4). This rate is compared to other values in the literature in Table 2.

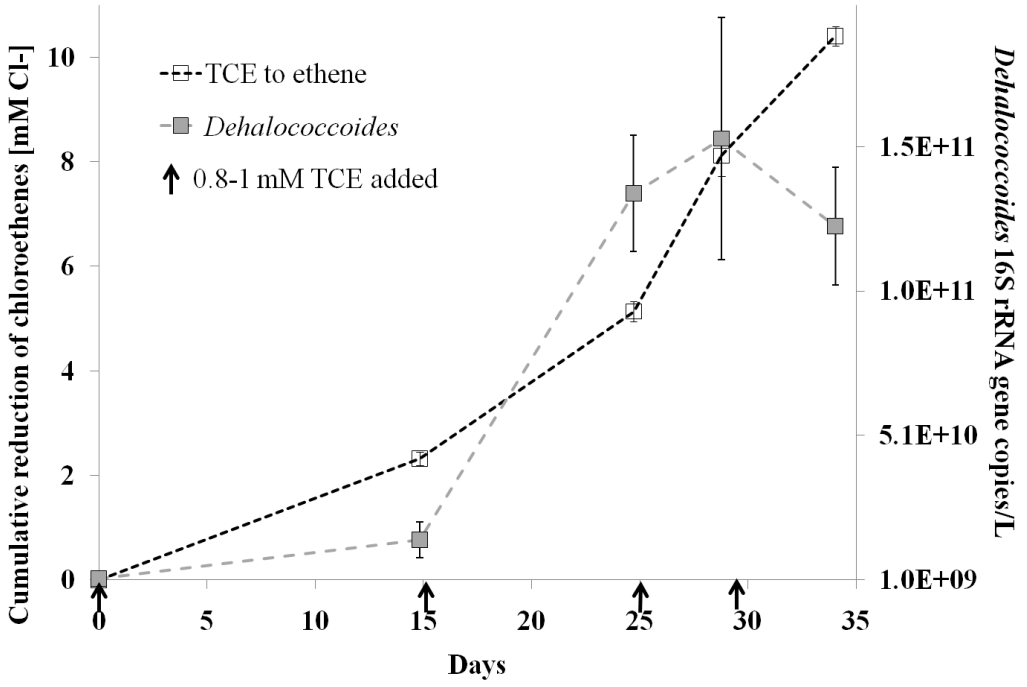


Figure 2.4. Kinetic experiment of successive TCE additions in triplicate enrichment cultures plotted in terms of the cumulative reduction of TCE to ethene, as mM Cl^- produced, and the concentration of *Dehalococcoides*. Note that 1 mM Cl^- is released during each step in the reductive dechlorination.

Table 2.2

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. The turnover rate per Dehalococcoides cell was only calculated when values for $(\Delta C\, \Delta t^{-1})_{max}$ and X_{Dhc} were available from the same source and where data were from the stationary phase. [a]*Vainberg et al. (2009), $(\Delta C\, \Delta t^{-1})_{max}$ was calculated from figure 4 and X_{Dhc} from Table 1.* [b]*Xiu et al.(2010).* [c]*Cupples et al. (2004).* [d]*Haest et al. (2010), $(\Delta C\, \Delta t^{-1})_{max}$ and X_{Dhc} were calculated from figure 2, and X_{Dhc} was the final concentration of cells.* [e]*Freeborn et al. (2005), $(\Delta C\, \Delta t^{-1})_{max}$ was from Figure 1 and X_{Dhc} from Table 3.* [f]*Richardson et al. (2002), calculated assuming 200 µmoles TCE/bottle were reduced in 10 days.* [g]*Amos et al. (2008), $(\Delta C\, \Delta t^{-1})_{max}$ was calculated from Figure 2 and X_{Dhc} was the final concentration of cells in Figure 1.*

Culture	$(\Delta C\ \Delta t^{-1})_{max}$ [mM Cl^- d^{-1}]	X_{Dhc} [cells L^{-1}]	$(\Delta C\, \Delta t^{-1})_{max}\ X_{Dhc}^{-1}$ [mmol Cl^- $cell^{-1}$ d^{-1}]
DehaloR^2	2.83 ± 0.60 (TCE)	$1.54 \pm 0.27 \times 10^{11}$	$1.9 \pm 0.11 \times 10^{-11}$
SDC-9[a]	2.9 (PCE)	1.4×10^{11}	2.1×10^{-11}
Unnamed[b]	0.96 (PCE)	--	--
VS[c]	0.31 (VC)	4.0×10^{11}	7.8×10^{-13}
KB1[d]	0.16 (TCE)	8×10^{10}	--
ANAS	0.006[e] (TCE), 0.05[f] (TCE)	$1.0 \pm 0.29 \times 10^{10}$ [e]	6×10^{-13}
BDI[g]	0.03 (TCE)	1×10^{11}	--

Discussion.

This work produced a novel microbial consortium, DehaloR^2, for potential use in bioaugmentation of chlorinated-ethene spill sites. Its enrichment history is of interest for the following three reasons. First, the origin was estuarine sediment. Second, whereas the chlorinated ethene-degrading microcosm from which DehaloR^2 was developed stalled at DCE with significant production of *trans*-DCE, complete dechlorination through *cis*-DCE was attained when sediment was precluded from the culture (Figure 1). This is the first report of achieving complete dechlorination to ethene after transferring an incompletely dechlorinating sediment consortium to a sediment-free culture medium. The immediate onset and rapid dechlorination to ethene suggests that

Dehalococcoides sp. capable of complete dechlorination to ethene were present in the microcosm, but were inhibited by sediment constituents. The cause for this inhibition and the accumulation of *trans*-DCE is presently unknown and continues to be the subject of investigation. Third, while many reductively dechlorinating cultures were developed from PCE and TCE spill sites (Duhamel & Edwards, 2006; Macbeth et al., 2004; Richardson et al., 2002; Schaefer et al., 2009), DehaloR^2 originated from sediment that, to our best knowledge, had not before been exposed to chlorinated ethene contaminants (Miller et al., 2008). The sediment utilized in this study has not yet been characterized extensively for its chemical composition, and only the presence of two antimicrobial agents, triclosan and triclocarban, have been reported, along with several dechlorination products of triclocarban.

We achieved a deep understanding of the community structure using an emerging, high-throughput sequencing technique, pyrosequencing, a powerful tool for microbial ecologists. To our knowledge pyrosequencing has only been used to characterize one other TCE dechlorinating microbial community (Zhang et al., 2010). While in Zhang et al. (2010) the target was the V6 region of the 16S rRNA gene which only allowed differentiation of the bacteria at the genus level, here we targeted the combined V2 and V3 regions, allowing us to identify organisms at the species level. We found that the pyrosequencing results correlated well with the microbial community in the clone library, providing over 3000 sequences compared to 73 in the clone library.

The relative abundance of *Dehalococcoides* sp. in DehaloR^2, determined at 26% in the clone library and 9-16% in the pyrosequencing of the enrichment cultures, was not surprising compared to other mixed cultures in the literature grown on similar concentrations of chlorinated ethenes (Duhamel & Edwards, 2006; Duhamel & Edwards, 2007; Richardson et al., 2002). However, it was surprising that the sum of *vcrA*, *tceA*, and *bvcA* obtained with qPCR was larger than the concentration of *Dehalococcoides*, as all described *Dehalococcoides* strains have only one of these reductive dehalogenase genes. qPCR data and the 16S rRNA gene clone library suggest the presence of novel *Dehalococcoides* strains. Laboratory efforts are underway to further characterize these potentially novel organisms all of which, based on sequence homology of the clone library and pyrosequencing, belong to the *Dehalococcoides* Cornell group.

Another interesting quality of the community structure was the relative abundance of specific homoacetogen genera and species when compared to previous research. Homoacetogens may affect rates of reductive dechlorination by providing cofactors to the *Dehalococcoides* reductive dehalogenase enzymes (Johnson et al., 2009*). Acetobacterium* was dominant in the pyroseqeuncing enrichment cultures (56-65%), and in the clone library they were 42.4%, with *Spirochaetes* contributing 5.5%. In an *in situ* remediation field study by Macbeth et al. (Macbeth et al., 2004) in which a TCE plume was amended with lactate as the electron donor, *Acetobacterium* comprised 34.4% of the Bacterial clone library community and *Spirochaetes* contributed 4.3%. Richardson et al. (2002) identified a relative abundance of 20% Eubacterium (the class containing *Acetobacterium*) and 1.1% *Spirochaetes* in a TCE-transforming mixed culture, ANAS, which utilizes lactate as the electron donor. In the TCE-transforming mixed culture KB1, *Acetobacterium* was not dominant when the consortium was maintained with methanol as the electron donor (Duhamel & Edwards, 2006; Duhamel & Edwards, 2007). Duhamel and Edwards (2006) initially reported a relative abundance of *Spirochaetes* at 10.8% as the only homoacetogen in KB1 and later reported a composition of 26% *Sporomusa,* 12% *Spirochaetes* (taxonomic class), and 1% *Acetobacterium* (Duhamel & Edwards, 2007). The comparable relative abundance of homoacetogens between mixed cultures is a function of providing mostly identical medium with similar concentrations of fermentable substrates, while the specific genera and species are reflective of the culture sediment, soil, or groundwater origin.

A key finding is that the maximum turnover rate of TCE to ethene $(\Delta C\ \Delta t^{-1})_{max}$ in DehaloR^2 was at least one order of magnitude larger than other cultures reported in the literature, except for culture SDC-9 (Table 2) (Amos et al., 2008; Cupples et al., 2004; Haest et al., 2010; Richardson et al., 2002; Vainberg et al., 2009; Xiu et al., 2010). The value of $(\Delta C\ \Delta t^{-1})_{max}$ is reached in the stationary phase when X_{Dhc} is at its maximum for a given culture, and so the maximum turnover rate per cell $(\Delta C\ \Delta t^{-1})_{max}\ X_{Dhc}^{-1}$ was calculated when data were available. X_{Dhc} for DehaloR^2 was the same order of magnitude as those of other cultures and pure strains (Table 2), but only SDC-9 matched the high value of $(\Delta C\ \Delta t^{-1})_{max}\ X_{Dhc}^{-1}$ in DehaloR^2, suggesting that the fast metabolic rates in DehaloR^2 are a function of enzymatic kinetics and not of its ability to reach higher densities of dechlorinators.

Conclusions

In summary, I describe here the characterization of the first dechlorinating culture from estuarine sediment able to reduce TCE to the harmless compound ethene, with rapid rates of reductive dechlorination that are not attributable to the concentration of *Dehalococcoides*. It will be worthwhile to investigate whether this is due to the presence of novel dechlorinators, novel reductive dehalogenase enzymes, optimal cofactors provided to the reductive dehalogenase enzymes from other members in the community, or a combination of the above.

Chapter 3

USING ELECTRON BALANCES AND MOLECULAR TECHNIQUES TO ASSESS TRICHOROETHENE INDCUED SHIFTS TO A DECHLORINATING MICROBIAL COMMUNITY

This chapter has been submitted in an altered format to *Biotechnology and Bioengineering.*

Introduction

As described in some detail in Chapter 1, four processes are essential in order for microbial communities to carry out reductive dechlorination in the environment. First is reductive dechlorination by *Dehalococcoides,* as this is the only organisms to date that can completely reduce DCE and VC to non-toxic ethene (He et al., 2003; Sung et al., 2006b), and by other dechlorinators that can reduce PCE and TCE to DCE (Taş et al., 2009). Second is provision of H_2, the obligate e^- donor for *Dehalococcoides* (Maymó-Gatell, 1999, He et al., 2003; Sung et al., 2006b). The most common approach is to ferment organic substrates to form H_2 (Smidt and de Vos, 2004), but it is also possible to provide H_2 by direct addition (Chung et al., 2008). Third is provision of acetate, since acetate is the obligate C-source for *Dehalococcoides* (Tang et al. 2010) and a possible e^- donor and C-source for other dechlorinators (Sung et al. 2006a; Maphosa et al., 2010). Acetate could be provided from: (1) fermentation of the organic electron donor, (2) autotrophic reactions by homoacetogens, (3) biomass decay processes by a wide group of organisms that can consume complex organics, or (4) direct delivery. The fourth requirement is growth factors, including the vitamin B_{12}

cofactor necessary for reductive dehalogenase enzymes of *Dehalococcoides* (He et al., 2007); vitamin B_{12} is presumably formed during these acetate-producing processes (Maymó-Gatell, 1995).

Tracking the distribution of electrons to these and to competing processes is a potentially effective strategy in understanding the dominant functions taking place in microbial communities carrying out reductive dechlorination (Aulenta et al., 2002; Fennel and Gossett 2003; Azizian et al. 2010). Molecular techniques also have been employed to elucidate the microorganisms present and active in these microbial communities, and these are summarized in a review article by Maphosa et al. (2010). Previous studies have correlated reductive dechlorination products (i.e., chlorinated ethenes and ethene) with the growth of *Dehalococcoides* and other dechlorinators, assessed with quantitative PCR (qPCR) (Duhamel and Edwards 2007, Veinberg et al. 2010, Haest et al. 2010). While various studies have also used molecular techniques to identify key microorganisms in reductive dechlorination communities (Richardson et al., 2002; Macbeth et al., 2004; Freeborn et al., 2005; Duhamel and Edwards, 2006; Azizian et al., 2010), they have not clearly demonstrated trends between electron flow and the relative abundance of dechlorinators and other microorganisms. Furthermore, no studies have attempted to estimate the microbial community structure in bioremediation systems based on electron balances.

Correlating predictions of microbial community structure based on electron balances with results obtained via molecular methods is a valuable approach that has been applied for other systems. For example, Lee et al. (2009)

combined these complementary techniques to better understand the pathways and microorganisms involved in fermentative bio-hydrogen production. Parameswaran et al. (2009) applied them to microbial electrochemical cells to reveal syntrophic and competitive interactions in the microbial community of the anode. In our study, we extended this approach to a reductive-dechlorination community in order to assess how stress from the chlorinated ethenes can alter the microbial community abundance and structure.

Chlorinated ethenes can induce stress either directly to the dechlorinators or indirectly to other members in the microbial community. Yu and Semprini (2004) demonstrated that sufficiently high concentrations of chlorinated ethenes can cause self-inhibition, also termed Haldane inhibition. Additionally, Yu and Semprini (2005) noted that chlorinated ethenes featuring a larger number of chlorine atoms can inhibit the reduction reactions of those with fewer chlorine atoms, termed competitive inhibition, e.g., PCE on conversion of TCE to DCE and DCE to VC. They indicated that chlorinated ethenes with fewer chlorine atoms also inhibit the dechlorination of compounds with higher numbers of chlorine atoms, but to a much lesser extent. These stressors may be due to thermodynamic limitations or to enzymatic inhibition, but both result in decreased kinetic activity. Haest et al. (2010) documented that, besides the concentration of the chlorinated ethenes, the density of the dechlorinating bacteria controlled the extent of inhibition. Recently, Sabalowsky and Semprini (2010a,b) demonstrated that high concentrations of chlorinated ethenes, >1 mM TCE and >3mM *cis*-DCE, as present in dense non-aqueous phase liquid (DNAPL) plumes, can cause a

decrease in the net yield of *Dehalococcoides*. They modeled this as an increase in the decay rate due to solubilization of the cell membrane, but they noted that it is also possible that a decreased biomass yield could have resulted from decoupling of biosynthesis from the electron transport chain.

Stress induced by chlorinated ethenes can affect the non-dechlorinating microorganisms in ways that benefit reductive dechlorination by reducing competition for electrons. For example, Yang and McCarty (2000) reported that inhibition to hydrogenotrophic methanogenesis and homoacetogenesis increased electron availability to dechlorination and resulted in higher dechlorination activity; the efficiency of H_2 utilization increased from 10% to 67% when the PCE concentration was increased from 0.1 mM to 1 mM. We hypothesize that suppressing the competing processes also has the potential to be detrimental to reductive dechlorination if the involved organisms provide growth factors or cofactors to the dechlorinators. For example, suppression of homoacetogens could decrease the acetate C-source and the vitamin B_{12} cofactor for *Dehalococcoides*, potentially leading to decreased kinetic activity of reductive dechlorination or to decoupling.

The focus of our study was to assess how varying the concentrations of chlorinated ethenes can induce shifts to the microbial community structure. We accomplished this goal by using the two complementary techniques described above. We operated fill-and-draw reactors over a wide range of TCE concentrations. For pseudo steady-state conditions, we analyzed the electron-flow distribution and used an electron-balance analysis to predict trends in the

microbial community structure. The electron-balance analysis estimates microbial cell concentrations using biomass yields and cell volumes from the literature. We then correlated these predictions with the microbial community structure assayed by qPCR and pyrosequencing. In interpreting our results, we applied the concept that the electron-balance analysis should predict the experimental trends from the molecular assays as long as stress to the community is exhibited through a decrease in the kinetic activity alone, either directly to the dechlorinators or indirectly to the competing or to the "helper" organisms. A deviation from the correspondence indicates that stress to the microbial community was manifest through a decreased biomass yield of the dechlorinators or other microorganisms, caused by increased decay or decoupling of the biomass yield from the electron transport chain.

Materials and Methods.

Set-up, inoculation, and operation of fill-and-draw reactors. The experimental study involved triplicate fill-and-draw reactors, each consisting of 120-mL serum bottles filled with 60 mL of liquid and sealed with black butyl rubber stoppers and crimped metal seals. To each bottle we provided one of five initial amounts of TCE: 14, 28, 56, 112, or 168 μmoles per bottle. The corresponding initial concentrations were 0.23, 0.47, 0.93, 1.87, and 2.80 mM or 30, 62, 122, 244, and 367 ppm, respectively. The inoculum was from a single bottle of DehaloR^2 (Ziv-El et al. 2011) effluent collected from a 1-L continuously stirred tank reactor that had been stored in the refrigerator at 4˚C for four months, but in a separate test showed rapid dechlorination activity when

inoculated into fresh media at 30˚C (data not shown). The liquid contents consisted of 58.5 mL of anaerobic medium as described in Ziv-El et al. (2011) amended with 0.48 mL of 1M lactate (8 mM), 50 µL neat methanol (20.5 mM), and neat TCE as indicated above. To each bottle we added 1 mL inoculum with a *Dehalococcoides* concentration of ~4 x 10^{11} cells/L, determined with qPCR. The bottles were stored in an incubator at 30˚C, oriented at a 45˚ angle so that the seal was submerged, and mixed on a platform shaker at 150 rpm in order to promote completely mixed conditions. We sampled the headspace gas periodically by drawing out 200 µL with a gas-tight syringe for determination of chlorinated ethenes, ethene, and methane concentrations.

After ≥80% conversion of TCE to ethene, we replaced 12 mL of liquid every two days in an anaerobic glove box (Coy Laboratory Products Inc., Grass Lakes, MI), drawing out the liquid with a syringe at the same time that new liquid was injected into the bottle; we shook each bottle just prior to replacing the liquid to ensure homogenous distribution of the bottle's contents. The solids retention time (SRT) in the bottles was thus maintained at 10 days. The desired total TCE mass was delivered in replacement media in 5 pulses spaced in 2-day intervals. Concentrations of the TCE amended every two days (referred to as "peak TCE concentrations") were 0.047, 0.093, 0.19, 0.37, 0.56 mM TCE (6-74 ppm). We analyzed the chlorinated ethenes, ethene, and methane before replacing the medium. Reactor pH was monitored every four days during fill-and-draw operation and adjusted to an optimum range (6.8-7.2) for reductive dechlorination (Middeldorp et al. 1999, Robinson et al. 2009) using 4.5 N NaOH.

We defined reactor pseudo steady-state as occurring when all of the following four criteria were met: (1) Operation longer than a minimum of three SRTs, 30 days. (2) Three consecutive stable measurements of chlorinated ethenes, ethene, and methane in the headspace, measured over six days. (3) Three stable measurements of the volatile fatty acids (VFAs) lactate, methanol, propionate, and acetate. (4) Three stable measurements of biomass.

Analysis of gas and liquid phase products. We analyzed the chlorinated ethenes, ethene, and methane by gas chromatography flame ionization detection (GC-FID), VFAs and methanol by high performance liquid chromatography ultraviolet (UV) and refractive index (RID) detection (HPLC-UV or HPLC-RID), and pH with a bench-top meter, all as described in Ziv-El et al. (2011). To improve cell lysis for the biomass analysis, we subjected effluent samples to a single 24-hour freeze/thaw cycle and then quantified protein in 10-fold dilutions using a Micro Bicinchoninic Acid (BCA) protein assay (Thermo Fisher Scientific, Rockford, IL) and a Varian Cary 50 Bio UV-Visible spectrophotometer (Agilent Technologies, Santa Clara, CA), following the kit manufacturer's protocol. To convert from protein to biomass, we assumed that biomass was comprised of 55% protein (Rittmann and McCarty 2001).

Electron-balance analysis. The electron flow for our dechlorinating consortia is illustrated in Figure 1, and the corresponding stoichiometric equations are in Table 1. The electron flow is based on our understanding of the metabolic groups and reactions in DehaloR^2 (Ziv-El et al., 2011): The initial electron donors were lactate and methanol, TCE and bicarbonate were the initial electron

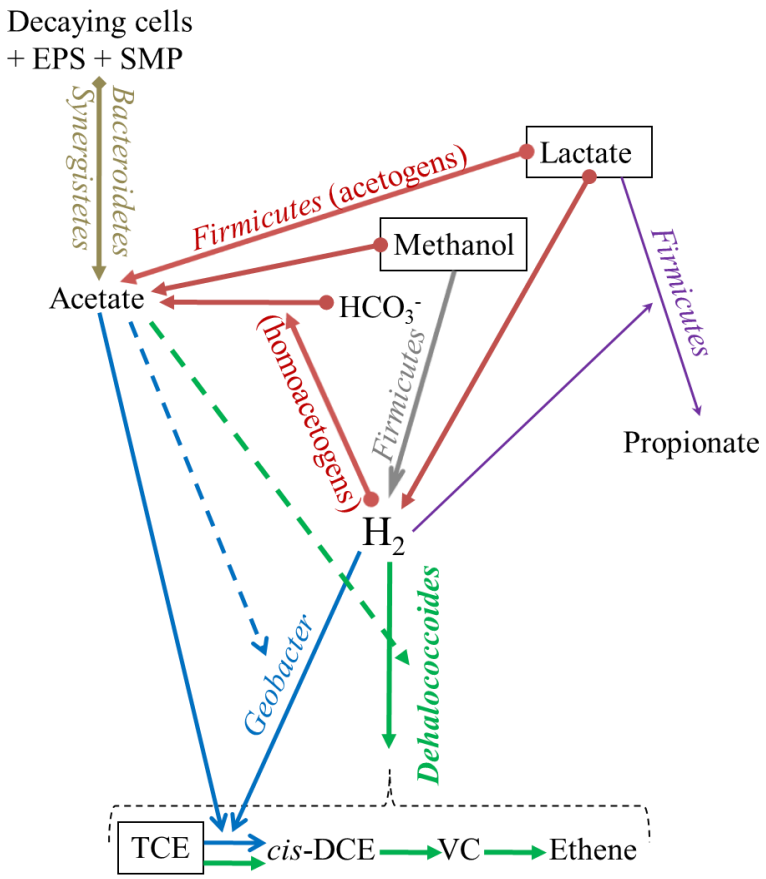


Figure 3.1. Dominant electron flows for DehaloR^2 grown on the combination of lactate and methanol as the electron donors and TCE as the electron acceptor. Initial electron donors and acceptor are in boxes. Products are in standard type, each distinct microbial group is in *italics*, unique dashed-lines represent use of C only, and unique solid lines represent electrons or electrons combined with C. Methanogenesis is not included, since it was negligible in the experiments at steady state. EPS stands for extracellular polymeric substances and SMP for soluble microbial products.

Table 3.1

Stoichiometric equations of the dominant biochemical reactions in a culture performing TCE reductive dechlorination with lactate, methanol, or both serving as the electron donor(s).

(1) TCE reductive dechlorination to ethene:

$C_2HCl_3 + 3H_2 \rightarrow C_2H_4 + 3Cl^- + 3H^+$

(2) Lactate dehydration to propionate:

$CH_3CH_2OCOOH + H_2 \rightarrow CH_3CH_2COOH + H_2O$

(3) Lactate fermentation to acetate:

$CH_3CH_2OCOOH + 2H_2O \rightarrow CH_3COOH + HCO_3^- + 2H_2 + H^+$

(4) Lactate fermentation to acetate and propionate (only if H_2 is not limiting):

$CH_3CH_2OCOOH \rightarrow \frac{1}{3} CH_3COOH + \frac{2}{3} CH_3CH_2COOH + \frac{1}{3} HCO_3^- + \frac{1}{3} H^+$

(5) Methanol fermentation:

$CH_3OH + 2H_2O \rightarrow 3H_2 + HCO_3^- + H^+$

(6) Methanol oxidation to acetate:

$CH_3OH + \frac{1}{2} HCO_3^- + \frac{1}{2} H^+ \rightarrow \frac{3}{4} CH_3COOH + H_2O$

(7) Autotrophic homoacetogenesis:

$HCO_3^- + 2H_2 + H^+ \rightarrow \frac{1}{2} CH_3COOH + 2H_2O$

(8) Hydrogenotrophic methanogenesis:

$HCO_3^- + 4H_2 + H^+ \rightarrow CH_4 + 3H_2O$

(9) Methylotrophic methanogenesis:

$4CH_3OH \rightarrow 3CH_4 + HCO_3^- + H_2O + H^+$

acceptors, and the major microbial groups were *Dehalococcoides*, *Geobacter*, and *Firmicutes* (Ziv-El et al. 2011).

The electron-balance analysis was of the major microbial processes at pseudo steady-state, defined by stability in the microbial functions and the relative abundance of the major microbial groups. A schematic of how the electron balance analysis was carried out is shown in Figure 2. Inputs were the end-product electron acceptors, and the electron-balance stoichiometry for each donor and product is in Table 2. Consumers of decaying cells, extracellular polymeric substances (EPS), and soluble microbial products (SMP) may have contributed to production of acetate (Figure 1), but these were not included in the electron balance (Table 2).

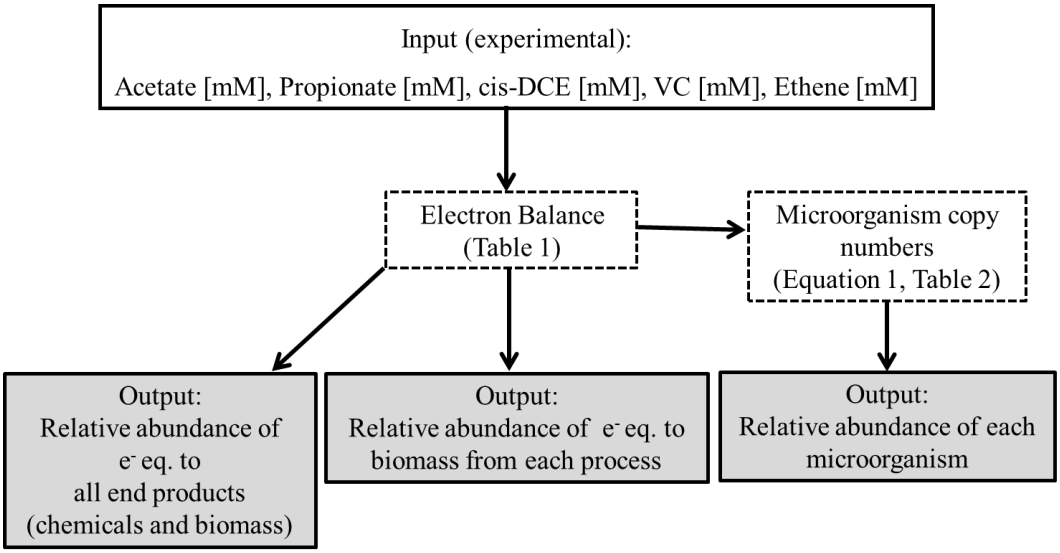


Figure 3.2. Steps to assess the electron distribution and microbial community abundance; the electron flow is in Figure 1, and the corresponding stoichiometric equations are in Table 1. Methane is not included, since it was negligible in the experiments at steady state.

Table 3.2

Electron-balance components based on electron flow (Figure 1), stoichiometry (Table 2), and f_s^0 values (Table 3).

steady-state end product [mM]	microbial process (Table S1 eqn. #)	microorganism	$\frac{me^- eq.}{mmol}$	steady-state end product $\left(\frac{me^- eq.}{L}\right)$	steady-state biomass[a] $\left(\frac{me^- eq.}{L}\right)$
[propionate]	(2)	fermenter	14	$[propionate]\times 14$	$\frac{(end\ product)}{1-f^0_{s,fermenter}}\times f^0_{s,fermenter}$
[acetate]	(3), (6), (7), biomass decay	fermenter	8	$[Ac^-]\times 8$	$\frac{(end\ product)+(Ac^-_{Geo})^b+(Ac^-_{Dhc})^c}{1-f^0_{s,fermenter}}\times f^0_{s,fermenter}$
[cis-DCE], [VC], [ethene]	(1)	*Geobacter*	2	$([cis-DCE]+[VC]+[ethene])\times 2\times(\%\ by\ Geo)^d$	$(end\ product)\times f^0_{s,Geo}$
	(1)	*Dehalococcoides*	2, 4, or 6	$([cis-DCE]\times 2+[VC]\times 4+[ethene]\times 6)-([cis-DCE]+[VC]+[ethene])\times 2\times(\%\ by\ Geo)^e$	$(end\ product)\times f^0_{s,Dhc}$

[a] f_s^0 values are in Table 3.

[b] (Ac^-_{Geo}) is the acetate used by *Geobacter* either as a carbon source, $\left(Ac^-_{Geo}\right)_{carbon}$, or as an e$^-$ donor, $\left(Ac^-_{Geo}\right)_{electrons}$.

$(Ac^-_{Geo})_{carbon} = (biomass)_{Geo} \times \left(\frac{mmol\ biomass}{20\,me^-\,eq.}\right) \times \left(\frac{5\,mmoles\ C}{mmol\ biomass}\right) \times \left(\frac{mmoles\ Ac^-}{2\,mmoles\ C}\right) \times \left(\frac{8\,me^-\,eq.}{mmoles\ Ac^-}\right)$, where biomass was assumed to be $C_5H_7O_2N$, and N was assumed to be from NH_4^+ so there were 20 me^- eq./mmol biomass (Rittmann and McCarty 2001).

$\left(Ac^-_{Geo}\right)_{electrons} = \left(end\ product\right)_{Geo} \times \left(\frac{mole\,cis-DCE}{2\,e^-\,eq.}\right) \times \left(\frac{8\,e^-\,eq.}{mole\,Ac^-}\right) \times \left(1-f^0_{s,Geo}\right)\left(\%\,e^-\,by\,Ac^-\right)$, where $\left(\%\,e^-\,by\,Ac^-\right)$ was the assumed fraction of electron donor from acetate; a sensitivity analysis was carried out on this parameter as described in the text.

[c] (Ac^-_{Dhc}) is the acetate used by *Dehalococcoides* as the carbon source. Assume biomass is $C_5H_7O_2N$ and N is from NH_4^+ so there are 20 me^- eq./mmol biomass (Rittmann and McCarty 2001), and 67% of carbon source is from acetate (Tang et al. 2009).

$(Ac^-_{Dhc}) = (biomass)_{Dhc} \times \left(\frac{mmol\ biomass}{20\,me^-\,eq.}\right) \times \left(\frac{5\,mmoles\ C}{mmol\ biomass}\right) \times (0.67) \times \left(\frac{mmoles\ Ac^-}{2\,mmoles\ C}\right) \times \left(\frac{8\,me^-\,eq.}{mmoles\ Ac^-}\right)$

[d] (% by *Geo*) was the assumed fraction of TCE to cis-DCE reduction carried out by *Geobacter*. A sensitivity analysis was carried out on this parameter as described in the text.

We computed the relative abundance of each of the expected microbial groups from the measurements of chemical end products, reaction stoichiometries in Table 1, and fractions ($f_s°$) of donor electrons that each microorganism sends to biomass synthesis; the $f_s°$ values are in Table 3 and represent true yields expressed in electron equivalents (Rittmann and McCarty, 2001). We then computed the relative abundance of each microorganism group, *i*, using the cell volumes in Table 3. Cells were assumed to be 20% dry weight by mass, with dry weight 90% organic by mass (Rittmann and McCarty 2001). Thus,

$$microorganism_i\left[\frac{cell\ copies}{L}\right] = \frac{biomass_i\left[\frac{e^- eq.}{L}\right] \times Yield_i\left[\frac{g\ dry\ org.bio.}{e^-\ eq.}\right] \times 10^{12}\left[\frac{\mu m^3}{g\ bio.}\right]}{f_{s,i}^0 \times 0.2[dry\ cell\ \ fraction] \times 0.9[org.cell\ \ fraction] \times volume\ cell_i[\mu m^3]} \quad (1)$$

Table 3.3.

Microorganism-specific parameters used in the electron-balance analysis.

Microorganism	Volume [µm^3]	Yield [g dry org. mass/e- eq.]	f_s^0
Firmicutes	1[a]	0.94[d]	0.18[f]
Dehalococcoides	0.02[b]	0.5[e]	0.10[f]
Geobacter	0.15[c]	1.4[e]	0.28[f]

[a] Madigan et al. (2009), Table 4.1
[b] Duhamel and Edwards (2004)
[c] Sung et al. (2006a)
[d] Using the yield from Rittmann and McCarty (2001), Table 3.1, the units were converted using
Y = (0.13 [g VSS/g BOD_L])*(8 [g BOD_L/e^- eq.])*(0.9 [g org. matter/g VSS])
[e] Duhamel and Edwards (2007)
[f] Calculated using f_s^0 = Y x (20 e^- eq./mol VSS)/(113 g VSS/ mol VSS)/(0.9 g org. matter/g VSS).

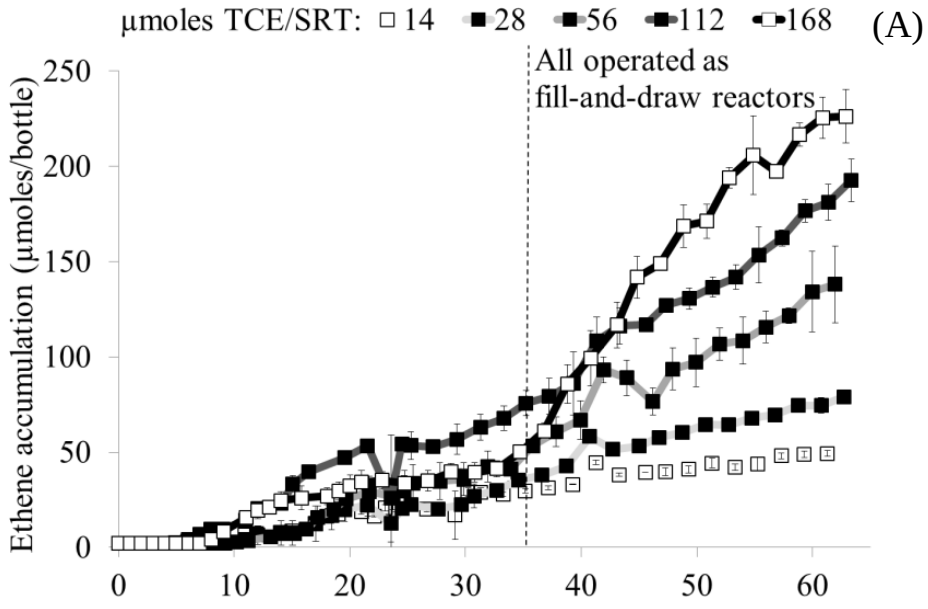
Molecular Techniques. We extracted DNA from biomass harvested from 1.5-mL effluent samples by centrifugation (20 min at 16.1 x 10^3 *g*) as described previously (Ziv-El et al. 2011). For all reactors, we quantified by qPCR using a TaqMan probe targeting the 16S rDNA of Bacteria, *Geobacteraceae* (the family within the sub-phylum *Deltaproteobacteria* containing the dechlorinating genus *Geobacter*), and *Dehalococcoides* as described in Ziv-El et al. (2011). SYBR Green I assays were used to quantify the *mcrA* gene of methanogens, following the protocol in Steinberg and Regan (2008), and for the formyltetrahydrofolate synthetase (*FTHFS*) gene of homoacetogens after a 1 in 10 dilution, following the protocols in Xu et al. (2009) and Parameswaran et al. (2011), except for the following minor modifications. For the *mcrA* and *FTHFS* assays, the reaction volume was 20 µL comprised of the following: 6.8 µL nuclease free water, 10 µL SYBR Realmastermix (5 Prime, CA), 0.6 µL each of the forward and reverse primers for a 0.3 µM final concentration, and 2 µL of template DNA or cDNA. All assays were carried out in triplicate along with a seven-point calibration curve with a ten-fold dilution factor of the standards.

For one bottle at each TCE pulsing rate, we assessed the bacterial community using 454 pyrosequencing targeting the combined V2 and V3 regions of the 16S rRNA gene as in Garcia-Peña et al. (2011), except for the following minor modifications. Before clustering, a maximum sequence cutoff length was not set, but sequences below 200 bp were removed. The resultant data included 1995 sequences with a median length of 311 bp for the biofilm sample and for the liquid sample 2513 sequences with a median length of 351 pb. The Ribosomal

Database Project classifier confidence threshold used for bacterial identification was 50% and the operational taxonomic unit (OTU) cut-off was a 3% difference.

Results and Discussion.

Batch and fill-and-draw operation, methanogen suppression, and experimental electron distribution. The consortia bottles were operated for 25-35 days in batch mode and then as fill-and-draw reactors for another 30-40 days (at least 3 SRTs). The accumulation of ethene for the triplicate bottles is shown in Figure 3A and for VC in Figure 3B. TCE and *cis*-DCE were not detected in any of the reactors during fill-and-draw operation. Figure 3C is the methane accumulation for one of the triplicate reactors at each TCE pulsing rate; the selected bottle had the greatest amount of methane production, but the trend was the same across all bottles. After the onset of ethene production at around day 8



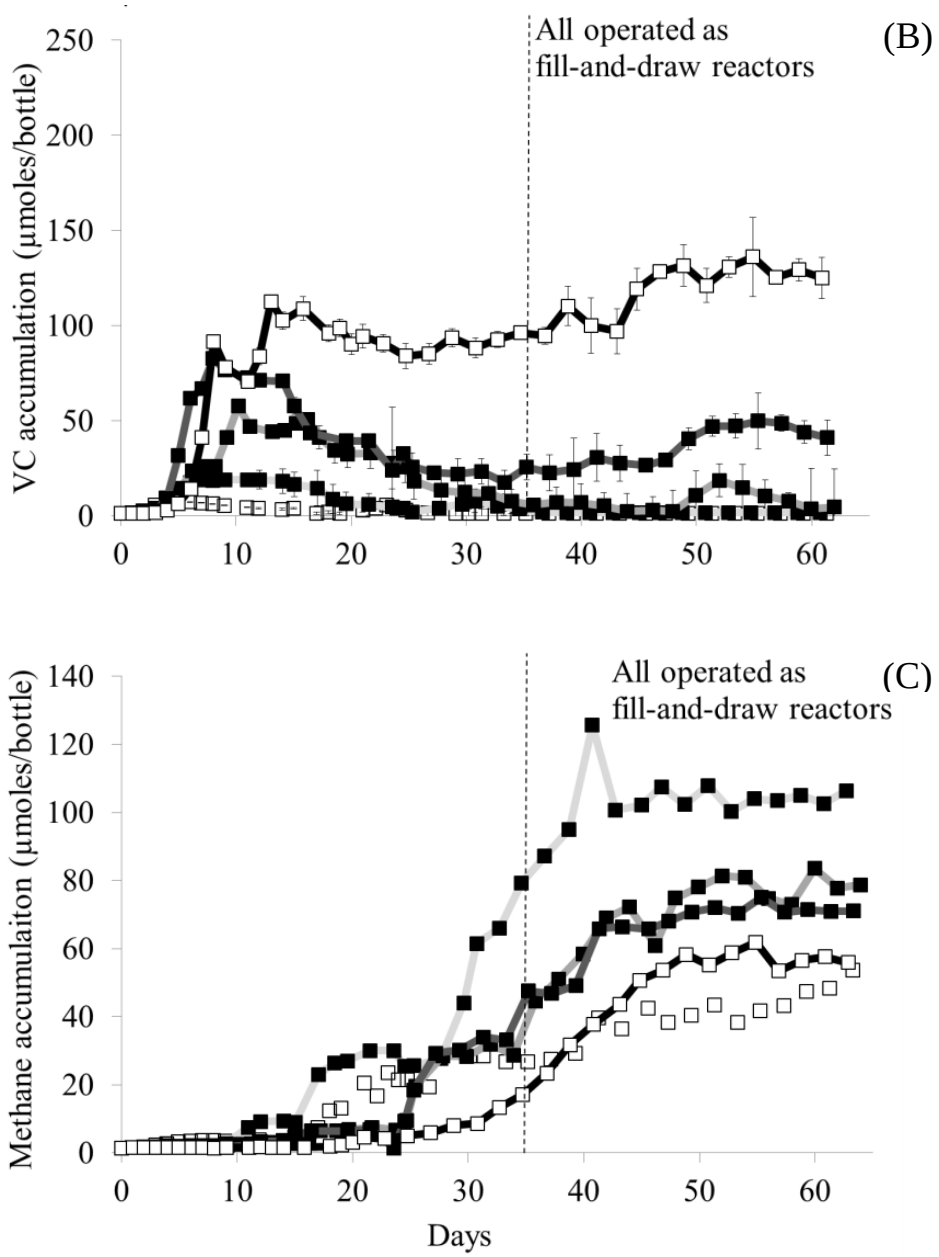


Figure 3.3. Experimental results from batch and fill-and-draw operation for each TCE pulsing rate in µmoles/SRT for triplicate bottles (with error bars) for (A) ethene and (B) VC, and (C) one set of bottles for methane. Only one bottle is presented for methane production, since the amounts varied widely between triplicate bottles. Fill-and-draw operation began between days 25-35 for all TCE-pulsing rates.

for all TCE-pulsing rates, ethene accumulated throughout batch and fill-and-draw operations. For the two highest TCE pulsing rates, VC accumulated in fill-and-draw operation until around day 50, at which time consumption matched production, i.e., a flat line. Methane production slowed significantly for all TCE pulsing rates, indicated by zero net production, from around day 45, ~1.5 SRTs of fill-and-draw operation.

In order to confirm minimal methanogenesis in fill-and-draw operation based on chemical analyses, we quantified the *mcrA* gene copy number for methanogens with qPCR and compared these values to the copy numbers for bacteria, *FTHFS* (homoacetogens), *Geobacteraceae*, and *Dehalococcoides*. The results in Figure 4A demonstrate that methanogen abundance was two to three orders of magnitude below that for the other organisms assayed and was an order of magnitude below the concentrations for the six methanogenic processes assayed in Steinberg and Regan (2009). We interpret that the low level of methanogenesis was a combined effect of TCE-induced stress and washout. Adding TCE every two days likely suppressed the methanogens due to frequent exposure to TCE or its dechlorination products, even for the lowest pulsing rate and peak concentration (14 μmoles/SRT, 0.05 mM). The slow maximum specific growth rate of methanogens, 1.2/day (Sakai et al. 2009) or 3-4/day (Yoochatchaval et al. 2008), was possibly slowed further by chlorinated-ethene stress, which did not allow net positive growth with the 10-day SRT. Therefore, methanogenesis was not a significant electron sink, and it did not need to be

considered in the experimental electron flow results or in the electron balance analysis.

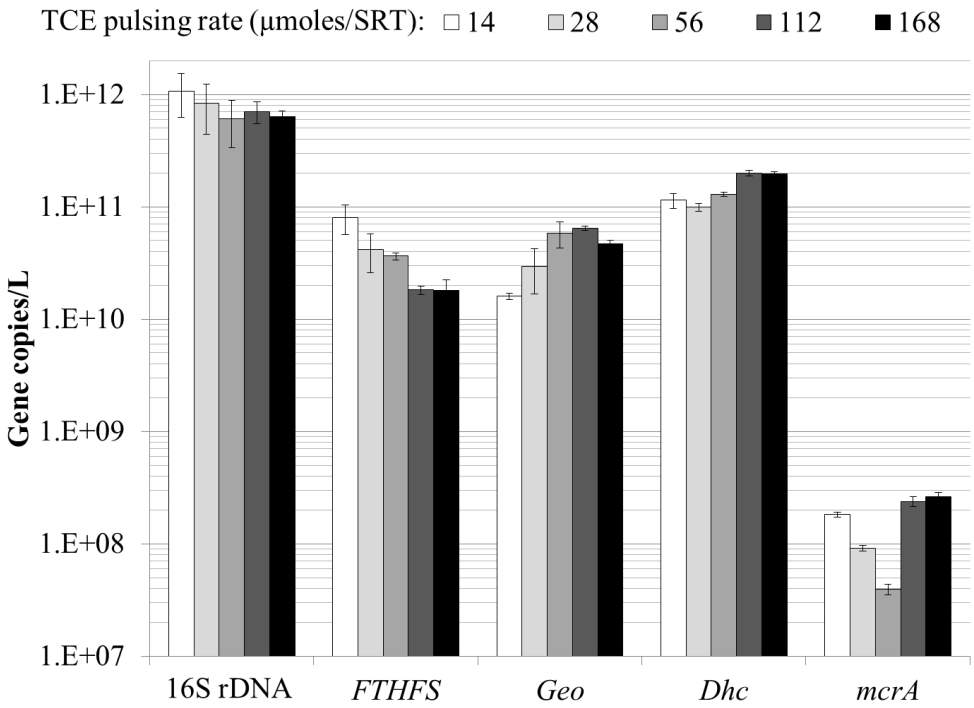


Figure 3.4. qPCR gene copy concentrations of bacteria (16S rDNA), homoacetogens (*FTHFS* gene), Geobacteraceae, Dehalococcoides, and methanogens (*mcrA* gene) for each TCE-pulsing rate.

The electron distributions for each reactor over three sampling days after at least 3 SRTs are in Table 4A. Methanol was fermented completely in all bottles, and the concentration of lactate was between 0-0.9 mM (data not shown). The relative standard deviations were below 10%, except for propionate, which was below 18%, but the propionate concentrations for all the reactors across all days were in the narrow and low range of 1.1-2.8 me^- eq./L. Additionally, systematic trends for the VFAs and biomass were absent across the three sampling days.

Table 3.4.

(A) Verification of steady-state operation by tracking the electron distribution for one reactor from each triplicate TCE pulsing rate. Methanol was fermented completely and the concentration of lactate was always below 1 mM. All samples were collected after at least 3 SRTs. The relative standard deviations (RSD) were <18% for all. (B) Electron equivalents for the triplicate bottles at the end of reactor operation, used to calculate the electron distribution relative abundance for Figure 5.

(A)

µmoles TCE/SRT	**14**	**28**	**56**	**112**	**168**
me- eq/bottle	**average, RSD (%)**				
acetate	9.1, 3	9.8, 5	9.6, 7	8.8, 8	7.0, 9
propionate	1.4, 4	1.3, 14	2.2, 24	1.9, 26	2.5, 10
biomass	2.6, 7	2.6, 6	2.8, 4	2.6, 15	2.3, 4
ethene	0.08	0.17	0.34	0.67	1.0
sum	13.2, 5	13.8, 9	14.9, 14	13.9, 18	12.8, 8

(B)

µmoles TCE/SRT	**14**	**28**	**56**	**112**	**168**
me- eq/bottle	**average, RSD (%)**				
acetate	9.8, 2	10.6, 2	9.3, 10	9.0, 7	7.0, 12
propionate	1.8, 10	1.2, 15	1.9, 18	1.5, 13	2.5, 13
biomass	2.4, 4	2.3, 4	2.6, 2	2.6, 2	1.9, 1
ethene	0.08	0.17	0.34	0.67	1.0
sum	14.1, 1	14.3, 1	14.1, 4	14.5, 3	12.4, 4

Together, the data support pseudo steady-state operation. Additionally, the sum of electron equivalents was statistically similar for all the TCE pulsing rates, except for the highest pulsing rate, where it was lower (Table 4A,B). This suggests that H_2 accumulated for the highest pulsing rate; although we did not

analyze for H_2, its accumulation is evident because no other products, i.e., VFAs, were detected.

The experimental electron distribution for the triplicate bottles at pseudo steady-state is presented in Figure 5 as fractions of the end products. Several trends are apparent. First, an increased TCE-pulsing rate led to increased

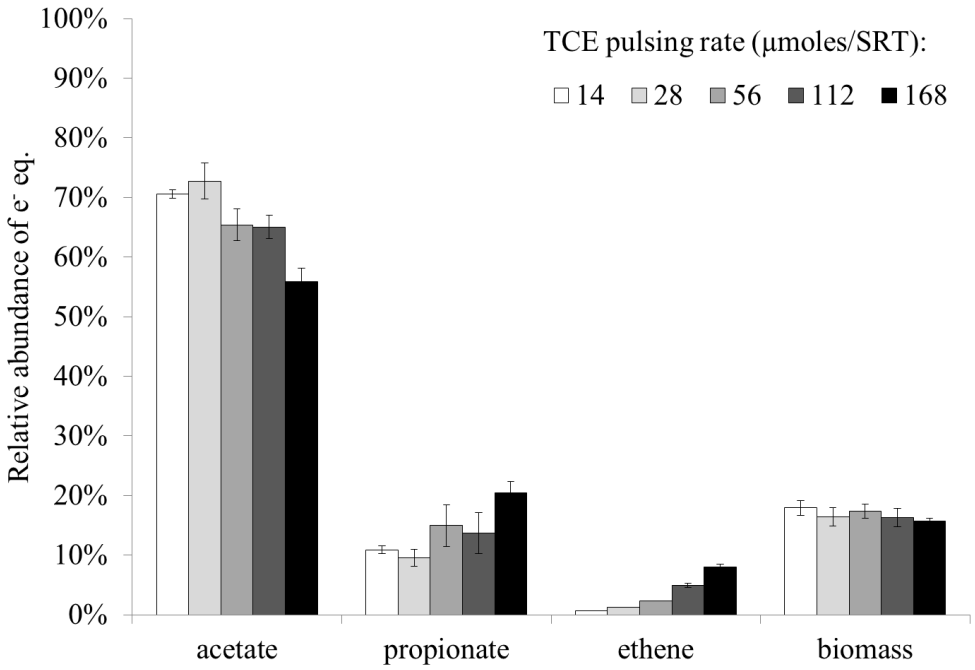


Figure 3.5. Experimental results of the electron-distribution relative abundance for each TCE-pulsing rate based on the fermented electron donors lactate and methanol. Error bars are for the triplicate reactors. To convert biomass to electron equivalents, we assumed that the biomass could be described by the molecular formula $C_5H_7O_2N$, and that the N was from NH_4^+ so there were 20 me-eq./mmol biomass. Other conversions were standard, in e- eq./mol: acetate, 8; propionate, 14; ethene (from TCE), 6. Absolute values are in Table 4B.

production of ethene. This is logical, since TCE is the ultimate source of ethene. Second, acetate had a downward trend and propionate an upward trend with increasing concentrations of TCE, and we discuss the cause later. Last, biomass consistently accounted for 16%-18% of the electron equivalents, suggesting dominance of the fermenters, whose $f_s°$ is generally around 0.18, rather than *Dehalococcoides* or *Geobacter*, which have very different $f_s°$ values (Table 3).

Evaluation of abundances predicted from the electron balances. We used the electron-balance analysis to estimate trends in the relative abundance of the major microbial groups. The two adjustable parameters were the relative contribution of *Geobacter* to total reductive dechlorination and the fraction of the reductive dechlorination by *Geobacter* for which acetate was the electron donor rather than H_2. For the predicted relative abundance in Figure 6, acetate was the only electron donor for *Geobacter,* and we adjusted the percent contribution of *Geobacter* to match their quantified relative abundance based on pyrosequencing in Figure 7A (discussed later).

Figure 6A provides the relative abundances of the bacterial groups as a fraction of biomass, and Figure 6B shows the relative abundances as fractions of cell numbers. The increasing abundance of *Dehalococcoides* with increasing TCE-pulsing rate, along with the corresponding decrease in *Firmicutes* abundance, is logical because the rate of reductive dechlorination also increased for each pulsing rate (Figure 5).

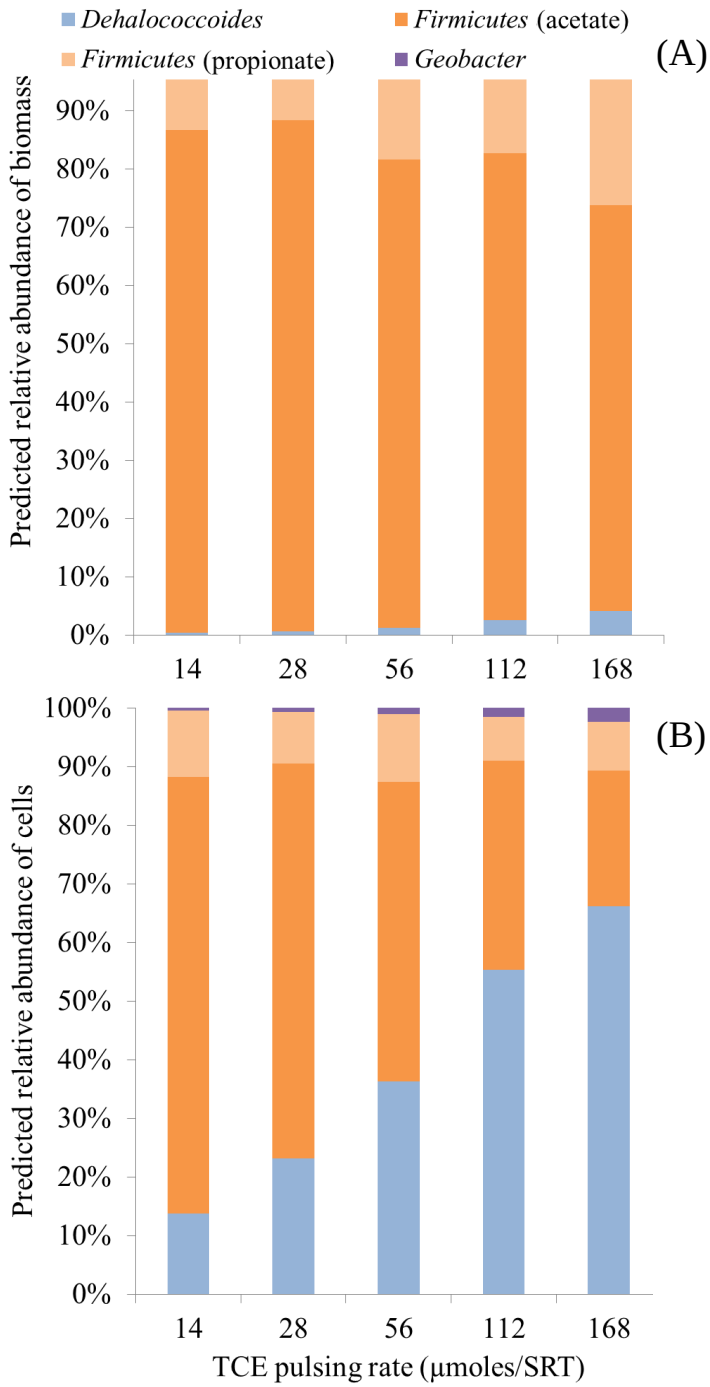


Figure 3.6. Relative abundances of bacteria by (A) biomass and (B) cell copy numbers predicted by the electron-balance analysis.

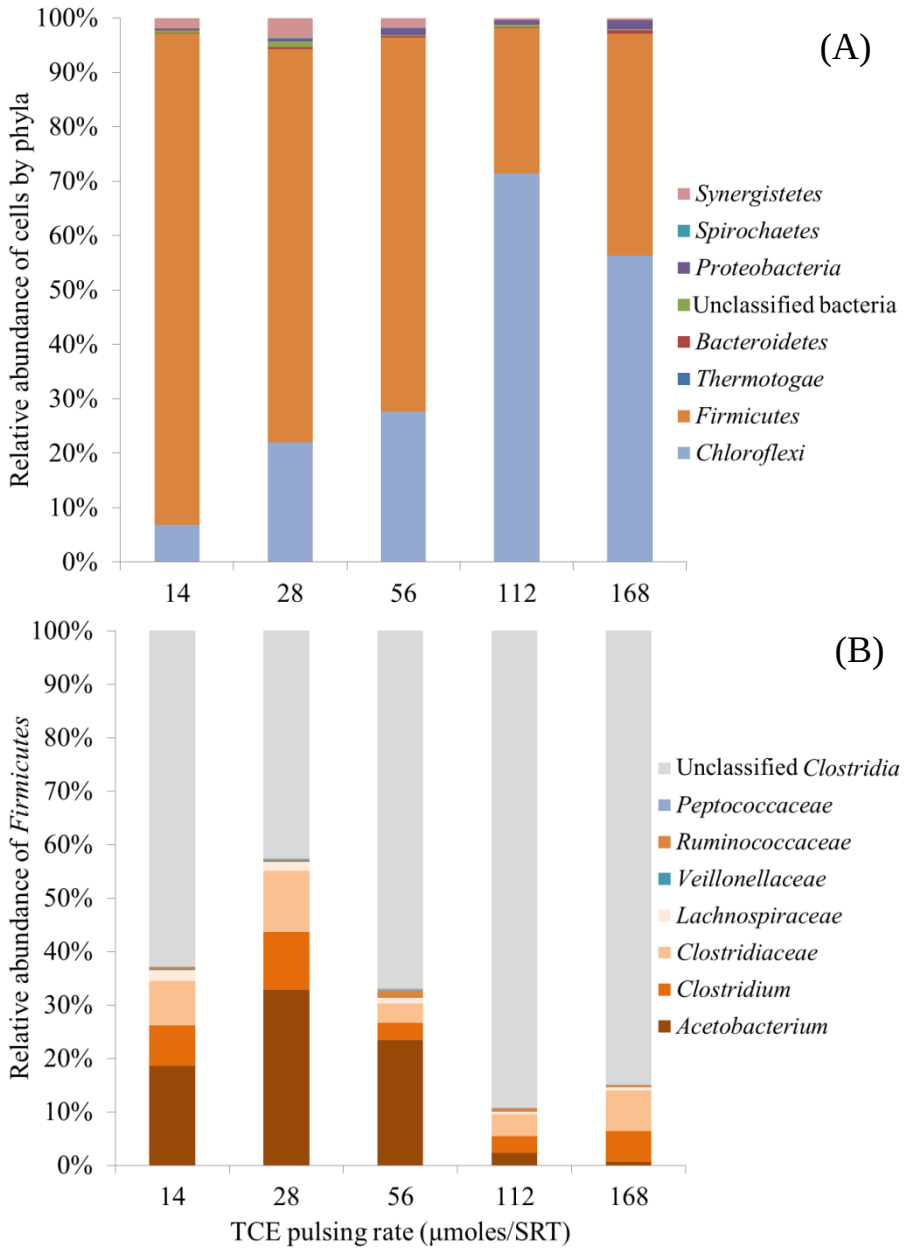


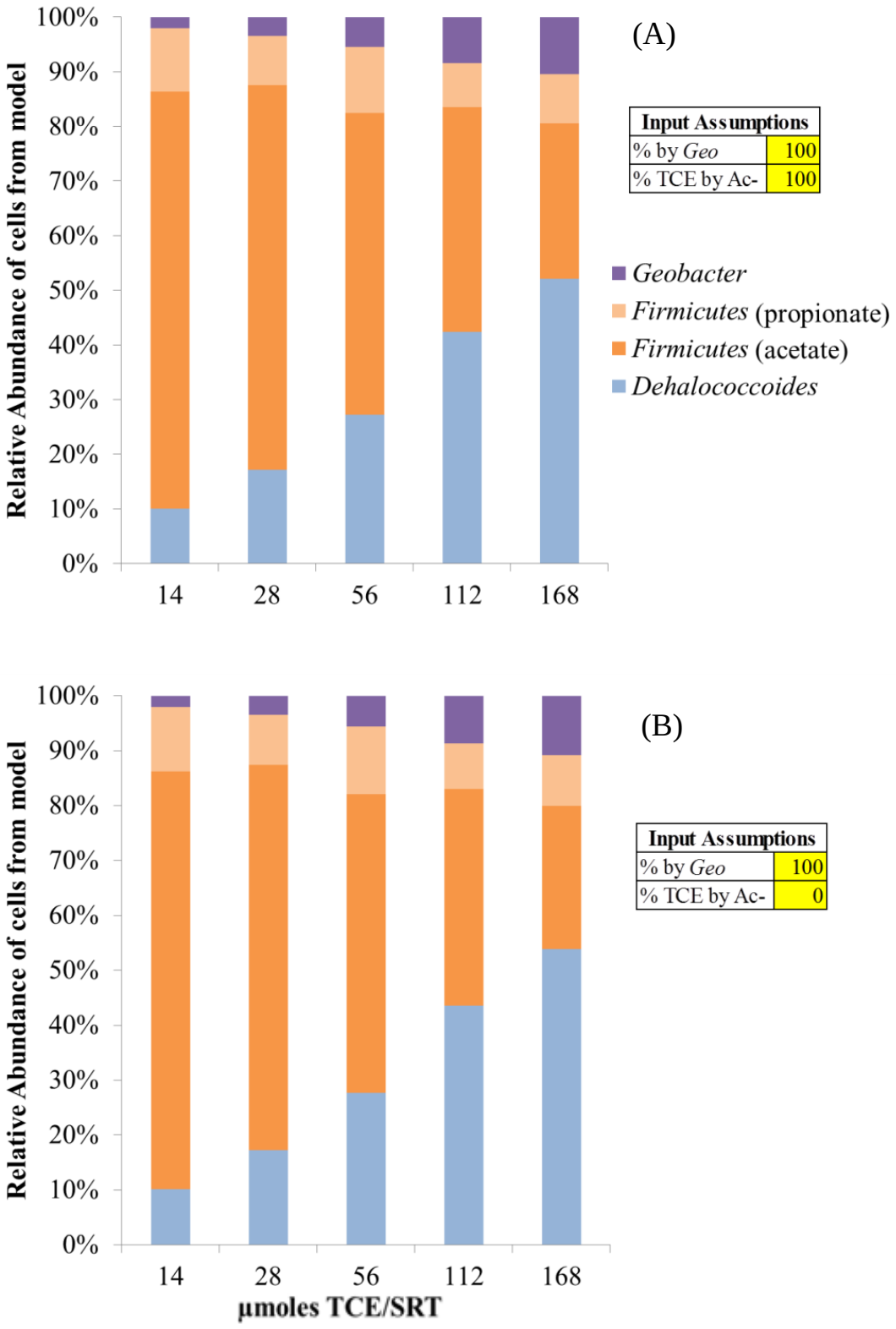
Figure 3.7. Relative abundance of (A) bacterial groups by phyla and (B) groups within the *Firmicute* phyla based on pyrosequencing of the combined V2 and V3 regions of the 16S rDNA. Bacteria copy numbers were calculated by assuming the number of 16S rDNA copies/cell indicated in Table 5. All values are from the rrn database, http://rrndb.mmg.msu.edu/search.php#result based on Klappenbach et al. (2001) and Lee et al. (2009b).

Table 3.5

Number of 16S rDNA copies/cell used to calculate the relative abundance of the pyrosequencing bacterial groups used to generate Figure 7(A). These copy numbers are based on those from the rrn database, http://rrndb.mmg.msu.edu/search.php#result, and based on Klappenbach et al. (2001) and Lee et al. (2009). Chloroflexi were 100% Dehalococcoides and Proteobacteria were 100% Geobacter; so those copy numbers were included here. For Firmicutes, Figure 7(A) in the main text had 5 16S rDNA copies/cell, and In Figure S1 we present the same figure using 3 or 10 copies/cell.

Phyla	**16S rDNA copies/cell**
Thermotogae	3
Bacteroidetes	3
Unclassified	3
Proteobacteria	2
Spirochaetes	3
Firmicutes	3, 5, or 10
Chloroflexi	1
Synergistetes	3

In Figure 8 is a sensitivity analysis that shows that the trends are the same even when *Geobacter* is a larger fraction of the community or for different assumptions regarding acetate utilization by *Geobacter*.


(A)
Input Assumptions
% by Geo 100
% TCE by Ac- 100
Geobacter
Firmicutes (propionate)
Firmicutes (acetate)
Dehalococcoides
Relative Abundance of cells from model
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
14
28
56
112
168
(B)
Input Assumptions
% by Geo 100
% TCE by Ac- 0
Relative Abundance of cells from model
µmoles TCE/SRT

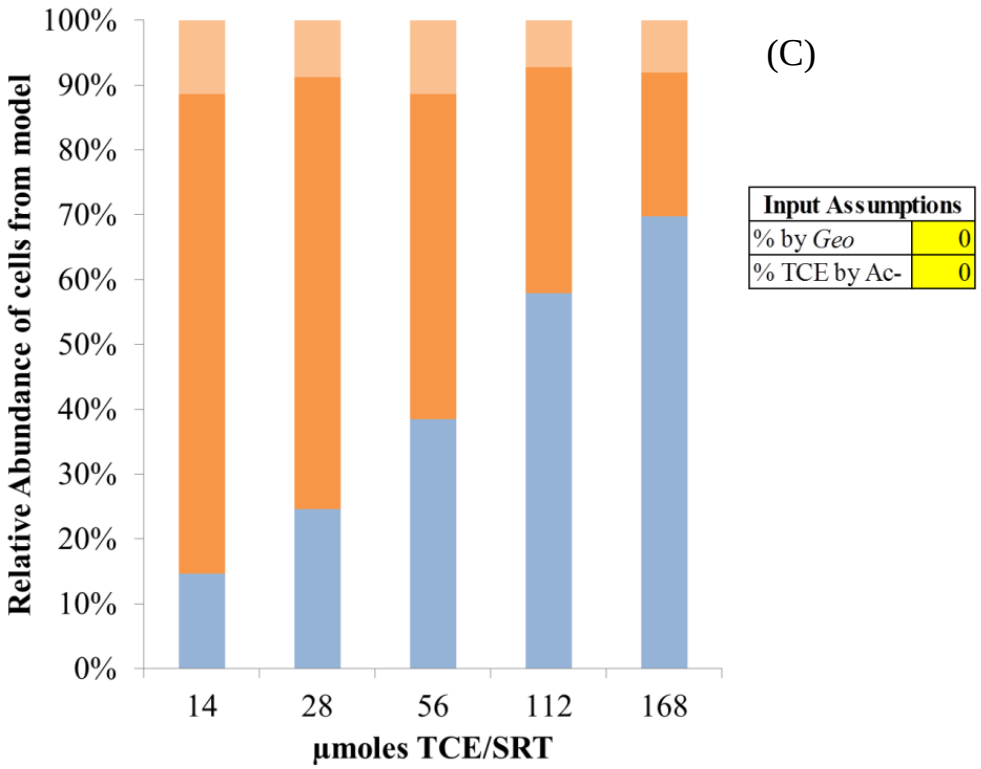


Figure 3.8. Impact of varying the contribution of *Geobacter* to TCE reduction (% by Geo) and the contribution of acetate as an electron donor for *Geobacter* (% by Ac^-).

The electron-balance analysis also provides quantitative insight for interpreting the role of *Dehalococcoides* in the microbial community structure of dechlorinating consortia. A comparison of Figures 6A and 6B demonstrates that, even when *Dehalococcoides* are a minor portion of the biomass, they can be a large fraction of the bacterial community in numbers. This is a direct result of the small relative volume of *Dehalococcoides* compared to the other bacterial groups (Table 2). More specifically, since the volume of a *Dehalococcoides* cell is at

least 25 times smaller than for cells of *Firmicutes*, *Dehalococcoides* produce many more cells for the same amount of electron equivalents.

Because of this difference in cell volume, small differences in concentration of TCE that is reductively dechlorinated can in some cases strongly alter the relative abundance of *Dehalococcoides* numbers within the bacterial community; however, *Dehalococcoides* biomass may not change as dramatically. This has been suggested previously (Haest et al., 2010), but now we show it quantitatively with our electron-balance analysis. qPCR and pyrosequencing obviate the masking effect of the fermenters by assessing and quantifying gene copies. The electron-balance analysis also underscores the importance of converting gene copies to cell numbers, since *Dehalococcoides* have only 1 16S rDNA copy/cell, while other commonly present organisms have between 2 to 10 16S rDNA copies/cell (Table 5 and Figure 10).

Concentration and relative abundance of bacterial community. We used qPCR and pyrosequencing to confirm the bacterial groups and trends in relative abundance predicted from the electron balance analysis. The concentrations of various bacterial groups quantified with qPCR are in Figure 4. For the three lowest TCE-pulsing rates, *Dehalococcoides* were ~1 x 10^{11} ± 1 x 10^{10} gene copies/L, and the concentrations for the two higher TCE-pulsing rates were ~2 x 10^{11} ± 1 x 10^{10} gene copies/L. *Geobacteraceae* had an upward trend for the first four TCE pulsing rates, ~1.5 x 10^{10} to ~6.5 x 10^{10} gene copies/L, but decreased for the highest pulsing rate. Within the *Firmicutes*, we only quantified

homoacetogens with qPCR, and they had a downward trend, ~8 x 10^{10} to ~2 x 10^{10} copies/L with the increasing TCE pulsing rate.

Pyrosequencing analyses were based on 95% similarity, corresponding to analysis of OTUs at the genera level (Ludwig et al., 1998). The number of qualified sequence tags for the five TCE pulsing rates were, respectively, 2381, 2128, 2622, 2448, and 2464. Presented in Figure 9 are the Chao1 estimates of unique operational taxonomic units (OTUs), the Shannon diversity index, and Shannon evenness index.

The Shannon diversity index, i.e. the unique number of organisms, and Shannon evenness index, i.e. the distribution of organisms, Figures S3B and S3C, decreased with increasing TCE-pulsing rate, except for the two highest pulsing rates. The second highest TCE-pulsing rate (22.2 µmoles TCE/SRT) had the lowest diversity and evenness, and the highest pulsing rate (32.2 µmoles TCE/SRT) had the next lowest values. Comparing the trends in diversity and evenness to the trends in *Dehalococcoides* relative abundance in Figure 7A it is evident that they have a negative correlation (i.e. lower diversity and evenness correspond to higher relative abundance of *Dehalococcoides*). This suggests that diversity and evenness were more strongly related to *Dehalococcoides* abundance than to inhibition of the microbial community as a whole.

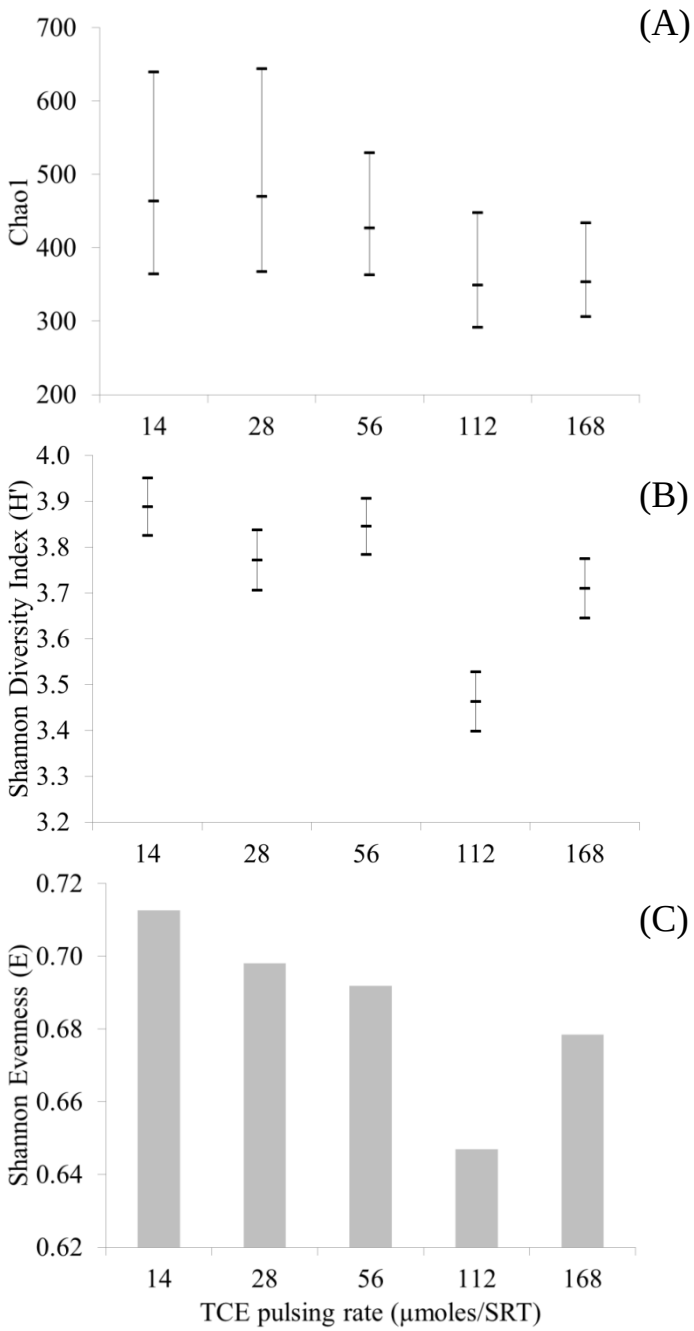
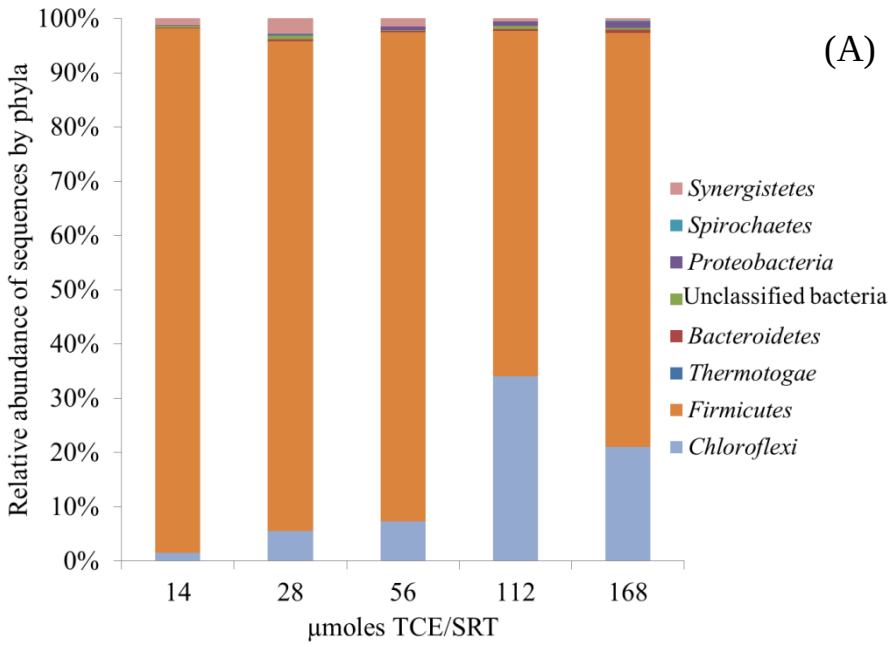
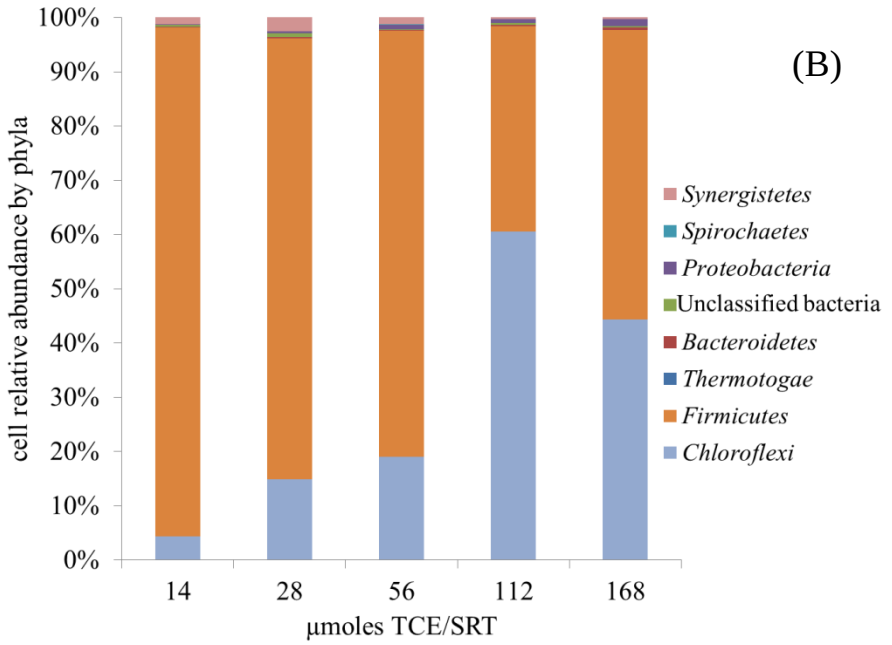


Figure 3.9. Pyrosequencing statistical parameters based on a 5% difference, corresponding to analysis of OTUs at the genera level. In (A) are the ranges of the Chao1 estimates of unique operational taxonomic units (OTUs), (B) are the ranges for the Shannon indices of diversity, in (C) are the Shannon evenness indices.

The relative abundance of the bacterial community is in Figure 7A; these results were determined using the number of 16S rDNA copies/cell listed in Table 5. For comparison, the relative abundance based on sequence copies is in Figure 10A. *Firmicutes* presented the most uncertainty in making the conversion from 16S rDNA copies to cells. Within the *Clostridia* class, to which all of the *Firmicutes* sequences belonged, the average number of 16S rDNA copies/cell based on currently isolated organisms is 6.8 (Klappenbach et al. 2001, Lee et al. 2009). Here, we assumed 5 16S rDNA copies/cell, and Figure 10B and 10C presents the results for 3 and 10 16S rDNA copies/cell; although the relative abundance differ, the trends are the same in all cases. *Chloroflexi* (100% of this phylum was *Dehalococcoides*) and *Firmicutes* together comprised at least 95% of the community for all the TCE-pulsing rates. The trend was that the relative abundance of *Dehalococcoides* increased with TCE-pulsing rate (in Figure 7A from ~10 to ~ 70%) except for the highest TCE-pulsing rate (~60%). *Proteobacteria*, which were 100% the *Geobacter* genus, were only 1-2% of the community. Various consumers of complex organics, i.e., *Thermotogae*, *Bacteroidetes*, and *Synergistetes*, accounted for the remaining 2-3%.


(A)
Relative abundance of sequences by phyla
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
14
28
56
112
168
µmoles TCE/SRT
Synergistetes
Spirochaetes
Proteobacteria
Unclassified bacteria
Bacteroidetes
Thermotogae
Firmicutes
Chloroflexi


(B)
cell relative abundance by phyla
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
14
28
56
112
168
µmoles TCE/SRT
Synergistetes
Spirochaetes
Proteobacteria
Unclassified bacteria
Bacteroidetes
Thermotogae
Firmicutes
Chloroflexi

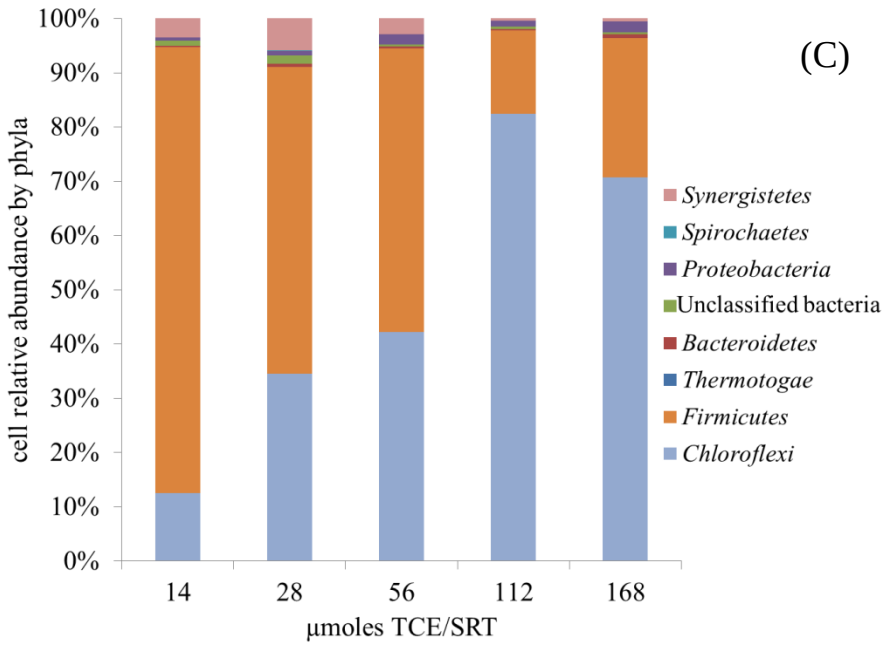


Figure 3.10. Relative abundances of the bacterial groups for each TCE-pulsing rate based on the same pyrosequencing as Figure 7(A) of the main text. Panel (A) is based on the sequence copies. The other two panels involve with the sequence conversions to cell copies based on values in Table 5, but with a different number of 16S rDNA copies/cell for the *Firmicutes.* Here 3 copies/cell were used for (B) and 10 copies/cell for (C).

Based on the pyrosequencing analysis, the only organisms not accounted for in the electron balance analysis were consumers of decaying biomass. The experimental relative abundance (Figure 7A) matched the trend observed in the electron balance analysis (Figure 6B) up to the largest TCE-pulsing rate, where the electron balance analysis gives a continued increase in relative abundance of *Dehalococcoides* (with *Firmicutes* decreasing to compensate).

Stress induced by chlorinated ethenes on 'Dehalococcoides', 'Geobacter', and 'Acetobacterium.' Two factors indicate that the dechlorinating

microorganisms experienced stress induced by chlorinated ethenes for the two highest TCE pulsing rates. Although chlorinated ethene consumption was equal to production by around day 50, the accumulation of VC (Figure 3B) indicated that the maximum rate of TCE conversion to ethene was that of the highest TCE-pulsing rate: 0.8 mM Cl^-/day. This value is well below the reported maximum turnover of 2.83 mM Cl^-/day for DehaloR^2 in Ziv-El et al. (2011) with a peak concentration was 0.8-1 mM, while it was 0.56 mM in this study. *Dehalococcoides* grew to similar concentrations for these two studies, ~2 x 10^{11} cells/L here compared to ~1.5 x 10^{11} cells/L in Ziv-El et al. (2011), suggesting that dechlorinator abundance was not the distinguishing feature. We hypothesize that what impaired the reductive dechlorination was the TCE-pulsing rate. In this study, 2.8 mM TCE was added during one 10-day SRT, whereas Ziv-El et al. (2011) added a total of 3 mM TCE to a batch reactor operated over 29 days.

The molecular data provide a second indicator of stress to the dechlorinators. The *Dehalococcoides* concentration based on qPCR (Figure 4) had an upward trend for the first four TCE pulsing rates, but did not increase further for the highest pulsing rate, and it decreased for *Geobacteraceae*. As a corollary, the pyrosequencing results for *Dehalococcoides* (Figure 7A) showed a decrease in their relative abundance for the highest TCE pulsing rate. The decrease in abundance and relative abundance of *Dehalococcoides* compared to that predicted from the electron balance analysis (Figure 6B), suggests that the TCE pulsing rate caused *Dehalococcoides* to have a decreased net yield.

The effluent concentrations of lactate and methanol showed no trends across the TCE-pulsing rates. Homoacetogens, however, were inhibited by the chlorinated ethenes, as indicated by a shift in the fermentation products. Within the VFAs, an increased TCE-pulsing rate led to an increasing relative abundance (Figure 5) and absolute production of propionate (shown in Table S2). *Clostridia*, which generally ferment lactate to acetate and propionate in a molar ratio of 1:2, obtain a higher metabolic energy when they partner with H_2 scavengers and ferment lactate to acetate only (Seeliger et al. 2002). The potential H_2 scavengers in this study were methanogens, *Dehalococcoides*, and the homoacetogenic genera *Acetobacterium*. We hypothesize that, when the H_2 scavengers were inhibited by TCE, lactate fermentation to propionate increased to compensate. H_2 likely accumulated for the highest TCE pulsing rate (discussed above) as a result of less homoacetogenic activity. The molecular data obtained in this study further confirms this hypothesis. The abundance of homoacetogens (*FTHFS* by qPCR, Figure 4A) had a downward trend with an increased TCE pulsing rate. Likewise, the relative abundance of *Acetobacterium*, within the *Firmicutes* phylum (Figure 7B), declined to 0.5-2% for the two highest TCE pulsing rates, compared with 18-32% for the other TCE pulsing rates. *Acetobacterium* and other acetogens produce necessary growth factors for *Dehalococcoides* (Maymó-Gatell et al. 1995), including the vitamin B_{12} cofactor for the reductive dehalogenase enzymes (He et al. 2007). Stress to *Acetobacterium* may have indirectly contributed to the accumulation of VC and the lower than expected TCE turnover rates (discussed above).

In summary, the highest TCE-pulsing rate and peak concentration (168 μmoles TCE/SRT, 0.56 mM TCE) caused stress to *Dehalococcoides* and *Geobacter*, while the second highest (112 μmoles TCE/SRT, 0.37 mM TCE/addition) and the highest pulsing rate and peak concentration also stressed *Acetobacterium* and possibly other homoacetogens. The high TCE pulsing rate may have directly decreased the kinetic activity of the dechlorinators, indirectly decreased their kinetic activity by stressing cofactor-producing *Acetobacterium*, decreased the net yield of *Dehalococcoides*, or caused a combination of these effects.

Conclusions.

Operating fill-and-draw reactors with a TCE-dechlorinating consortium demonstrated a direct correlation between microbial community function (e.g., TCE reduction to ethene) and structure (e.g., the fraction and abundance of *Dehalococcoides* and *Acetobacterium*) as the TCE-pulsing rate was increased. An electron-balance analysis predicted the community structure based on measured concentrations of products and constant net yields for each microorganism. The predictions corresponded to observed trends in the microbial community structure based on qPCR and pyrosequencing up to the second-highest TCE pulsing rate (112 μmoles TCE/SRT); deviations from the trend for the highest pulsing rate (168 μmoles TCE/SRT) can be attributed to either direct stress to *Dehalococcoides* or to "helper organisms." Methanogens were washed out during fill-and-draw operation for all TCE pulsing rates, probably due to their low growth rate and being further decreased due to stress from chlorinated

ethenes. This study highlights the potential value in using electron balances in combination with molecular analyses to understand bioremediation systems; here, it demonstrated how chlorinated ethenes can induce shifts in the performance and abundance of dechlorinators, fermenters, homoacetogens, and methanogens in reductive dechlorination communities.

Chapter 4

DETERMINATION OF CHLORINATED ETHENES AND ETHENE USING AUTOMATED HEAD-SPACE SOLID PHASE MICROEXTRACTION FOLLOWED BY GAS CHROMATOGRAPHY AND FLAME IONIZATION DETECTION (HS SPME GC-FID)

This chapter has been submitted in an altered format to the *Journal of Chromatography A*.

Introduction

In order to carry out a mass balance to monitor anaerobic reductive dechlorination of chlorinated ethenes it is advantageous to have a method capable of simultaneous detection of the parental chlorinated ethenes, their dechlorination intermediates, and the end product, ethene. In Chapters 2 and 3 I described the GC-FID method that I used for headspace injection of cultures grown in batch serum bottles. Here I describe the method I developed for sampling of liquid for the MBfR studies in Chapter 5.

The U.S. Environmental Protection Agency (U.S. EPA) maximum contaminant levels (MCLs) for chlorinated ethenes and ethene are all in the low µg/L range. In order to obtain sufficiently low method detection limits (MDLs) with headspace injection, the minimum liquid volume using U.S. EPA Method 5021A is 10 mL. U.S. EPA Method 624 involves the sample concentration approach called purge and trap, reducing the liquid volume requirement to 5 mL. Whereas this volume is only a fraction of the volume of a standard EPA Volatile Organic Analysis (VOA) 40-mL vial (U.S. EPA, 2008), it may still represent a

challenge when repeatedly analyzing lab-scale reactors during remediation feasibility studies that do not contain a headspace. This is the case in my labscale MBfR study (Chapter 5) and in Enzien et al. (1994), Shen et al. (2007), Azizian et al. (2008), and Chung et al. (2008a,b).

Various studies describe the concentration technique solid phase microextraction in the sample headspace (HS SPME) combined with gas chromatography (GC) and either a flame ionization detector (FID), electron capture detector, or mass spectrometer for the analysis of PCE, TCE, DCE, or a combination of these (Popp et al., 1997; Lee et al., 2002; Poli et al., 2005; Avila et al., 2007). None of these methods, however, include analysis of vinyl chloride and ethene. In addition, most of these methods use liquid volumes of 5 mL or greater and require below room temperatures for the extraction.

Song et al. (2002), Wymore et al. (2006), and Chung et al. (2008a,b) note the use of HS SPME GC-FID for analysis of TCE, *cis*-DCE, VC, and ethene from groundwater samples, but none describe the method in detail. Song et al. (2002) state that the analyses were carried out with a 75-µm carboxen-PDMS fiber, but a full description of the method was not published. Wymore et al. (2006) validated their method by sending various samples to off-site laboratories that used U.S. EPA methods and receiving blind samples from an independent vendor to analyze on-site; they too do not further describe their method.

Chung et al. (2008a) provide the greatest detail on their method, e.g. fiber type, adsorption time, column, and temperature profile. In additional they describe a small liquid volume requirement of 2 mL, and note that the SPME was

carried out manually. Their calibration approach, however, was conducted in 160-mL serum bottles with 100 mL of liquid, whereas the samples were analyzed in 2.5 mL vials with 2 mL of liquid. It may be possible to scale the analyses for different liquid and headspace volumes, but this would impinge on achieving the desired low detection limits.

The methodology described here describes the simultaneous detection of PCE, TCE, *cis*-, *trans*-, and 1,1-DCE, vinyl chloride, and ethene using HS SPME GC-FID. It has been applied successfully in the lab-scale MBfR described in Chapter 5. This method is completely automated and requires a sample volume of only 1 mL.

Materials and Methods.

Sample preparation. 1-mL liquid samples were analyzed in 2-mL crimp top vials with a magnetic cap and silicon/PTFE septa (MicroSOLV Eatontown, NJ). The samples were vortexed inverted for 1 minute to promote rapid mass transfer of the chemicals into the vial headspace.

Analysis. The samples were processed with an AOC-5000 autosampler as follows (Shimadzu, Columbia, MD). The vials were heated and vortexed in an agitator oven at 30˚C for 1 minute, followed by a 5-minute adsorption period by solid-phase microextraction, using an 85-µm Carboxen-PDMS fiber (Supelco, Bellefonte, PA), followed by 5 minutes of desorption into a gas chromatograph equipped with a flame ionization detector (Shimadzu GC-2010, Columbia, MD). The compounds were separated with an Rt-QSPLOT column of 30 m length, 0.32 mm ID, and 10 µm film thickness (Restek, Bellefonte, PA). The split injection

port was held at 240˚C and the temperature profile was 110˚C for 1 minute, a ramp of 50˚C/minute to 200˚C, and another ramp of 20˚C/minute to 220˚C and held for 3 minutes, for a total analysis time of 9.15 minutes.

Calibration curves, limits of detection, and linearity. The calibrations were carried out with neat PCE (Sigma Aldrich, St. Louis, MO), TCE (Sigma Aldrich, St. Louis, MO), *cis*-, *trans*-, and 1,1-DCE (Supelco, Bellefonte, PA), gaseous VC at 99.5% (Fluka, Milwaukee, WI), gaseous ethene at 99.5% (Matheson Tri-gas, Basking Ridge, NJ).

For PCE, TCE, *cis*-DCE, *trans*-1,2-DCE, and 1,1-DCE a stock solution, described in Table 1A of the supporting information, was prepared in 245-mL serum bottles containing a Teflon-lined stirring bar and filled with deionized water, leaving no headspace, capped with a polytetrafluoroethylene (PTFE)-lined stopper and crimped. The solution was stirred at room temperature for four hours before use. A second stock solution was prepared by a two orders of magnitude dilution into the same serum bottle setup described above. Details of the VC and ethene stock concentrations are provided in Table 1B. The concentrations were prepared in 120-mL serum bottles holding a PTFE-lined stirring bar, capped with a PTFE-lined stopper, and crimped. The mixtures were stirred for 4 hours before use.

Varying volumes of the liquid stock solutions were added to the 2-mL sampling vials with a gas-tight syringe containing deionized water to provide a final volume of 1 mL; the final concentrations and preparation details are in Table 1C. The gaseous stock solutions were then added as listed in Table 1D. Before

Table 4.1

Preparation of stock solutions for (A) PCE, TCE, and the three DCE isomers, and (B) VC and ethene. All were prepared in serum bottles with a stirring bar and closed with a PTFE-lined stopper and crimped; the bottle volume for the stock solution in (A) was 240 mL and it was 120 mL for all other stock solutions. Tor the compounds in (A) the bottles were filled completely with deionized water and for those in (B) no liquid was added. The calibration standard samples for (C) PCE, TCE, and the three DCE isomers and (D) VC and ethene, were prepared in 2 mL vials augmented with deionized water to bring the total liquid volume to 1 mL. Before adding the stock solutions, the equivalent volume of liquid and gas were removed from the vials. Handling of the chlorinated ethenes and ethene was with gas tight syringes.

(A)

Compound	MW (g/mol)	density (g/mL)	$V_{chlorinated\ ethene}$ (µL)	V_{water} (mL)	C_{stock} [µM]
PCE	165.83	1.622	12	240	479
TCE	131.39	1.46	10	240	453
cis-1,2-DCE	96.95	1.28	8	240	431
trans-1,2-DCE	96.95	1.26	8	240	424
1,1-DCE	96.94	1.213	8	240	408

(B)

VC and ethene		
C_{stock} (%)	$C_{VC\ or\ ethene}$	V_{stock} (mL)
5.00	99.5%	6
1.00	99.5%	1.2
0.10	99.5%	0.12
0.01	1.00%	1.2

(C)

Compound	PCE		TCE		cis-1,2-DCE		trans-1,2-DCE		1,1-DCE	
V_{stock} (µL)	C_{stock} [µM]	C_{final} [µM]	C_{stock} [µM]	C_{final} [µM]	C_{stock} [µM]	C_{final} [µM]	C_{stock} [µM]	C_{final} [µM]	C_{stock} [µM]	C_{final} [µM]
10	4.79	0.05	4.53	0.05	4.33	0.04	4.24	0.04	4.08	0.04
50	4.79	0.24	4.53	0.23	4.33	0.22	4.24	0.21	4.08	0.20
100	4.79	0.48	4.53	0.45	4.33	0.43	4.24	0.42	4.08	0.41
500	4.79	2.40	4.53	2.26	4.33	2.16	4.24	2.12	4.08	2.04
10	479	4.79	453	4.53	433	4.31	424	4.24	408	4.08
50	479	24.0	453	22.6	433	21.6	424	21.2	408	20.4
100	479	47.9	453	45.3	433	43.1	424	42.4	408	40.8
200	479	95.8	453	90.5	433	86.2	424	84.8	408	81.7
500	479	240	453	226	433	216	424	212	408	204
1000	479	479	453	453	433	433	424	424	408	408

(D)

VC and ethene		
V_{stock} (µL)	C_{stock} (%)	C_{final} [µM]
10	0.01	0.04
40	0.01	0.16
10	0.10	0.4
40	0.10	1.6
10	1.00	4
10	5.00	20
20	5.00	40
40	5.00	80

adding the stock solutions of liquid and gas, the volume equivalent to that being added was removed from the vial headspace so as to minimize chemical losses due to over-pressurization.

The method detection limits (MDLs) were determined as described previously (Halden et al., 2001). Seven blank samples were analyzed and the signal mean and standard deviation were determined. The MDL was the lowest concentration analyzed for which the signal for seven samples was always larger than three standard deviations above the mean of the blanks.

The limits of linearity were determined as the concentration range for which the correlation coefficient (r^2) was greater than 0.99. A seven point calibration was then carried out in triplicate.

Recoveries of Arizona groundwater and anaerobic mineral medium. The recoveries of the compounds (n = 4) were tested in Arizona groundwater and in anaerobic mineral media (Löeffler et al., 2005), typical of that used for anaerobic reductive dechlorination, with spiked samples containing target analytes at concentrations between 9-36 µM. The values for each in µM and ppb are in Table 3, described later.

Maximum holding time for abiotic samples. The maximum time that samples can be stored, was defined as the time for which the compound recoveries remained in the range of 90 to 110%. Triplicate samples were tested by spiking deionized water with target analytes to final concentrations of 10-30 µM and subsequent analysis after temporal storage in two conditions: upright in

the autosampler at room temperature and inverted at 4˚C, to minimize losses through the cap.

Sediment flow-through column study. The utility of the SPME-GC-FID method was assessed in a bench-scale feasibility study design typical of the groundwater remediation industry. A sediment column, flow-through microcosm was inoculated with the mixed-microbial consortium and fed with groundwater containing TCE at a concentration ~50 ppb. The influent-feed cycle was 56 µL for 90 seconds followed by a 240 second pause, resulting in an effective feed-rate of 0.91 mL/hour. Influent and effluent samples were taken periodically with a gas-tight syringe by sampling 0.2 mL and diluting in 0.8 mL of deionized water, and analyzed as described above.

Results and Discussion.

All seven compounds – PCE, TCE, *cis*-DCE, *trans*-DCE, 1,1-DCE, VC, and ethene – could be separated and analyzed simultaneously, as seen in the chromatogram in Figure 1. While four studies using SPME-GC for studies on groundwater bioremediation reported simultaneous detection of TCE, *cis*-DCE, VC, and ethene (Song et al., 2002; Wymore et al., 2006; Chung et al., 2008a,b), these studies do not provide method details and no other studies analyzed for ethene.

The MDLs for all the compounds, listed in Table 2, were at or below the U.S. Environmental Protection Agency maximum contaminant levels (MCL), except for VC where the MDL was slightly (0.5 ppb) above the MCL. Page and Lacrois (2000) demonstrated that lower detection limits can be achieved with a

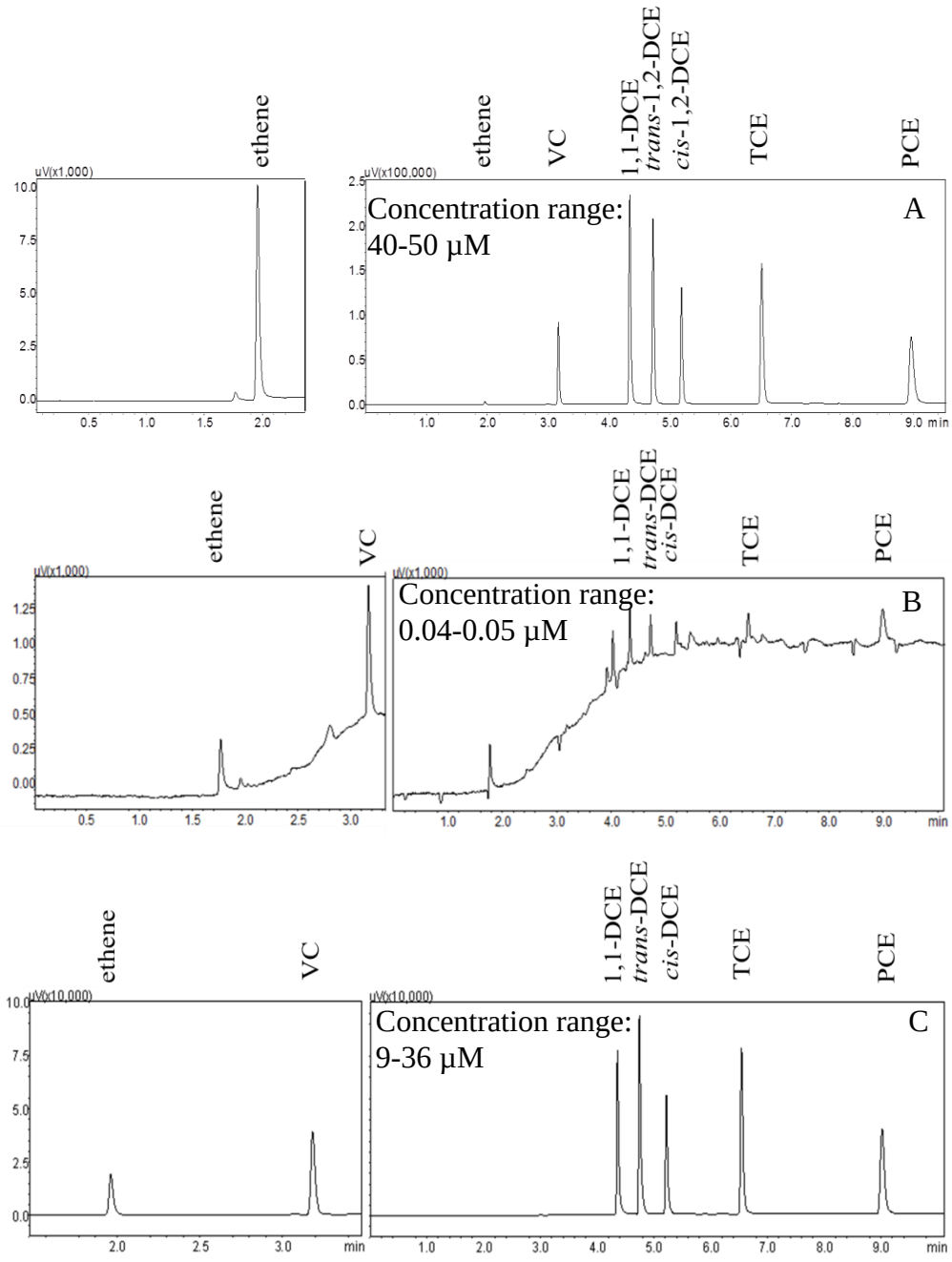


Figure 4.1. Example chromatogram in DI water for (A) upper linearity values (Table 1) and (B) near the MDLs (Table 1), and in (C) spiked groundwater at the recovery concentrations listed in Table 3.

Table 4.2

Compound retention times, limits of linearity for the simultaneous detection of triplicate concentrations, and method detection limits determined with seven replicates.

Compound	Retention Time (min)	Linear Range	r^2	MDL	MCL[1]
PCE	8.99	0.049-49 µM	0.990	0.03 µM (5 ppb)	5 ppb
TCE	6.52	0.046-46 µM	0.999	0.01 µM (1.3 ppb)	5 ppb
cis-1,2-DCE	5.19	0.044-44 µM	0.998	0.02 µM (2.0 ppb)	70 ppb
trans-1,2-DCE	4.73	0.043-43 µM	0.997	0.03 µM (2.9 ppb)	7 ppb
1,1-DCE	4.34	0.042-42 µM	0.992	0.03 µM (2.9 ppb)	100 ppb
VC	3.17	0.04-40 µM	0.999	0.04 µM (2.5 ppb)	2 ppb
ethene	1.96	0.04-40 µM	0.992	0.2 µM (6.0 ppb)	NA[2]

[1]MCL = Maximum Contaminant Level specified by the Safe Drinking Water Act; [1]NA = not applicable

fiber coated with carbonxen/PDMS as opposed to PDMS alone. Furthermore, analyzing TCE in municipal sewage, Wejnerowska and Gaca (2008) report a four times lower detection limit using an ECD detector (0.005 ppb) compared to an FID detector (6 ppb), and this may improve the detection limits for the method described in this study. However, such modifications are non-essential for feasibility studies (Enzien et al., 1994; Shen et al., 2007; Azizian et al., 2008) and would be beneficial mostly if the method is applied to environmental monitoring for compliance purposes. For remediation feasibility studies, replacement of the FID with a halogen-responsive ECD is counter-productive, as it makes impossible the simultaneous detection of the fully dechlorinated product ethene along with its chlorinated parental compounds.

The linear range of this method extended across three orders of magnitude for all seven compounds when monitored jointly (Table 2) and could be extended further for at least one order of magnitude when analytes were assayed individually (data not shown). As a comparison, Poli et al. (2005) reported a linear range of four orders of magnitude for PCE and TCE analysis in urine with an MS detector. Fabbi et al. (2007) observed linearity across three orders of magnitude in concentration for olive oil samples. Wejnerowska and Gaca (2008) achieved linearity across a single order of magnitude using an FID detector and twice that when using an ECD detector.

Analyte recovery were insensitive to the aqueous sample matrix assayed (Table 2). Results obtained for spiked Arizona groundwater and spiked mineral

medium commonly used in laboratory settings and in remedial design feasibility studies compared favorably to those for spiked deionized water (Table 3). The recoveries of PCE, TCE, and three DCE isomers were 99-108% for groundwater and 94-100% for anaerobic mineral medium; these recoveries were similar to those reported by Wu et al. (2002) who analyzed industrial wastewater samples. The recoveries for VC and ethene were lower: ~90% in groundwater and ~80% in the mineral medium, respectively. Other studies have analyzed samples from a diverse group of liquids including vegetable oil (Page et al., 2000), olive oil (Arrebola et al., 2005), municipal sewage (Wejnerowska and Gaca, 2008), urine (Poli et al., 2005), and rat blood (Dixon et al., 2005). No studies have reported on method development for these compounds in groundwater or mineral medium, and the studies referenced above did not assess compound recoveries.

Table 4.3

Recovery rates obtained for different matrices (n=4).

Compound	Spiked Concentration µM (ppb)	Recovery % Arizona Groundwater	Recovery % Mineral Media
PCE	23 ± 2 (0.14)	103 ± 5	99 ± 8
TCE	23 ± 1 (0.18)	108 ± 3	98 ± 4
cis-DCE	21 ± 0.1 (0.22)	104 ± 3	100 ± 2
trans-1,2-DCE	21 ± 1 (0.22)	103 ± 3	94 ± 3
1,1-DCE	19 ± 0.5 (0.20)	99 ± 1	99 ± 1
VC	9 ± 0.2 (0.14)	92 ± 5	78 ± 6
ethene	36 ± 1 (1.28)	89 ± 9	79 ± 10

We further studied the impact of sample storage for vials kept upright at room temperature in the autosampler and inverted at 4˚C, seen in Figure 2, and calculated the corresponding maximum holding times, listed in Table 4. Samples were stable for up to 1 hour in the autosampler and at least 47 hours at 4˚C. Other studies using autosamplers (Arrebola et al., 2005; Chung et al., 2008a,b) do not report holding times. Use of a chilled autosampler could potentially extend the here reported sample holding times but this equipment is not widely available in typical lab settings.

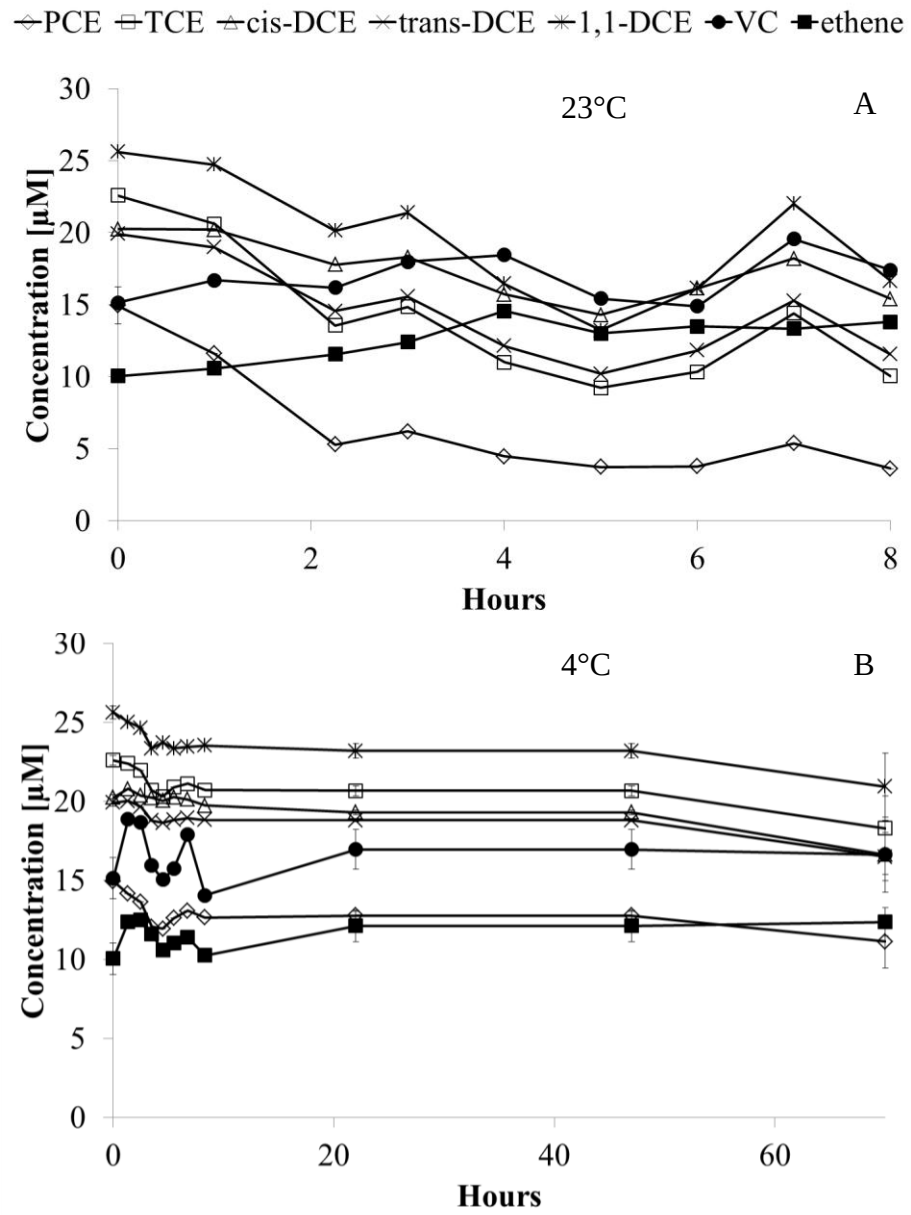


Figure 4.2. Compound concentrations of samples in deionized water held (A) upright in the auto-sampler at room temperature and (B) inverted at 4°C. Triplicate samples were analyzed for the initial concentrations for both holding temperatures and for times 22 hours, 47 hours, and 70 hours at 4°C. The maximum holding times are summarized in Table 3.

Table 4.4

Maximum holding times, defined as recovery between 90-110%, for triplicate samples in deionized water with a concentration in the high range of the calibration curve. Samples were held upright in the autosampler or inverted at 4˚C.

Compound	Maximum holding time in autosampler (hours)	Maximum holding time at 4˚C (hours)
PCE	1	47
TCE	1	47
cis-1,2-DCE	1	47
trans-1,2-DCE	1	47
1,1-DCE	1	47
VC	2	47
ethene	2	47

Finally, we applied the method to the analysis of a lab-scale sediment flow-through column experiment to examine the time course of biological reductive dechlorination of TCE to ethene, as seen in Figure 3. The method was successful in tracking reduction of TCE to *cis*-DCE, VC, and ethene over an 80-day span with starting concentrations in the low ppb range. The sum of the products at any point (i.e., the mass balance) fluctuated by at most 40%. These fluctuations are indicative of sorption phenomena taking place in the experimental setup.

Based on the results obtained, this method appears best suited for laboratory feasibility studies, such as small-scale sediment columns or water treatment technologies, e.g., the MBfR, where sample volume is limited and

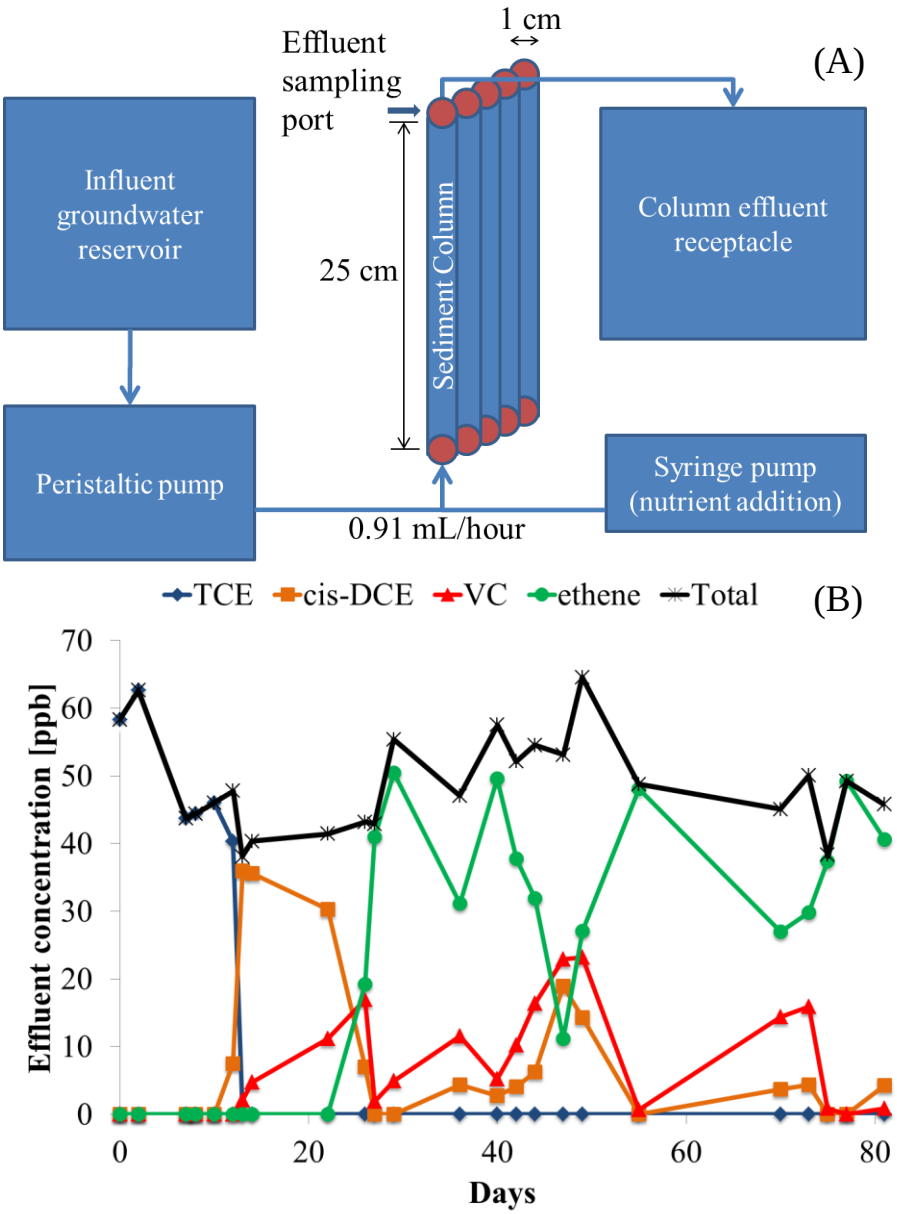


Figure 4.3. Application of SPME-GC-FID method. A schematic of the multi-channel lab-scale sediment flow-through columns is in panel (A). In panel (B) are the effluent reductive dechlorination products from a column with the mixed-microbial consortium DehaloR^2 and an influent TCE-feed in groundwater.

samples taken are processed immediately to obtain an optimal mass balance. The method also may serve for compliance monitoring when sample volumes are limited and prevent the use of conventional EPA standard methods.

Conclusions.

A rapid, simple, and replicable method was developed for the simultaneous detection of chlorinated ethenes and ethene quantification with a linear range across at least three orders of magnitude and detection at or below the EPA MCLs. This method is advantageous for analysis of biological reductive dechlorination where detection of ethene is essential to detect and monitor the desired outcome. Additionally, the small (1 mL) required sample volume and its ability to simultaneously track chloroethene conversion and ethene evolution make it particularly well suited for bench-scale experiments where the reactor volume is often small.

Chapter 5

MANAGING METHANOGENS AND HOMOACETOGENS TO PROMOTE REDUCTIVE DECHLORINATION OF TRICHLOROETHENE WITH DIRECT DELIVERY OF H_2 IN A MEMBRANE BIOFILM REACTOR

This chapter was submitted in an altered format to *Biotechnology and Bioengineering*.

Introduction

While i*n-situ* remediation in combination with pump and treat systems is the typical approach for treatment of chlorinated-ethene plumes (LaGrega et al., 2001), pump and treat systems may be a viable stand-alone option for difficult to remediate sites or if the ultimate use is for drinking water. The MBfR may be such a system.

As described in Chapter 1, *Dehalococcoides* are the only microorganisms known to be capable of metabolic conversion of chlorinated ethenes to non-toxic ethene, and they use molecular H_2 as the electron donor for anaerobic reductive dechlorination (Maymó-Gatell, 1999; He et al., 2003; He et al., 2005; Sung et al., 2006a):

$$C_2HCl_3 + 3H_2 \rightarrow C_2H_4 + 3Cl^- + 3H^+ \quad (1)$$

A commonly used *in-situ* bioremediation strategy is to provide H_2 through slow-releasing fermentation processes rather than by direct addition (Fennel et al., 1997; Yang and McCarty, 1998). One reason for this is that H_2 has a very low solubility in water, making it difficult to deliver by sparging (Rittmann, 2007). The second reason is that slow-release of H_2 provides a competitive advantage to

Dehalococcoides, who are slow growers, but excellent scavengers of H_2, based on H_2 half-saturation rate constants, K_s (H_2) (Smatlak et al., 1996; Fennel and Gossett, 1998). Direct delivery of H_2 may overcome a disadvantage inherent to fermentation: The major fraction of electron equivalents is routed to organic products, not to H_2 (Fennell and Gossett, 1997; Aulenta et al., 2007). The organic products may exceed the requirement for reductive dechlorination, thereby generating biochemical oxygen demand (BOD) in the water.

A limited amount of experience illustrates the pitfalls and possibilities for achieving the ideal situation for direct delivery of H_2. Carr and Hughes (1998) compared direct addition of H_2 to the headspace versus addition of fermentable substrates to completely-mixed, continuous-flow columns, and Aulenta et al. (2005) to fill-and-draw reactors. Rates of reductive dechlorination in the H_2-fed reactors were at least as high as those fed fermentable substrates. However, methanogens, which are good scavengers of H_2 and carry out autotrophic methanogenesis according to:

$$HCO_3^- + 4H_2 + H^+ \rightarrow CH_4 + 3H_2O \quad (2)$$

consumed the major fraction of H_2 in both studies, ~60% and 80% respectively. Homoacetogens, which are facultative autotrophs that can utilize H_2 to generate acetate:

$$2HCO_3^- + 4H_2 + H^+ \rightarrow CH_3COO^- + 4H_2O \quad (3)$$

or can produce acetate heterotrophically via fermentation, were presumed to account for the next highest fraction of H_2 equivalents in Carr and Hughes (1998),

although it was not measured, and in Aulenta et al. (2005) consumed ~15% of the H_2.

Based on a concept first proposed by Fang et al. (2002), Ma et al. (2003, 2006) provided H_2 through pressurized hollow-fiber membranes to continuous-flow sediment columns carrying out PCE reductive dechlorination. Here too methanogenesis accounted for ~90% of the H_2 equivalents. However, the plug-flow reactors had H_2 concentration gradients along the flow path, and dechlorinators could out-compete methanogens in regions of H_2 limitation. Thus, PCE was reduced to less chlorinated products, and ethene was produced near the column effluent, where methanogenesis was absent.

These studies showed that having too much available H_2 caused significant growth of methanogens and homoacetogens, but these studies did not use molecular analyses to confirm the major microbial groups. Clapp et al. (2003) modeled the results of Ma et al. (2003), confirming the experimental results, and suggested that methanogenesis could be decreased by using a lower H_2 pressure.

Allowing abundant methanogenesis to occur can lead to undesirable impacts on reductive dechlorination. Besides wasting H_2 reducing equivalents (above), if H_2 is limiting methanogens can divert electrons away from reductive dechlorination and overwhelm *Dehalococcoides* if the concentration of H_2 is high and the concentration of chlorinated ethenes is not toxic to methanogens (Smatlak et al., 1996; Fennell and Gossett, 1998; Yang and McCarty, 2000). In addition, methane is an undesirable end product, since it can create an explosive environment. However, a small diversion of electrons to methanogenesis may be

valuable for reductive dechlorination if methanogens produce the vitamin B_{12} cofactor required for the *Dehalococcoides* reductive-dehalogenase enzymes (Maymó-Gatell et al., 1995; He et al., 2007; Johnson et al., 2009). The research supporting this benefit is not conclusive, and previous studies indicated that methanogens can only produce precursor corrinoids to vitamin B_{12} (Ryzhkova et al., 2003).

While large-scale diversion of electrons from H_2 to acetate also may be disadvantageous, a small amount of homoacetogenesis can be beneficial to dechlorinators. The main reason is that the produced acetate is the carbon source for *Dehalococcoides* (Tang et al., 2009). In addition, homoacetogens, including *Acetobacterium*, conclusively produce vitamin B_{12} (Stupperich, 1988) and possibly other cofactors required by dechlorinators (Maymó-Gatell et al., 1995).

The interactions among reductive dechlorination, autotrophic homoacetogenesis, and autotrophic methanogenesis are further complicated by pH effects, particularly since *Dehalococcoides* function optimally in a narrow pH range of 6.8-7.8 (Middeldorp et al. 1999; Robinson et al., 2009; Popat and Deshusses, 2009). Equation 1 indicates that reductive dechlorination is a proton-producing process, equation 2 (methanogenesis) is a proton-consuming reaction, and equation 3 shows that autotrophic homoacetogenesis replaces bicarbonate with acetate while consuming protons. For the same amount of bicarbonate, methanogenesis consumes over twice the number of protons as does homoacetogenesis, indicating that the former has the potential to more significantly increase the pH. Thus, homoacetogens, rather than methanogens, are

potentially the better partners for dechlorinators, as they provide growth factors and acetate to *Dehalococcoides* while consuming less than half the molar concentration of protons for the same amount of bicarbonate. Ideally, H_2 should be delivered in a stoichiometric amount to support reductive dechlorination, plus just enough for a minimal extent of homoacetogenesis to supply acetate and necessary cofactors for to *Dehalococcoides*.

Above-ground reactors seem to be a more feasible approach for direct addition of H_2, as reactor conditions can be controlled more readily (e.g., to preclude methanogenesis). As described in Chapter 1, Chung and co-workers used the MBfR to study reductive dechlorination of trichloroethene (TCE) (Chung et al., 2008a) and a combination of TCE, chloroform, and trichloroethane (Chung et al., 2008b). In MBfRs performing the reduction of chlorinated solvents, denitrification (Chung et al., 2008a) or denitrification and sulfate reduction (Chung et al., 2008b) consumed over 95% of the H_2 electron equivalents.

Methanogens were not detected in either study using the MBfR for reductive dechlorination (Zhang et al., 2010). This is an expected result because of strong competition from denitrifiers and sulfate reducers and because bicarbonate was not provided in the influent media. However, homoacetogens were detected in these experiments, although in low relative abundance (Chung et al., 2008b; Zhang et al., 2010).

Although two previous studies (Chung et al. (2008a,b) were successful in converting TCE to ethene as a co-contaminant, the start-up strategy did not turn out to be replicable. Thus, before moving to further studies involving TCE as a

co-contaminant it was necessary for us to understand how to identify and control the core microbial community. In contrast to previous MBfR studies with TCE, the influent in the present study contained TCE as the sole electron-accepting contaminant, and it also contained bicarbonate, which is common and abundant in groundwater. This means that methanogens or homoacetogens could not be readily suppressed by strong competition from denitrifiers or sulfate reducers or by the absence of bicarbonate; thus, *Dehalococcoides* had to compete directly for H_2 against homoacetogens and methanogens.

The first objective of this study was to assess whether methanogenesis and homoacetogenesis can be managed so that TCE reduction is favored when using direct delivery of H_2 in the MBfR. We tested the hypothesis that, by controlling pH, reductive dechlorinators can favorably compete for H_2 during batch startup, allowing the dechlorinators to continue to compete successfully during continuous operation. The second objective was to define the microbial community in the MBfR during sustained reductive dechlorination, which provides additional information to corroborate the hypothesis.

Materials and Methods.

MBfR set up, inoculation, and operation. Experiments were carried out in four MBfRs specially modified for TCE reduction and operated chronologically; a schematic of the set-up is shown in Figure S1 (Supplemental Information). Each 70-mL MBfR consisted of two glass columns connected with rigid Teflon tubing, caps, and connectors. MBfR 1, MBfR 2, and MBfR 3 held a bundle of 32 fibers in one column (MHF 200TL, Mitsubishi Rayon, Otake,

Japan), and MBfR 4 held 50 fibers. Each fiber was ~25 cm in length, for a total fiber surface area of 72 cm^2 for the MBfRs 1, 2, and 3 and 112 cm^2 for MBfR 4. All fibers were pressurized on both ends with ultra high purity (UHP) H_2 at a pressure of 2.5 psig (0.17 atm.) and delivered via stainless steel tubing. The fiber pressure was periodically switched to UHP N_2 in MBfR 3 and MBfR 4.

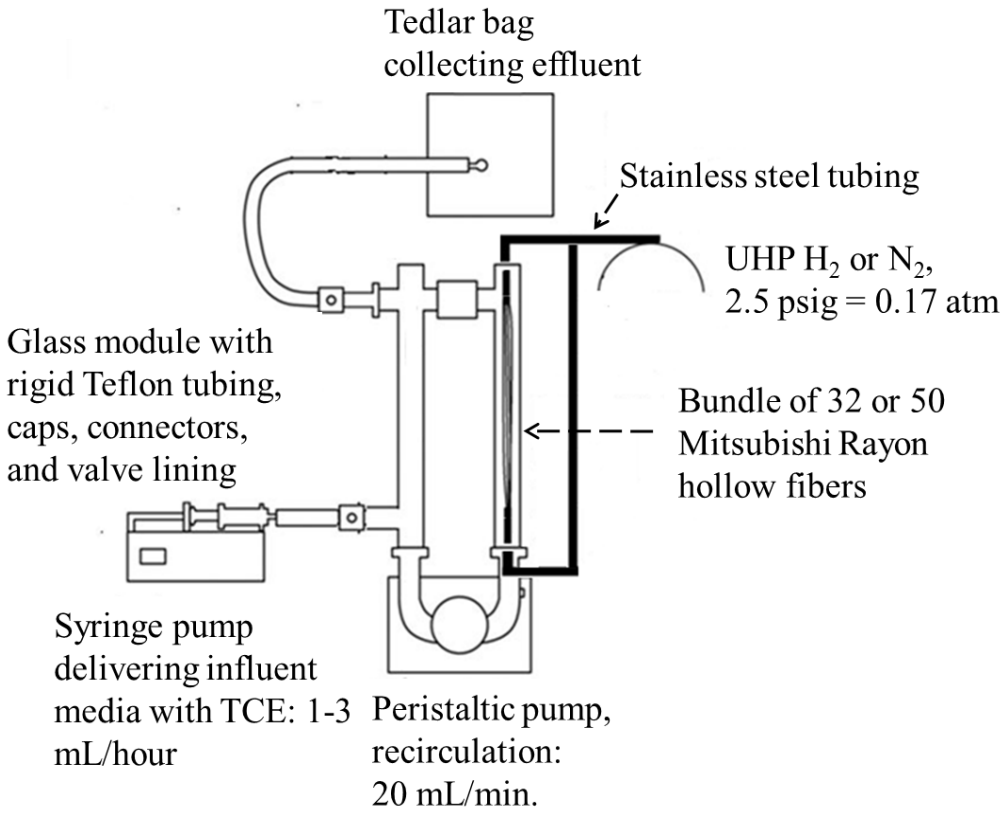


Figure 5.1. Schematic of the MBfR set up for research with TCE.

The anaerobic medium was that of Ziv-El et al. (2011), except for the buffer. In MBfR 1, the buffer was 30 mM bicarbonate during batch operation and was 10 mM phosphate plus either 15 or 7.5 mM bicarbonate. For MBfRs 2, 3,

and 4, the buffer was 10 mM phosphate and 7.5 mM bicarbonate for a final pH of 6.8. The medium was fed into the reactor with a syringe pump (NE-300, New Era Syringe Pumps, Wantagh, NY) at a flow rate of 1 ml/hour for MBfRs 1, 2, and 3 and 3 ml/hour for MBfR 4, corresponding to hydraulic retention times (HRTs) of ~3 and 1 days, respectively. Medium was recirculated inside the reactor at 20 ml/min to promote mixed conditions using an L/S high-pressure peristaltic pump and an L/S rigid PTFE pump head (Cole-Parmer, Vernon Hill, IL).

In all MBfRs, the inoculum was the mixed microbial consortium DehaloR^2, characterized by Ziv-El et al. (2011). The inoculation procedure involved adding ~30 mL inoculum to the MBfR and then maintaining it as a batch reactor for between 2-33 days before switching to continuous flow. Lactate was added in each MBfR during this startup phase, and methanogen overgrowth was partially controlled during startup of MBfR 3 and MBfR 4 by addition of the methanogen-specific inhibitor 2-bromoethanesulfonate (BES) at 4mM. We used MBfR 1 to determine the combination of phosphate and bicarbonate buffer that would allow for a stable pH and MBfR 2 to assess how to achieve reduction to ethene during batch start-up. We then employed these principles in operating MBfR 3 and MBfR 4.

Analytics. The chlorinated ethenes and ethene were monitored in liquid samples using a solid-phase micro-extraction (SPME) fiber (85 µm Carboxen/PDMS StableFlex, Supelco, Bellefonte, PA) followed by a gas chromatograph (GC) with an Rt-QSPLOT column (30 m x 0.32 mm x 10 µm, Restek, Bellefonte, PA) equipped with a flame ionization detector (GC-2010,

Shimadzu, Columbia, MD). Methane could not be detected with this set-up. Details of sample preparation and GC operation are provided in Chapter 4.

Concentrations of lactate, acetate, propionate, and other volatile fatty acids (VFAs) were quantified with high performance liquid chromatography, as described in Ziv-El et al. (2011). The pH was assayed with an Orion 2-Star Benchtop Meter (Thermo Scientific, Waltham, MA) and an Accumet MicroProbe (Cole Parmer, Vernon Hills, IL).

Molecular analyses. DNA and RNA were extracted from the liquid and fibers at the end of operation of MBfR 4. We refer to the concentrated biomass as pellets. Duplicate pellets for DNA extractions from the liquid were made from 5 mL by centrifuging in 1.5 mL centrifuge tubes at 13.2 rpm for 20 minutes and then decanting the liquid. Duplicate pellets for RNA extractions from the liquid were made in 1.5 mL centrifuge tubes by mixing 0.5 mL of liquid with 1 mL of RNAprotect Bacteria Reagent (Qiagen, Germany) incubating at room temperature for 5 minutes, centrifuging at 13.2 rpm for 10 minutes, and decanting the liquid. For the fibers, 48 of the 50 in the bundle were cut into five sections, and within each section a pellet was made for DNA and RNA extractions using half the fibers for each. For the DNA pellets, the fibers were put into a 1.5-mL centrifuge tube with 1.3 mL of DNase-free water, vortexed on a flat-bed for 5 minutes to promote detachment of the microorganisms from the fibers, centrifuged at 13.2 rpm for 20 minutes, and then the liquid was decanted. The RNA pellets were made similarly, but using RNAprotect Bacteria Reagent with a 10-minute centrifugation.

DNA extraction followed the protocol described in Ziv-El et al. (2011). RNA extraction, DNase treatment, and reverse transcription to cDNA followed the protocol in Parameswaran et al. (2011), except that an additional DNase step was carried out using the product from the first treatment.

454 pyrosequencing targeting the combined V2 and V3 regions of the 16S rRNA gene was performed on one DNA sample from the liquid, using a mixture of the two extracted samples, and one from the biofilm, using a mixture of the five extracted samples. The analysis protocol and data processing followed that described in Garcia-Peña et al. (2011) except for the following modifications. Before clustering, a maximum sequence cutoff length was not set, but sequences below 200 bp were removed. The resultant data included 1995 sequences with a median length of 311 bp for the biofilm sample and for the liquid sample 2513 sequences with a median length of 351 pb. The Ribosomal Database Project classifier confidence threshold used for bacterial identification was 50% and the operational taxonomic unit (OTU) cut-off was a 3% difference. Sequence tags were converted to cell copies using the number of 16S rRNA gene copies per cell indicated in Table 1.

Quantitative PCR (qPCR) assays were carried out as described in Ziv-El et al. (2011), using a TaqMan probe to target the 16S rRNA gene of bacteria, the 16S rRNA gene of *Geobacteraceae*, the 16S rRNA gene of *Dehalococcoides*, and the identified genes encoding for reductive dehalogenases of *Dehalococcoides* involved in TCE to ethene reduction: *tceA* (TCE to *cis*-dichloroethene (*cis*-DCE)), *vcrA* (TCE to ethene), and *bvcA* (vinyl chloride (VC) to ethene). The

Table 5.1

16S rRNA copies/cell used to obtain the relative abundance of the biofilm and liquid microbial communities in Figure 5. Sub-tables (A), (B), and (C) correspond with the same sub-tables in Figure 5. The values are from the rrn database, http://rrndb.mmg.msu.edu/search.php#result, which is based on Klappenbach et al. (2001) and Lee et al. (2009). For (A), all values were taken directly from the database, except for 'Chloroflexi,' where the copy number was that of 'Dehalococcoides,' and 'Proteobacteria,' which was calculated based on the values in (B).

(A)

Phylum	16S copies/cell		amplicons		cells	
	biofilm	liquid	biofilm	liquid	biofilm	liquid
Proteobacteria	3.5	2.8	1220	2392	349	854
Firmicutes	6.6	6.6	350	48	53	7
Chloroflexi	1	1	123	15	123	15
Actinobacteria	3.1	3.1	102	13	33	4
Bacteroidetes	3.5	3.46	81	8	23	2
Synergistetes	3	3.3	1	7	0	2
unclassified_Bacteria	4.2	4.2	118	30	28	7
TOTAL			1995	2513	609	892

(B)

Proteobacterial Class	16S copies/cell	sequence copies		cells	
		biofilm	liquid	biofilm	liquid
Alphaproteobacteria	2.4	2	0	1	0
Betaproteobacteria	3.85	501	43	130	11
Gammaproteobacteria	5.8	345	127	59	22
Deltaproteobacteria	2	234	416	117	208
Epsilonproteobacteria	3	132	1784	44	595
unclassified	4.1	6	22	1	5
TOTAL				353	841

(C)

Genus	16S copies/cell	sequence copies		Genus unavailable	cells	
		biofilm	liquid		biofilm	liquid
Acetivibrio	7.5	1	1		0	0
Acetobacterium	5	247	1		49	0
Anaerovorax	7.5	2	1		0	0
Azoarcus	4	225	22		56	6
Bradyrhizobium	2.4	1	0		0	0
Brevundimonas	2.4	1	0		0	0
Clostridium	8.7	0	1		0	0
Comamonas	3	102	7		34	2
Dehalococcoides	1	123	15		123	15
Desulfomicrobium	4	4	2	family: *Porphyromonadaceae*	1	1
Desulfovibrio	4.5	6	7	family: *Comamonadaceae*	1	2
Dysgonomonas	5.5	4	5		1	1
Geobacter	2	218	401	family: *Porphyromonadaceae*	109	201
Hydrogenophaga	2.8	7	8	order: *Desulfovibrionales*	3	3
Oscillibacter	7.5	1	0	family: *Porphyromonadaceae*	0	0
Paludibacter	5.5	28	2	family: *Clostridiales*	5	0
Proteiniphilum	5.5	3	1	family: *Clostridiales*	1	0
Pseudomonas	5.3	215	98	family: *Clostridiales*	41	18
Sedimentibacter	7.5	1	8	family: *Clostridiales*	0	1

Genus	16S copies/cell	sequence copies		Genus unavailable	cells	
		biofilm	liquid		biofilm	liquid
Stenotrophomonas	4	29	6	phylum: *Alphaproteobacteria*	7	2
Unclassified *Betaproteobacteria*	3.8	19	0	phylum: *Alphaproteobacteria*	5	0
Unclassified *Clostridia*	6.9	33	0		5	0
Unclassified *Gammaproteobacteria*	5.8	103	0		18	0
unclassified *Rhodocyclaceae*	4	139	6		35	2
Wolinella	3	132	1783		44	594
other	4.2	351	138		84	33
TOTAL		1995	2513		622	880

cDNA samples were similarly assayed for expression of reductive dehalogenase genes of *Dehalococcoides*.

SYBR Green assays were used to quantify the *mcrA* gene of methanogens in the DNA and cDNA, following the protocol in Steinberg and Regan (2008), and for the formyltetrahydrofolate synthetase (*FTHFS*) gene of homoacetogens after a 1 in 10 dilution, following the protocols in Xu et al. (2009) and Parameswaran et al. (2011). The 16S rRNA gene of acetoclastic methanogens was targeted using the family specific TaqMan primers and probe for *Methanosarcinaceae* and *Methanosaetaceae* (Yu et al., 2005). For the *mcrA* and FTHFS assays, the reaction volume was 20 µL comprised of the following: 6.8 µL nuclease free water, 10 µL SYBR Realmastermix (5 Prime, CA), 0.6 µL each of the forward and reverse primers for a 0.3 µM final concentration, and 2 µL of template DNA or cDNA. The reaction volume for the *Methanosarcinaceae* and *Methanosaetaceae* assays was 10 µL comprised of the following: 1.37 µL nuclease free water, 4 µL SYBR Realmastermix (5 Prime, CA), 0.3 µL each of the forward and reverse primers for a 0.3 µM final concentration, 0.03 µL of the probe, and 4 µL of template DNA. All assays were carried out in triplicate along with a seven-point calibration curve with a ten-fold dilution factor of the standards. The standard concentrations ranged from 10^{-7}-10^{-1} ng/µL. Melting curves were analyzed for all SYBR Green assays to assess for nonspecific amplification.

Scanning electron microscopy imaging. One fiber from MBfR 4, cut into the same five sections as above, was prepared for SEM imaging. Fixation

involved a sequential two-step chemical immersion for one hour each, first in 2% glutaraldehyde and then in 1% osmium tetroxide, both at pH 7.2 in 0.1M NaPO4. Gradual dehydration in acetone involved 8-10 minute immersions in 20%, 40%, 60%, and 80% solutions, and then three consecutive solutions of 100% acetone. The fibers were then critical-point dried using CO_2 (Balzers CPD020 unit), after which they were mounted on stubs and sputter-coated with gold-palladium (Technics Hummer II unit). SEM imaging was carried out with an XL-30 Environmental Scanning Electron Microscope (SEM) with a Schottky Field Emission Source.

Results and Discussion.

MBfR performance. Our repeated attempts at operating an MBfR with a 30-mM bicarbonate buffer caused the pH to rise above 8 and, at best, resulted in reduction of TCE only to *cis*-DCE (data not shown). We hypothesized that the pH increase was from proton-consuming methanogenesis (equation 2) and homoacetogenesis (equation 3). In Figure 2 are the reductive dechlorination, pH, and acetate results for MBfR 1. Decreasing the bicarbonate concentration from 15 to 7.5 mM reduced the acetate concentration by half, i.e., reduced homoacetogenesis, and caused the pH to stabilize at ~7.4. However, TCE reductive dechlorination still stopped at *cis*-DCE during continuous flow, which we attribute to the pH initially rising above 8 when the bicarbonate concentration was 15 mM. Based on this result, we used a 10 mM phosphate buffer plus 7.5 mM bicarbonate for subsequent experiments.

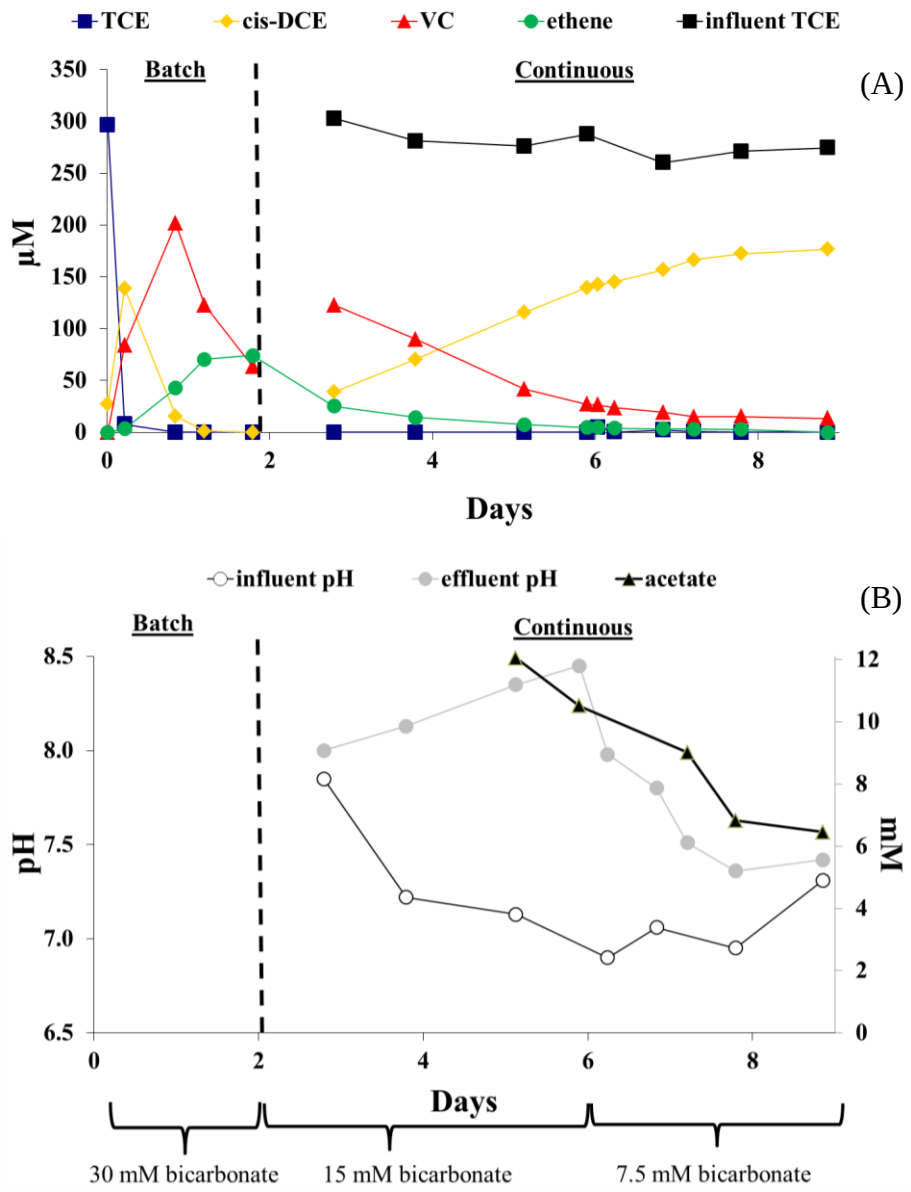


Figure 5.2. MBfR 1 was inoculated with a 30 mM bicarbonate pH buffer for 2 days, but the buffer was changed during continuous flow (after day 2) to 10 mM phosphate plus either 15 or 7.5 mM bicarbonate, as noted along the horizontal axis. 7.5 mM bicarbonate led to a drop in the concentration of acetate, from ~12 to 6 mM, and resulted in a pH drop to ~7.4, a value still within the optimal range for *Dehalococcoides*.

Shown in Figure 3 are the reductive dechlorination and pH results for MBfR 2, in which the pH was well maintained, but nonetheless reductive dechlorination did not progress to ethene, even after three inoculations with DehaloR^2. Ethene production became significant only on the fourth inoculation, which was accompanied by eliminating H_2 delivery and providing lactate as the ultimate electron donor. These results suggested that obtaining complete dechlorination to ethene may require H_2 limitation during reactor start-up.

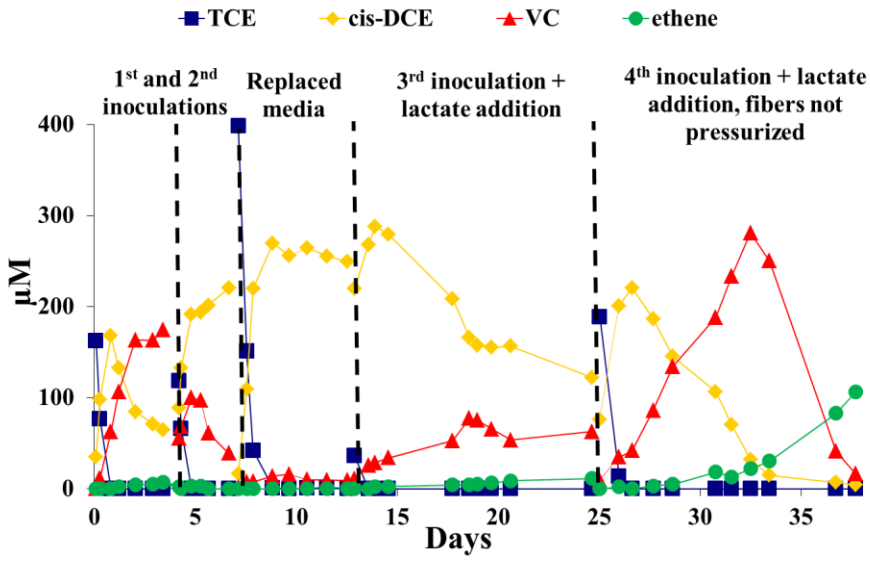
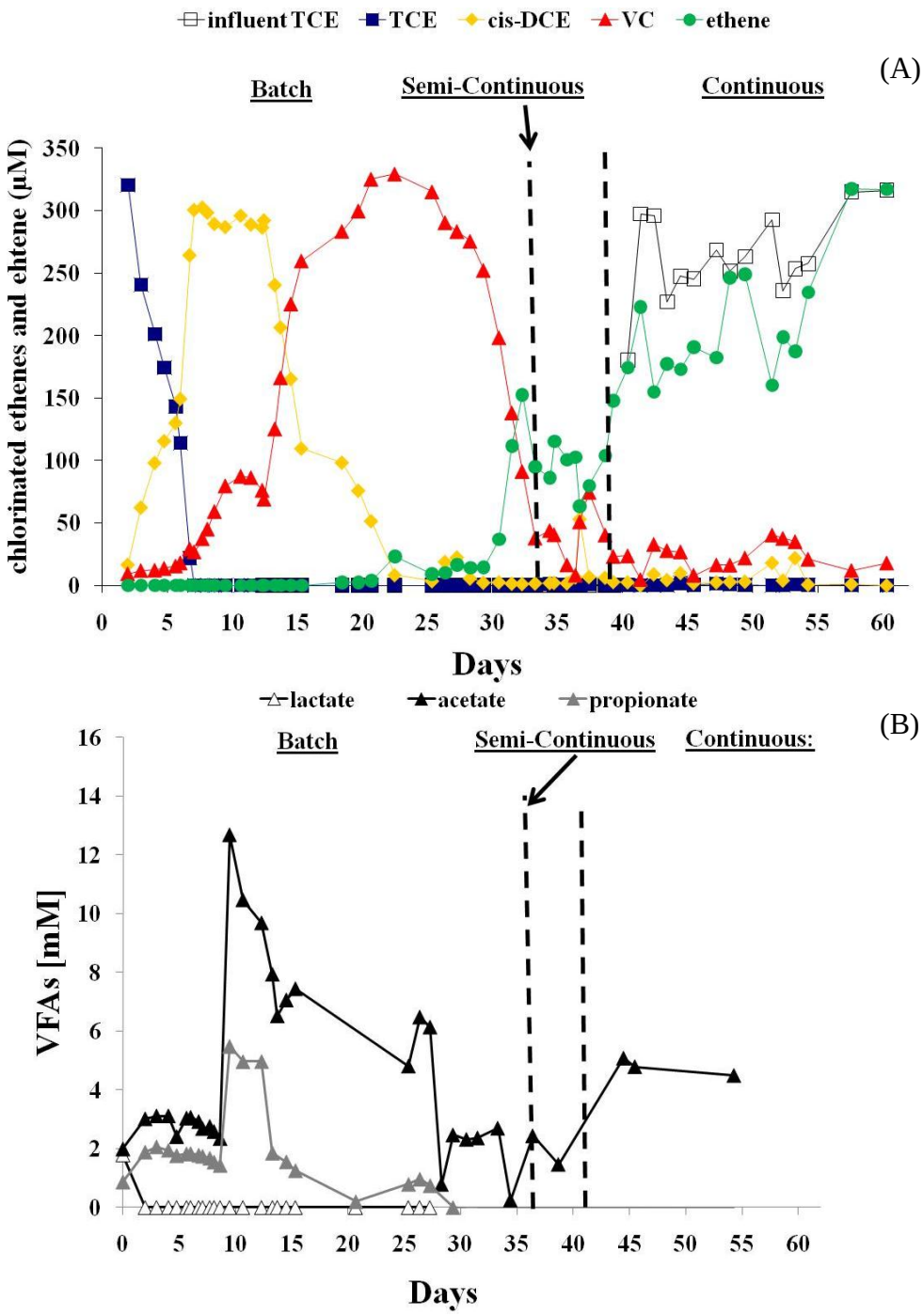


Figure 5.3. MBfR 2 was operated as a batch reactor with the 10 mM phosphate and 7.5 mM bicarbonate combination determined from MBfR 1. The pH was maintained throughout operation between 6.6-7.2, but reductive dechlorination did not progress to ethene until the reactor was H_2-limited in inoculation 4. This was achieved by removing the H2 pressure and providing lactate as the electron donor.

Based on our experience with MBfRs 1 and 2, we imposed more stringent startup conditions for MBfRs 3 and 4 to allow for sustained reductive dechlorination to ethene. During batch operation, we switched the fiber pressure from UHP H_2 to UHP N_2 at the onset of a pH increase, as seen in Figures 4c and 5c. This allowed for *Dehalococcoides*, which are good H_2 scavengers, to have a competitive advantage, thereby minimizing the other H_2-oxidizing processes that can consume protons and raise the pH. As additional precautions to aid in selecting for *Dehalococcoides*, when we added the inoculum we also added lactate in order to provide possible growth factors from the fermentation reactions and inhibited methanogens with BES. In continuous flow, the 7.5-mM bicarbonate concentration plus 10-mM phosphate buffer maintained the pH in both reactors between 6.4-7.5, values within the reported optimal range for *Dehalococcoides* (Middeldorp et al., 1999; Robinson et al., 2009; Popat and Deshusses, 2009).

Performance data for MBfR 3 and MBfR 4 are shown, respectively, in Figure 4 and Figure 5. Other than a two times higher TCE loading and removal flux for MBfR 4, reported in Table 1, the trends were similar for both reactors. Since molecular analyses were carried out only on MBfR 4, we focus more closely on results from MBfR 4. During startup, when the MBfRs were operated as batch reactors, it took ~30 days to see significant production of ethene (Figures 4a and 5a). During continuous flow, TCE reduction was sustained to primarily ethene (~90%) in MBfR 3 and to ethene (20-80%) and VC (20-75%) in MBfR 4. Lactate was added during the startup of both reactors, and it was fermented to


influent TCE
TCE
cis-DCE
VC
ethene
(A)
Batch
Semi-Continuous
Continuous
chlorinated ethenes and ehtene (µM)
Days
lactate
acetate
propionate
(B)
Batch
Semi-Continuous
Continuous:
VFAs [mM]
Days

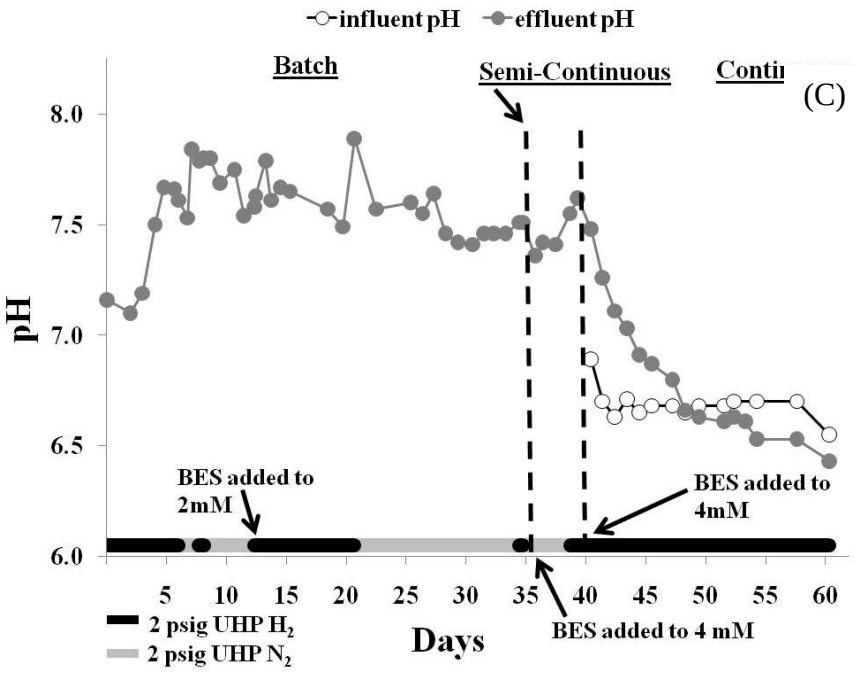
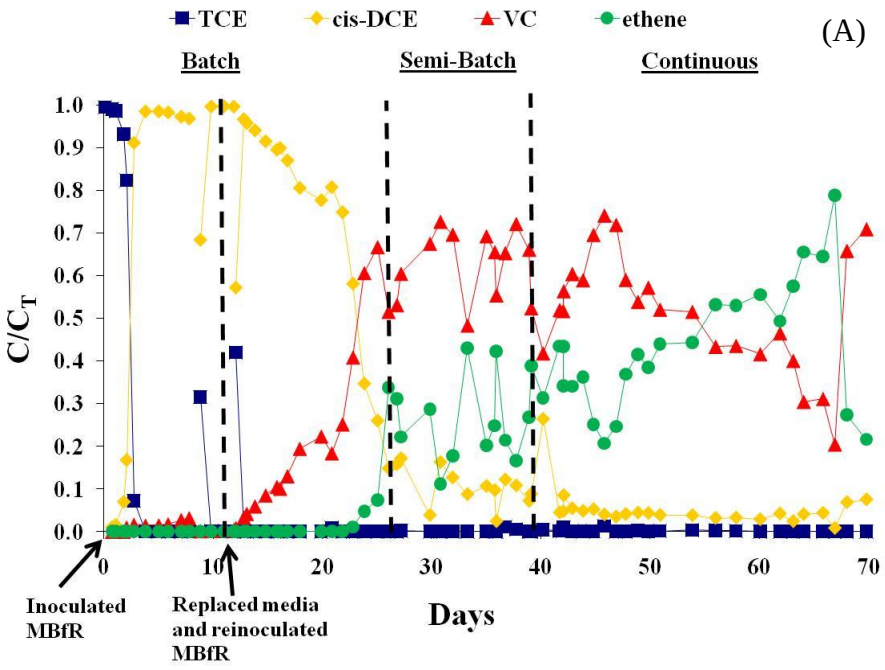
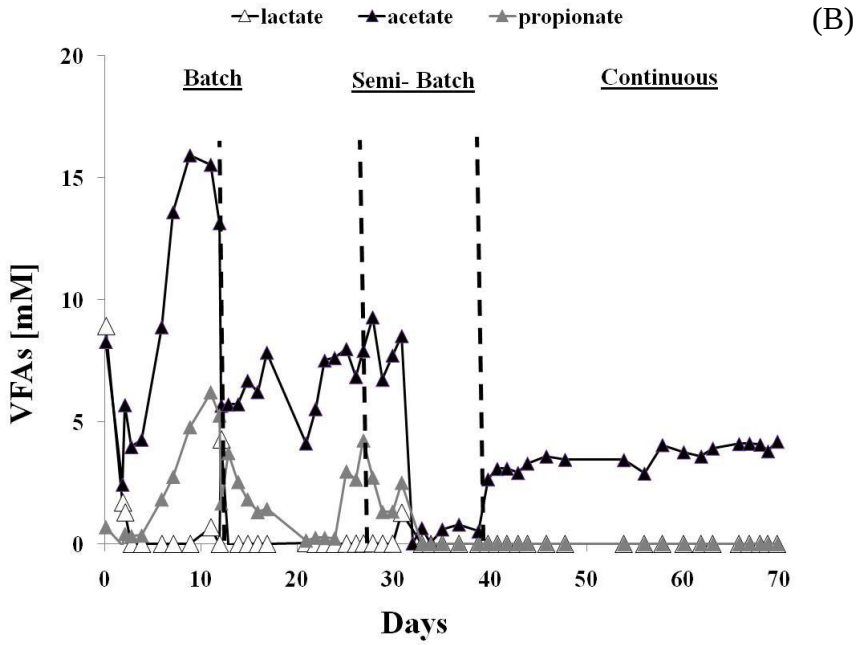


Figure 5.4.

(A) Concentrations of TCE and its reductive dechlorination products, (B) VFAs, and (C) pH in MBfR 3, for which the influent TCE concentration was 325 μM and the HRT was 3 days.


TCE
cis-DCE
VC
ethene
(A)
Batch
Semi-Batch
Continuous
C/C_T
Inoculated MBfR
Replaced media and reinoculated MBfR
Days


lactate
acetate
propionate
(B)
Batch
Semi- Batch
Continuous
VFAs [mM]
Days

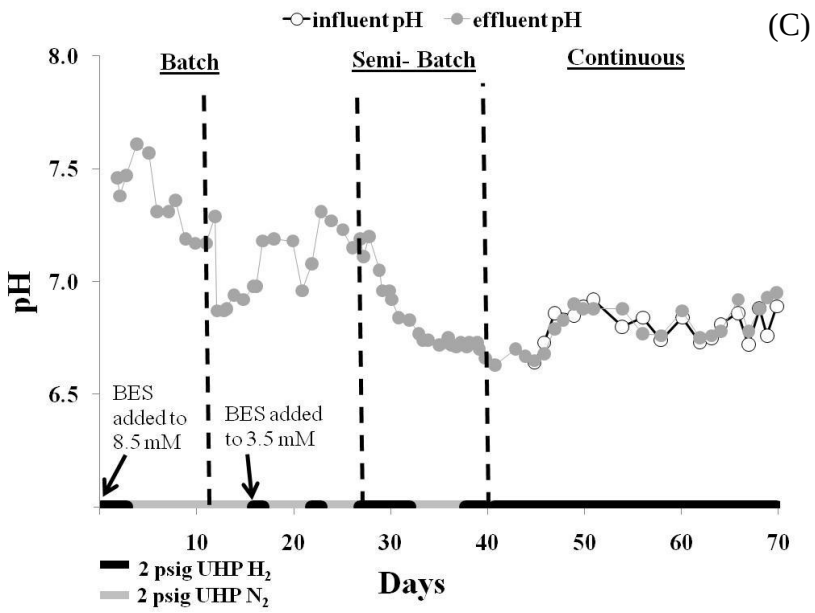


Figure 5.5. (A) Reductive dechlorination, (B) VFAs, and (C) pH in MBfR 4, for which the influent TCE concentration was 325 µM and the HRT was 1 day.

propionate and acetate (Figures 3b and 4b). During continuous flow, H_2 was the only electron donor, TCE was the electron acceptor for *Dehalococcoides*, and bicarbonate was the electron acceptor available for homoacetogens and methanogens. The steady-state effluent acetate concentrations were 4.1 mM in MBfR 3 and 4.5 mM in MBfR 4, which demonstrate that homoacetogens were active.

Table 2 summarizes TCE removal fluxes and distribution of chlorinated ethenes and ethene in the effluent of MBfRs 3 and 4. It also compares these values to the previous MBfR studies active in reductive dechlorination of TCE (Chung et al., 2008a,b) and to other bench-scale systems active in reductive dechlorination of TCE or PCE. Volumetric loadings and removal fluxes are

Table 5.2

Comparison of chlorinated-ethene removal rates and effluent distributions in MBfR studies for TCE reduction and for other bench-scale systems removing TCE or PCE. The values correspond to the largest observed fraction of ethene in the effluent for that study.

Study	bench-scale system	chlorinated ethene fed	electron donor	PCE or TCE surface area or volumetric removal rate		% distribution of chlorinated ethene and ethene in effluent				
				µmoles/m^2-d	µmoles/L-d	PCE	TCE	DCE	VC	ethene
MBfR-1 (this study)	membrane biofilm reactor	TCE	H_2	1130	108	--	0	0	4	96
MBfR-2 (this study)	membrane biofilm reactor	TCE	H_2	2190	325	--	0	4	31	65
Chung et al. (2008a)	membrane biofilm reactor	TCE	H_2	1400	425	--	7	0	0	93
Chung et al. (2008b)	membrane biofilm reactor	TCE	H_2	3280	996	--	13	0	0	87
Popat and Deshusses (2009)	gas phase biofilm reactor	TCE	lactate	--	278	--	20	24	7	49
Ma et al. (2003)	membrane sediment column	PCE	H_2	--	19.8	0	0	11	11	78
Azizian et al. (2008)	sediment column	PCE	lactate	--	69.2	0	0	0	33	67
Azizian et al. (2010)	sediment column	TCE	lactate, formate	--	43.8	0	0	0	6	94

provided for all the studies; for the MBfR studies, surface area loadings and removal fluxes (per fiber area) also are presented, as they are useful design criteria for scale-up. Despite very different inputs of electron-acceptor substrates, all of the MBfR systems gave similar TCE removal fluxes. The volumetric loadings in the MBfRs of this study and the biofilm study of Popat and Deshusses (2009) were two-fold to two orders of magnitude greater than any of the sediment columns, whose contaminant loading rates were presumably selected to mimic the subsurface. This highlights an advantage of *ex-situ* treatment: the ability to achieve much higher volumetric loading rates.

Even though we could not analyze for methane, the acetate results make it evident that homoacetogenesis outcompeted methanogens for bicarbonate at steady state. Equation (3) indicates that the maximum production of acetate from the 7.5 mM bicarbonate in the influent medium, without considering biomass production, was 3.75 mM. Since the effluent concentrations in both reactors were consistently above this amount (Figures 3B and 4B), we hypothesize that most of the bicarbonate was consumed by homoacetogenesis, not methanogenesis, and additional acetate was produced by fermentation of decaying biomass and extracellular polymeric substances (EPS) remaining from reactor startup. These decay processes may have supplied *Dehalococcoides* with essential growth factors, including vitamin B_{12}, otherwise supplied through fermentation processes (DiStefano et al., 1992; He et al., 2007; Aulenta et al., 2007).

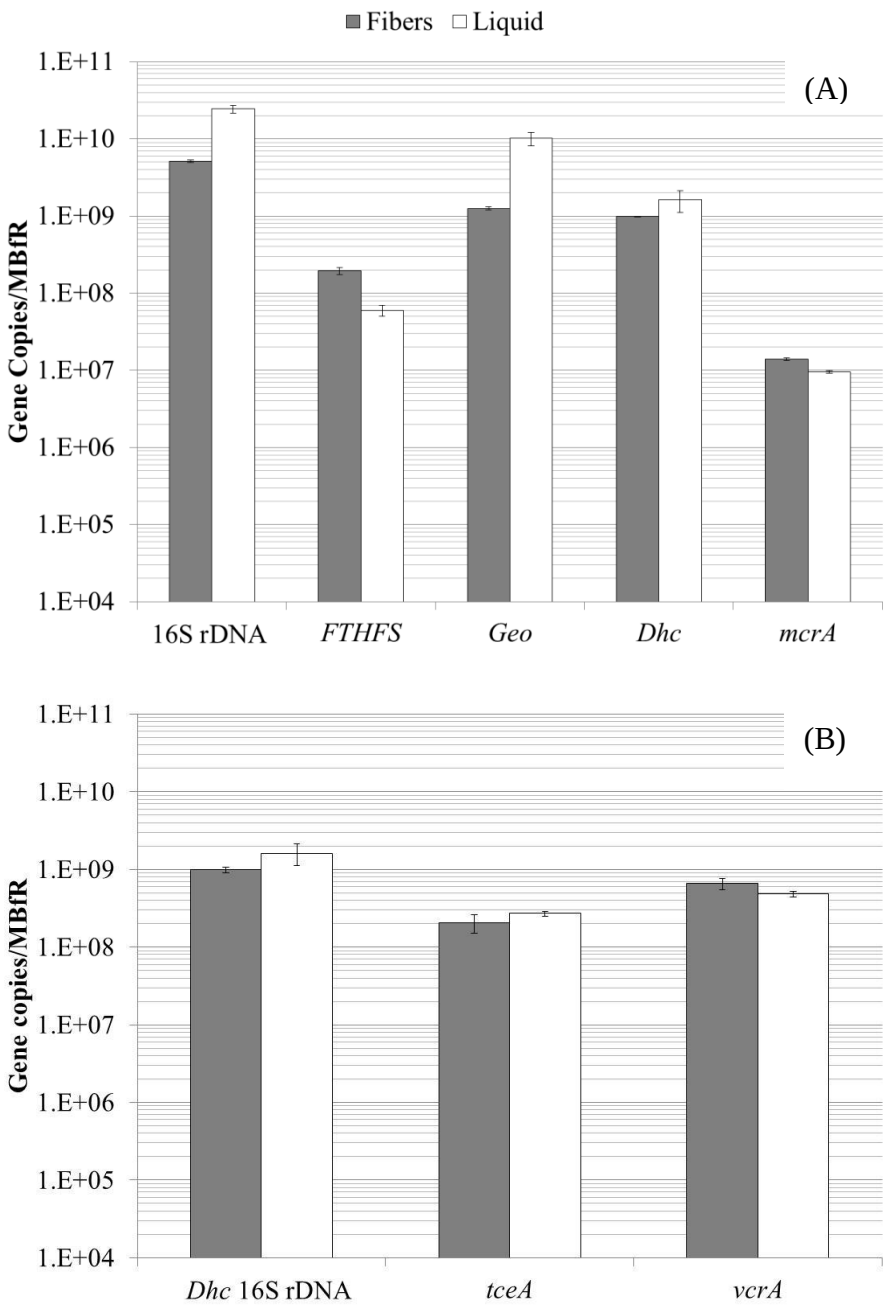
Considering that homoacetogenesis consumed most of the available 7.5 mM bicarbonate, ~2.7 e^- eq./m^2-d was channeled to acetate (8 me^- eq./mole of

acetate and using the fiber surface area of 0.0112 m^2) compared to a maximum ~0.16 e^- eq./m^2-d to TCE reduction (Table 1 and using 2, 4, and 6 e^- eq./mole for reduction to *cis*-DCE, VC, and ethene, respectively). The effluent concentration of acetate was even greater. Clearly, homoacetogenesis exceeded the required minimum acetate supply necessary as a C source for *Dehalococcoides*. Using hollow-fiber membranes with lower rates of H_2 diffusion or supplying gas mixtures containing lower H_2 concentrations, it may be possible to control the available H_2 and, in turn, methanogenesis and homoacetogenesis; this will require further investigation.

All MBfR studies in Table 1 achieved significant TCE reductive dechlorination and similar removal rates, even though differences exist between our study and the previous MBfR studies. While the inoculum here was a mixed consortium specifically enriched for reduction of TCE to ethene, Chung et al. (Chung et al., 2008a,b) used an inoculum from a pilot-MBfR reducing nitrate and perchlorate. Furthermore, Chung et al. (2008a,b) added TCE to the influent only after denitrification was stable. Over 90% conversion to ethene was reached in Chung et al. (2008a) after an additional 120 days of operation and in Chung et al. (2008b) after 60 days; these lengths of time to reach ethene production were significantly longer than the ones reported in this study. In contrast to these previous studies, the MBfRs in our study were first operated as batch reactors until ethene was the dominant end product, and we added lactate during the startup phase to aid in initial reductive dechlorination. Last, bicarbonate was not added to the influent of the previous studies, and this should have minimized

competition for H_2 from methanogens and homoacetogens. No methanogens were detected in the previous studies (Zhung et al., 2010), but homoacetogens were found in a clone library of Chung et al. (2008a) and in the pyrosequencing performed by Zhang et al. (2010), although in small relative abundance. Chung et al. (2008a) hypothesized that acetate was produced in autotrophic reactions involving H_2 and CO_2 (which had partitioned from the atmosphere) or via soluble microbial products.

Dominant bacteria and presence of methanogens. In order to quantify the abundance of microorganisms involved in the key processes, we used qPCR to target bacteria, *Dehalococcoides*, *Geobacteraceae*, homoacetogens, and methanogens; the numbers of each in the entire MBfR are presented in Figure 6a. The breakdown for the *tceA* and *vcrA* reductases is given in Figure 6b; *bvcA* was not detected. The high copy numbers in the liquid (Figure 6a,b) may be a result of the long HRT, which did not wash out suspended bacteria. Gene copies for homoacetogens were higher in the biofilm than in suspension, while the opposite was true for the dechlorinators (Figure 6a,b); since dechlorinators are good scavengers of H_2 (Smatlak et al, 1996), they may have benefited from the low concentrations in the liquid relative to that expected in the biofilm. The concentration of *Dehalococcoides* on the fibers was $1.76 \pm 0.07 \times 10^7$ gene copies/cm^2, a value comparable to the 1×10^7 gene copies/cm^2 in Chung et al. (2008a). The sum of *tceA* and *vcrA* gene copies was in the same order of magnitude, but lower than that for *Dehalococcoides*. However, a comparison of *Dehalococcoides* to total bacteria indicates a higher relative abundance in the


Fibers
Liquid
(A)
Gene Copies/MBfR
1.E+11
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
16S rDNA
FTHFS
Geo
Dhc
mcrA
(B)
Gene copies/MBfR
1.E+11
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
Dhc 16S rDNA
tceA
vcrA

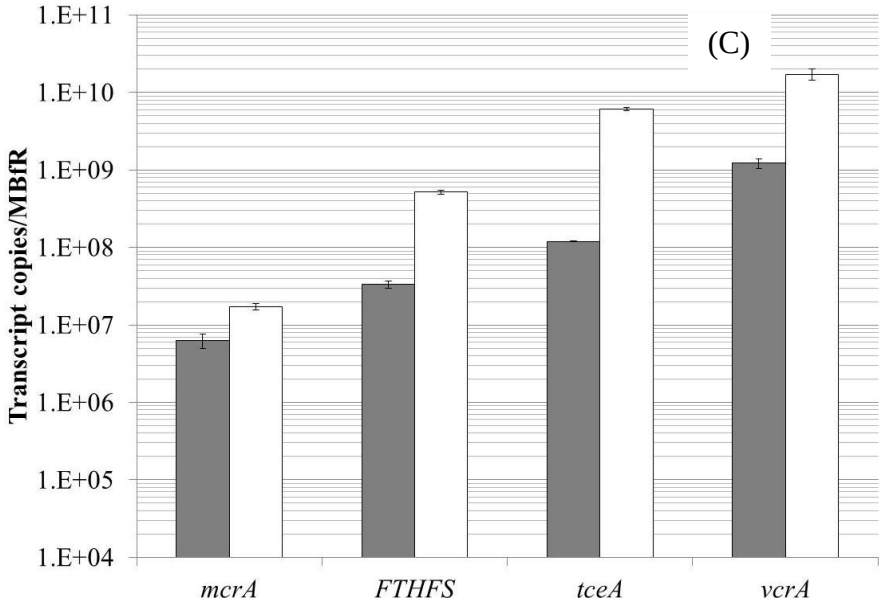
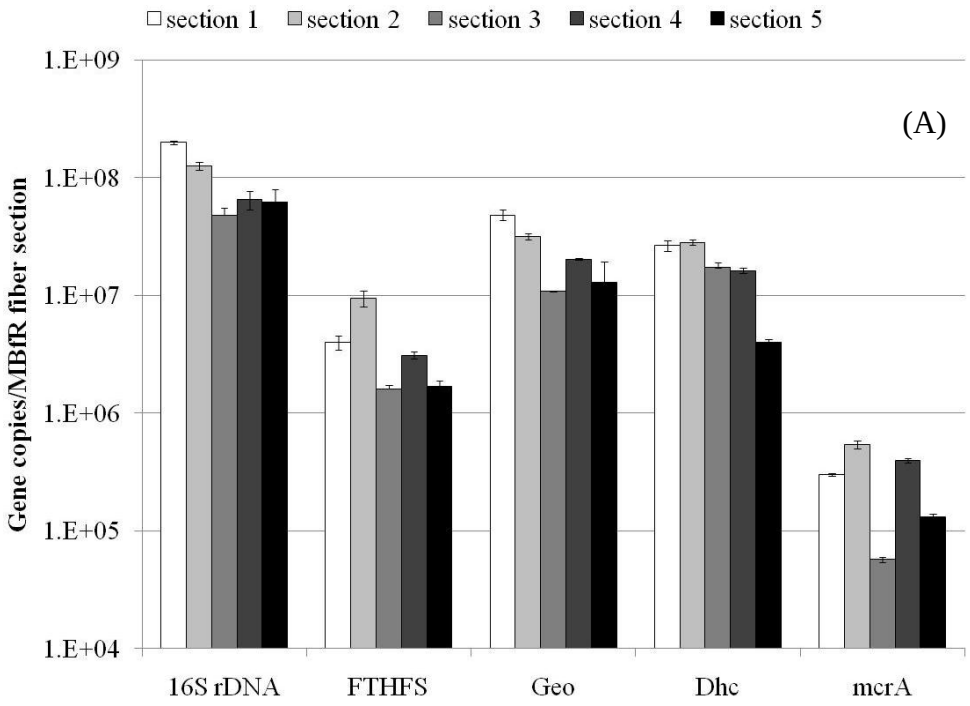


Figure 5.6. Gene and transcript copies for the liquid and fibers at the end of operation of MBfR 4. (A) Genes of total bacterial 16S rRNA gene, FTHFS (homoacetogens), Geobacteracea, Dehalococcoides, and mcrA (methanogens). (B) Genes of total Dehalococcoides and breakdown for the tceA and vcrA reductases. (C) Transcripts of mcrA (methanogens), FTHFS (homoacetogens), and tceA and vcrA (Dehalococcoides).

biofilm compared to the liquid. The same trend is apparent for homoacetogens, while the relative abundance of *Geobacter* was similar in the biofilm and liquid. Transcript copies also were assessed to address directly whether homoacetogens and *Dehalococcoides* were active throughout the reactor. In all cases, overall transcript copies were larger in the liquid than in the biofilm (Figure 6c), but these values should not be compared to each other quantitatively, since the extraction efficiencies were not necessarily the same. Transcript and gene copies along the

fiber lengths, shown in Figure 7, suggest non-homogeneous abundance; the primary significance is that homoacetogens and *Dehalococcoides* were present and active over all of the fibers.



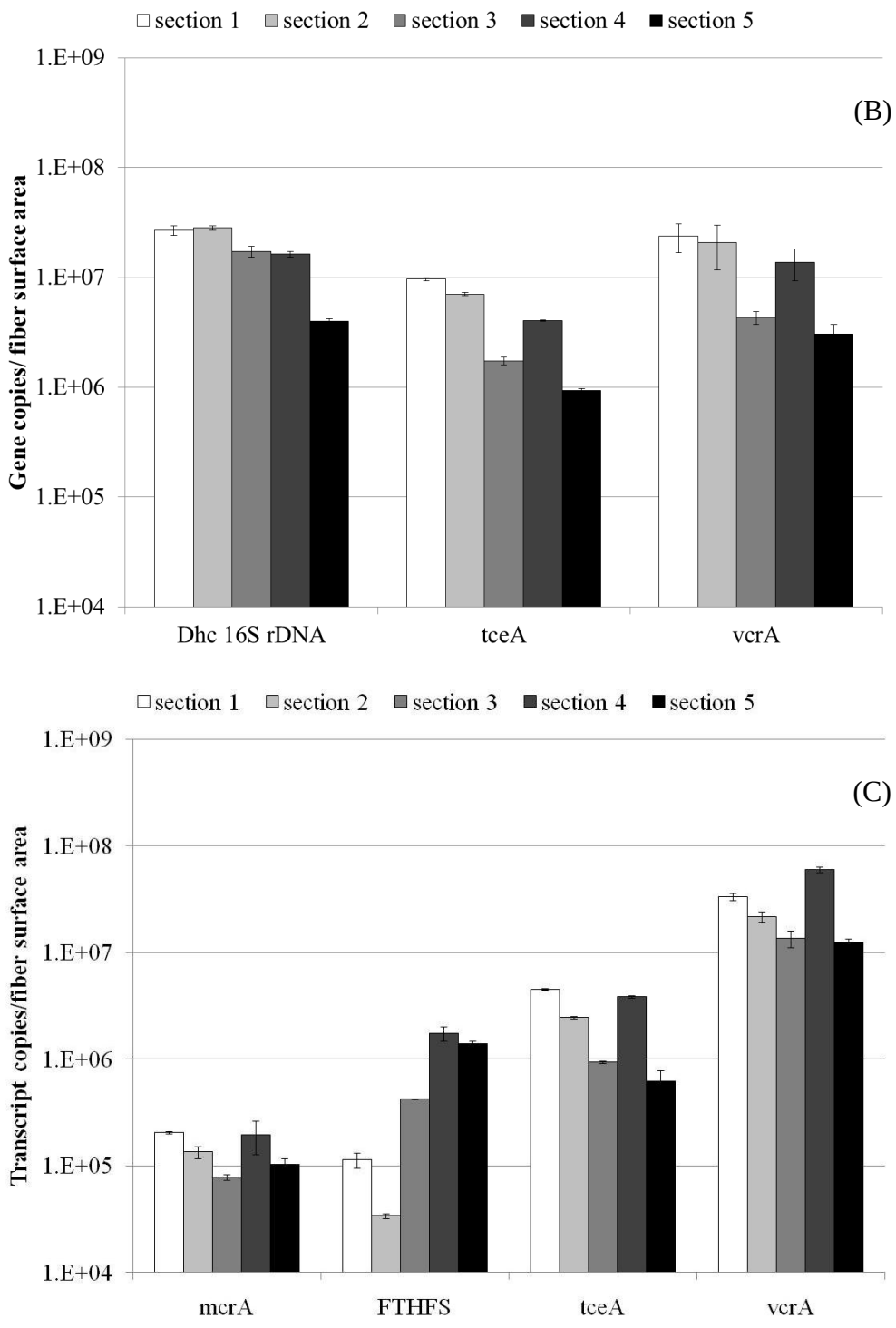


Figure 5.7. Gene and transcript copies on MBfR 4 fibers divided into five sections.

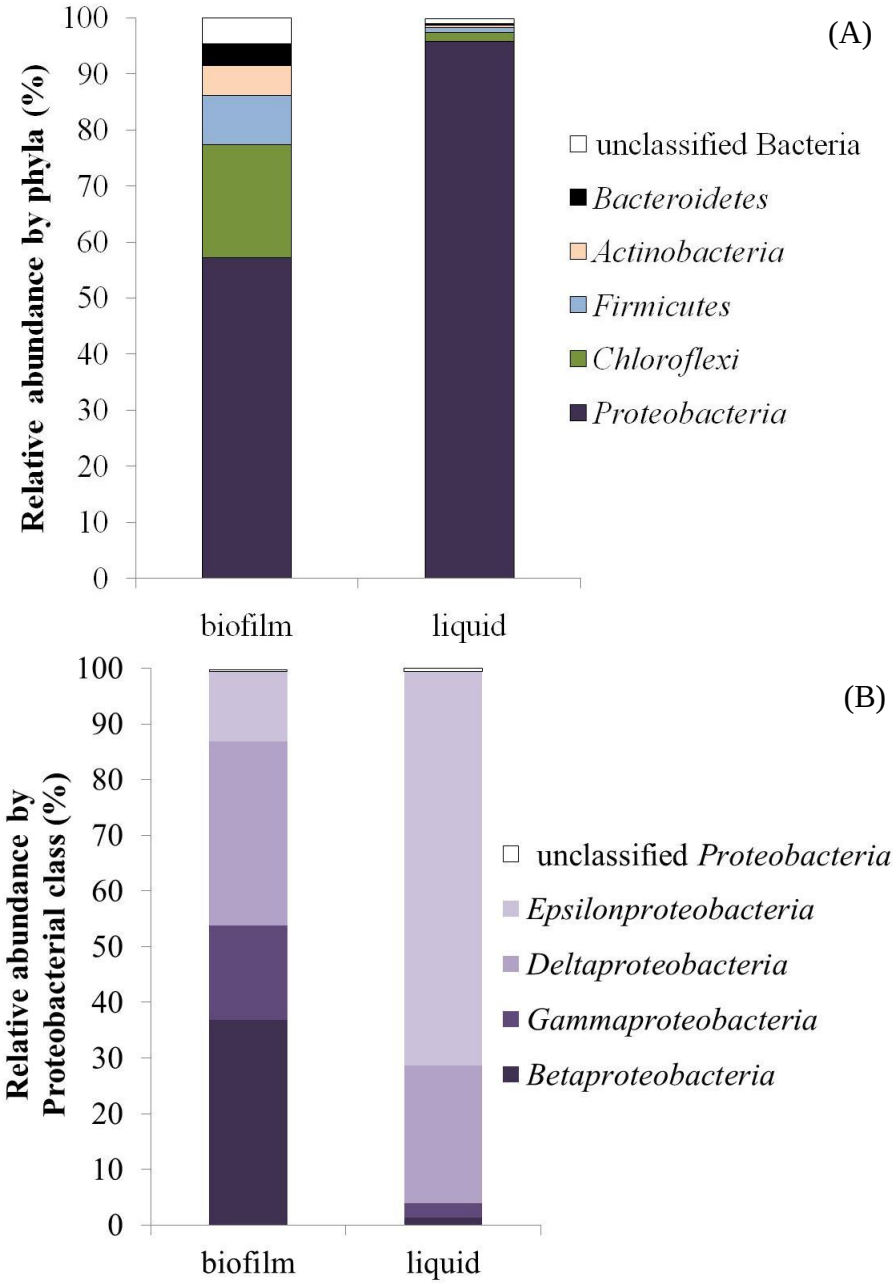
Methanogens were present and active on the fibers and in the liquid, since the *mcrA* qPCR gene and transcript copies were detected (Figure 5A and C). However, the numbers were orders of magnitude smaller than for other activities assayed by qPCR. Methanogens were intentionally suppressed only during startup, but remained at a low level after more than 30 days (~30 HRTs) of continuous flow operation. Methanogenesis always was active when H_2 was added directly to DehaloR^2 grown in batch serum bottles (data not shown), indicating that the MBfR inoculum contained methanogens. Acetoclastic methanogens were not detected.

In the previous two studies of the MBfR for TCE reduction (Chung et al., 2008a,b), methanogens were not detected (Zhang et al., 2010). The lack of methanogens was logical in that case, since no bicarbonate was present in the influent, except that from atmospheric CO_2 or from mineralization of autotrophic biomass. In addition, denitrification or denitrification and sulfate reduction were the major electron-acceptor reactions and competed strongly with methanogenesis. In our study, although bicarbonate was provided in the influent medium at a concentration of 7.5 mM, methanogens were out-competed by homoacetogens during continuous flow. We interpret that, since the MBfR was bicarbonate-limited in continuous flow (i.e., homoacetogens consumed all of the inorganic C, since the acetate concentration exceeded that available based on the stoichiometry of equation (3)), methanogens were less successful at competing for the small bicarbonate loading against the organsims already active from startup.

Bacterial community composition and diversity. A comparison of the relative abundance of the bacterial community in the biofilm and in the liquid is shown in Figure 7. The Chao1 estimates of OTUs were between 670-870 for the biofilm compared to 430-570 for the liquid, and the Shannon diversity Indices were between 5.0-5.2 and 4.0-4.1, respectively. Both statistical parameters reinforce greater diversity in the biofilm.

The biofilm bacterial community was dominated by ~20% *Dehalococcoides*, which is in the phylum *Chloroflexi*, and ~18% *Geobacter* in the *Deltaproteobacterial* class. *Geobacter lovleyi* is known to dechlorinate TCE to *cis*-DCE with acetate as the carbon source and either acetate or H_2 as the electron donor (Sung et al., 2006). Also significant was the homoacetogen *Acetobacterium*, a *Firmicute* representing ~8% of the community. The phyla *Bacteroidetes* and *Actinobacteria*, both of which can metabolize complex organic molecules (e.g. decaying biomass) through fermentation, in part to acetate (Madigan, 2009), together accounted for ~10%. The remaining fractions of organisms (~38%) were in the *Beta-*, *Gamma-*, and *Epsilon- Proteobacterial* classes, but we choose not to speculate on the metabolism of these organisms.

The community suspended in the liquid was less diverse and dominated by the *Proteobacterial* phylum (~96%), of which ~25% belonged to the *Deltaproteobacteria* class, mostly *Geobacter* sp., and the remainder were *Wolinella* sp. of the *Epsilonproteobacteria* class. Based on the literature, we have no reason to believe that *Wolinella is a homoacetogen* or dechlorinator. The long hydraulic retention time (HRT) of one day allowed a unique suspended


(A)
Relative abundance by phyla (%)
100
90
80
70
60
50
40
30
20
10
0
biofilm
liquid
unclassified Bacteria
Bacteroidetes
Actinobacteria
Firmicutes
Chloroflexi
Proteobacteria
(B)
Relative abundance by Proteobacterial class (%)
100
90
80
70
60
50
40
30
20
10
0
biofilm
liquid
unclassified Proteobacteria
Epsilonproteobacteria
Deltaproteobacteria
Gammaproteobacteria
Betaproteobacteria

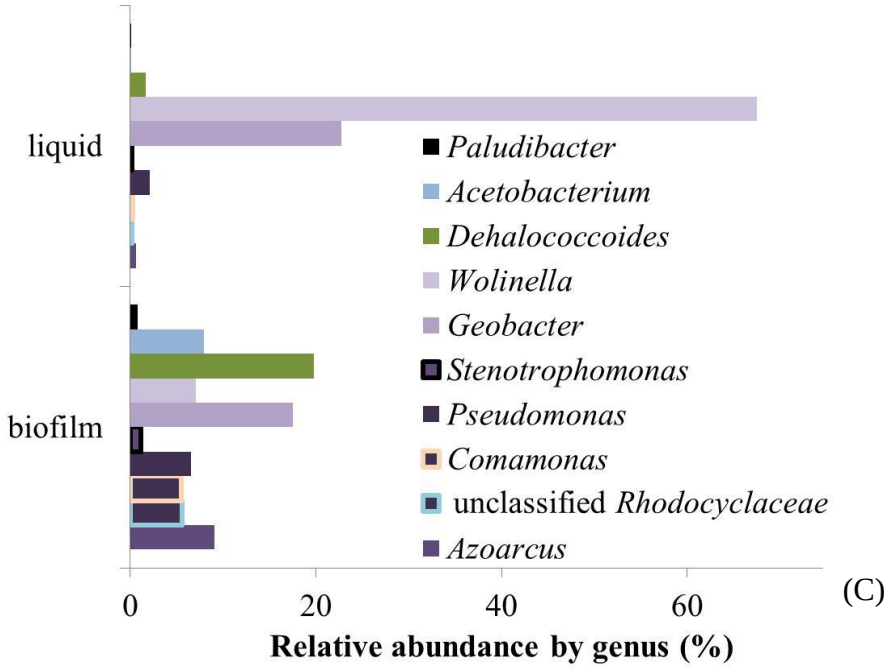


Figure 5.8. MBfR 4 relative abundance of cells by (A) phyla, (B) Proteobacterial class, and (C) genera using pyrosequencing targeting the combined V2 and V3 regions of the 16S rRNA gene of bacteria. Sequence tag copies were converted to cells using 16S rRNA copy numbers per cell from the rrnDB database, http://rrndb.mmg.msu.edu/search.php#result based on Klappenbach et al. (2001) and Lee et al. (2009). Details are in Table 1.

community to develop. A shorter HRT would have washed out suspended microorganisms, probably causing the suspended community to better reflect that of the biofilm. In summary, the same trends in relative abundance were observed from the pyrosequencing results as from the qPCR results: greater relative abundance of *Dehalococcoides* and homoacetogens in the biofilm than in the liquid, and similar abundance of *Geobacter*.

SEM images of the fibers, shown in Figure 8, reveal varying degrees of microbial coverage and a diverse community. The wide-view in panel (A) makes it evident that biomass was not distributed uniformly along the fiber, confirming the qPCR results in Figure 5. A representative region of high accumulation is seen in the patch of biofilm in panel (B) with a closer view in panel (C). Representative regions with low accumulation, where the microorganisms appear to have attached directly to the fibers, are in panels (D)-(F).

Rod-shaped microorganisms of various sizes could belong to the *Firmicute* or *Proteobacterial* phylum, with the smaller rods of ~1 μm length and 0.5 μm diameter possibly matching the morphology of the genus *Geobacter* (Sung et al, 2006a). EPS-like material was observed in the biofilms (panel (C)). Thin filamentous organisms with diameter ~0.25 μm and at least 3 μm in length were seen throughout (panels C and D), in addition to abundant branching, rod-shaped organisms (panel E). A cocccoid, disk-like morphology of diameter 0.5-1 μm, resembling that previously described for *Dehalococcoides* (He et al, 2003; Sung et al., 2006b), is apparent in the mixed communities of panels (C)-(E) and dominates in panel (F).

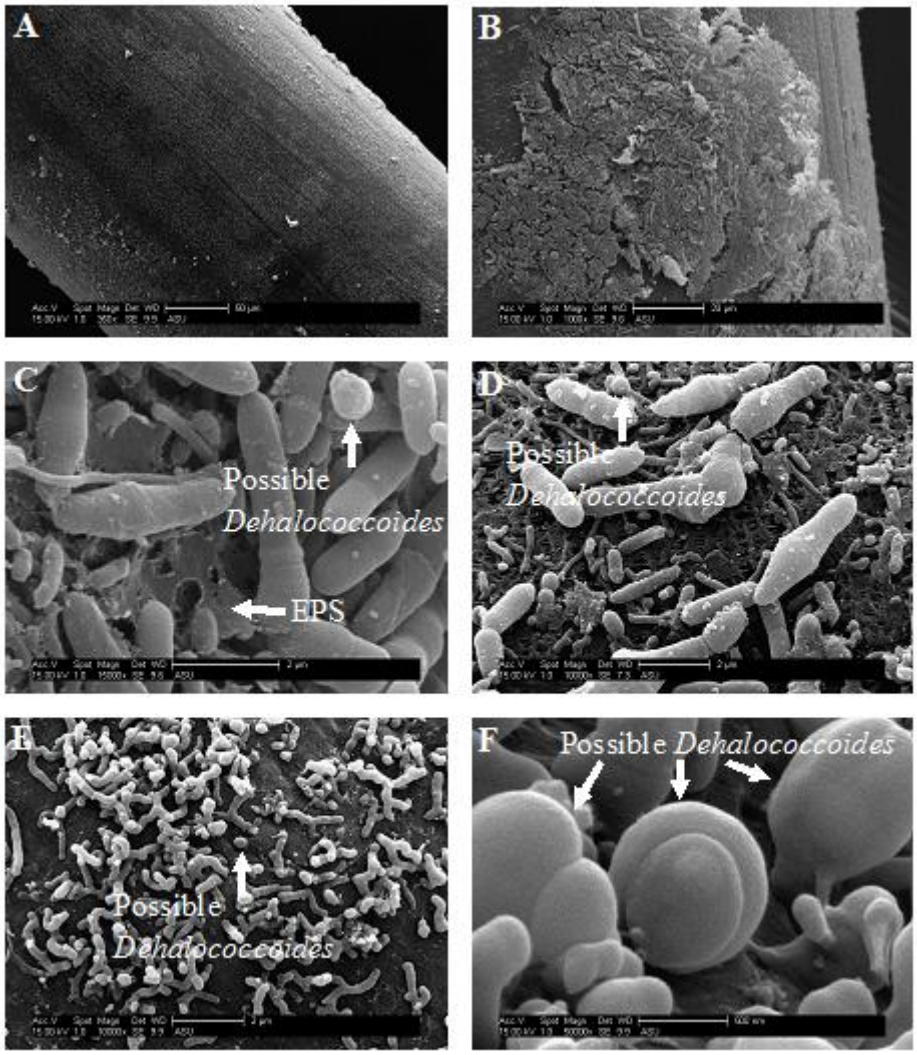


Figure 5.9. The diverse microbial community on the fibers of MBfR 4 imaged with SEM. Panel (A) shows varying degrees of microbial coverage and accumulation. Panel (B) is a patch of biofilm and (C) is a closer view of that biofilm; Panels (D)-(F) display regions of lower accumulation.

Figure 10, similar to that in Ma et al. (2006) synthesizes the microbial processes relating to reductive dechlorination in the H_2-based MBfR. Homoacetogenesis and decaying biomass/EPS were major contributors to acetate

production; the acetate was used as a carbon source and electron donor by the dechlorinators, and the bacteria involved in acetate production may have also provided the dechlorinators valuable growth factors. Methanogens were present and active, but were not significant compared to the other microorganisms. *Proteobacterial* organisms other than *Geobacteraceae,* found with the pyrosequencing analysis, are not included in this schematic as their functions are only speculative.

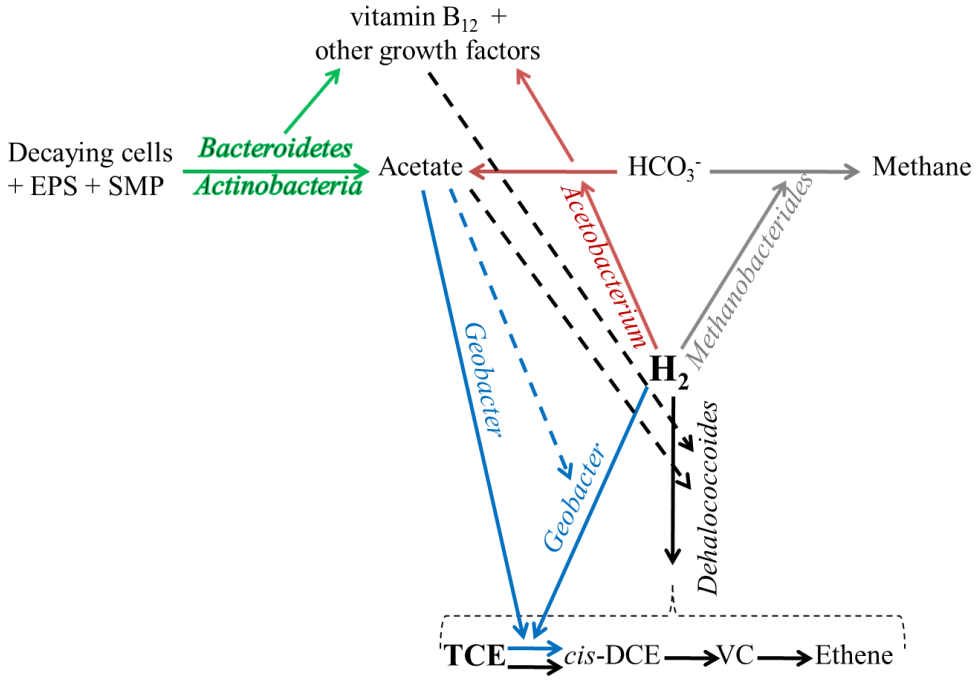


Figure 5.10. Suggested major microbial processes in the MBfR carrying out reductive dechlorination of TCE. Indicated microbial groups are based on the molecular analyses. Dominant flows of electrons are solid arrows. Carbon sources and other growth factors are dashed arrows. *Proteobacteria* other than *Geobacter* are not included, since their metabolism in this MBfR was not determined.

Conclusions

This study supports our hypothesis that, even with bicarbonate present in the medium, direct addition of H_2 in the MBfR allowed for TCE reduction as long as the microbial community was managed to control the pH and, by extension, methanogenesis and homoacetogenesis. First, after inoculation, the only electron donor for the microbial biomass was the H_2 provided through the gas-transfer fibers. Second, the qPCR and pyrosequencing results demonstrated that a biofilm community developed on the fibers and that it contained the microorganisms key to a dechlorinating microbial community, i.e.. *Dehalococcoides*, *Geobacter*, and homoacetogens. Third, the SEM images confirmed the presence of a mixed microbial community on the fibers. In order for dechlorination to proceed to ethene reliably, it was necessary to create H_2 limitation during batch start-up. In this study, methanogens presumably were minimized during continuous flow operation by a limitation in bicarbonate resulting from strong homoacetogenic activity. Homoacetogenesis was limited by the influent bicarbonate concentration, and pH was controlled with a phosphate buffer. A strategy to minimize homoacetogenesis in practice would involve creating a H_2-limitation by using a different fiber type that allows for better control of the H_2 pressure.

Chapter 6

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

My research expands the understanding of dechlorinating microbial communities that has been elucidated over the past two decades, and it provides new insights on how to manage these communities in engineered systems.

In the first stage of my research, I characterized a new anaerobic dechlorinating community developed in our laboratory, named DehaloR^2, in terms of chlorinated ethene turnover rates and assessed its microbial community composition (Chapter 1). TCE-to-ethene reductive dechlorination rates in batch serum bottles compared favorably to other enrichment cultures described in the literature, identifying DehaloR^2 as a promising consortium for use in bioremediation of chlorinated ethene-impacted environments. I used a clone library, quantitative PCR, and pyrosequencing to identify the dominant microbial community composition in DehaloR^2 as *Dehalococcoides*, *Geobacter*, *Acetobacterium*, and various other bacteria from the *Firmicutes* phylum. I also determined that the only active methanogen was from the hydrogenotrophic order *Methanobacteriales*.

Armed with my understanding of DehaloR^2, I carried out an experiment to correlate performance and community structure for the trichloroethene (TCE)-dechlorinating microbial consortia. I used an electron-balance analysis to predict the community structure based on the measured concentrations of products and constant net yields for each microorganism. Using fill-and-draw reactors inoculated with DehaloR^2, I demonstrated, for the first time, a direct correlation

between microbial community function and structure as the TCE-pulsing rate was increased. The electron-balance analysis predicted the community structure based on measured concentrations of products and constant net yields for each microorganism. The predictions corresponded to trends in the community structure based on pyrosequencing and qPCR up to the highest TCE pulsing rate, where deviations to the trend resulted from stress by the chlorinated ethenes. The approach I used in this study has potential for application in predicting and monitoring *in-situ* and *ex-situ* bioremediation.

I then developed a SPME-GC-FID method (Chapter 4) for simultaneous detection of chlorinated ethenes and ethene at or below the Environmental Protection Agency maximum contaminant levels for groundwater. This method is ideal for monitoring biological reductive dechlorination in groundwater where ethene is the ultimate end product. The major advantage of this method is that it uses a small sample volume of 1 mL, making it ideally suited for bench-scale feasibility studies, such as the MBfR, or for flow-through columns performed during the remedial design stage of groundwater cleanup projects.

Last, I developed a reliable start-up and operation strategy for TCE reductive dechlorination in the MBfR (Chapter 5). The study sheds light into how H_2-fed biofilms, when operated to manage methanogenic and homoacetogenic activity, can be used for *ex-situ* bioremediation of chlorinated ethenes. Successful operation relied on controlling the pH-increase effects of methanogenesis and homoacetogenesis, along with creating H_2 limitation during start-up to allow dechlorinators to compete against other microorgansims. Methanogens were

additionally minimized during continuous flow operation by a limitation in bicarbonate resulting from strong homoacetogenic activity.

I now suggest several additional steps that would be required before implementing the MBfR technology for TCE reductive dechlorination at the pilot and full-scale levels. These studies build off of my research presented in Chapter 5. I also provide details on a batch reactor study using carbon monoxide (CO) as a strategy for methanogen inhibition that may be valuable for TCE reduction in the MBfR. This strategy was initially proposed by Prathap Parameswaran (personal communication) and in this chapter I provide background on this topic and suggest an experimental plan to assess its feasibility.

As discussed in Chapters 1 and 5, pH is a critical factor to control in reductive dechlorination. In these proposed experiments, I suggest holding the pH constant using sufficient phosphate buffer, in addition to bicarbonate buffer, in order to eliminate pH as a cause of reactor failure or experimental uncertainty. The order in which I present the MBfR studies is significant, because each study builds off of the previous studies.

Study 1: Decreasing the hydraulic retention time in the MBfR reducing TCE.

The MBfR characterized in chapter 5, having a hydraulic retention time (HRT) of 1 day, was dominated by the *Proteobacterial* phylum (95%), according to pyrosequencing results. In the liquid, 65% of the bacterial community was the genus *Wolinela*, an *Epsilonproteobacteria*, and 23% was the *Deltaproteobacterial* dechlorinator *Geobacter*. Around 56.5% of the biofilm microbial community was the *Betaproteobacteria Comamonas* (5.5%) and *Azoarcus* (9.0%), the

Gammaproteobacteria Pseudomonas (17.5%), and *Wolinella* (7.1%), and the *Deltaproteobacteria Geobacter* (17.5%). With regard to other members in the microbial community, *Dehalococcoides* accounted for ~2% of the liquid and ~20% of the biofilm and *Acetobacterium* was 0% and 8%, respectively. Thus, the relative abundance of the dominant members in the liquid microbial community did not reflect that in the biofilm. According to the qPCR results, the dechlorinators *Dehalococcoides* and *Geobacter* were in greater absolute abundance in the liquid than in the biofilm. The reactor would be more efficient if these organisms were in greater abundance in the biofilm. These two factors suggest disadvantages of operating the MBfR with a long HRT.

If the HRT is significantly decreased, I hypothesize the following will occur due to wash-out: (1) The liquid microbial community will be minimized. (2) The liquid microbial community will closely resemble that in the biofilm, since biofilm detachment will be the primary source for suspended microorganisms.

I propose the following to test these hypotheses. I will first devise an influent feed set-up uses a peristaltic pump rather than a syringe pump, so that it can reach much higher flow rates. Additionally, I will devise a reactor sampling module that allows for fiber sampling during reactor operation. Steady-state conditions for this study and the others that I propose will be defined as chemical stability of the reduced products and pH during continuous flow.

The startup will resemble that presented in Chapter 5, where the HRT was 24 hours. As in Chapter 5, I will regularly analyze for chlorinated ethenes,

ethene, acetate and other VFAs, and pH. Since the SPME-GC-FID method described in Chapter 4 does not allow for analysis of methanogens, I would instead analyze for bicarbonate in the influent and effluent of the reactor, and back-calculate potential production of methane. After maintaining steady-state conditions for around one month to provide enough time for a mature biofilm to develop, the HRT will be decreased stepwise to 12, 6, 3 , and 1 hour, using the same TCE loading for all HRTs. The TCE loading and H_2 pressure will be close to that from MBfR 1 in Chapter 5, 1.1. mmoles/m^2-d and 2 psig (0.17 atm). At each condition, after reaching a steady-state, I will maintain that condition for two weeks to allow sufficient time to alter the bacterial community.

At the end of each operating condition, DNA will be extracted from biofilm and liquid samples. qPCR and pyrosequencing will be carried out on all samples as described in Chapter 5.

Study 2: Maximum, sustained TCE loading in the MBfR.

The two MBfRs described in Chapter 5 had TCE removal fluxes of 1.1 and 2.2 mmoles/m^2-d, with 96% and 65% reduction to ethene, respectively (Chapter 5 Table 1). Both of these reactors were operated in continuous flow for around 30 days with at least 3 HRTs for the first loading and 10 HRTs for the second. Causes for incomplete dechlorination in this set-up could have been from reaching the enzymatic limit of the dechlorinators or from lacking one or more growth factors essential to the dechlorinators. A shortcoming of the study in Chapter 5 was that the reactors were operated for only one month. The objective of this proposed study are: (1) Assess the *Dehalococcoides* enzymatic upper limit

for reductive dechlorination in the MBfR biofilm. (2) Assess whether reductive dechlorination can be sustained in the MBfR for several months, or if periodic addition of fermentable substrates is required in order to provide additional growth factors.

To accomplish these goals I will proceed as follows. I will carry out the start-up as described in Chapter 5 with the modifications to the influent feed and medium described in Study 1. Once in continuous flow, I will transition the reactor to the TCE loading and HRT found to work well in Study 1. Chlorinated ethenes, ethene, acetate and other VFAs, and pH will be monitored as described in Chapter 5

In order to address the first objective, I will keep constant this HRT and H_2 pressure and will incrementally increase the TCE loading by increasing the TCE concentration, maintaining the reactor at each TCE loading for two weeks after reaching each steady-state. This two week period should be sufficiently long to allow for changes to the biofilm community. At some loading, TCE reduction to ethene will deteriorate, by stalling at intermediate products, and this will signify that the maximum TCE loading has been surpassed. I will return the MBfR to the highest loading that allowed for nearly complete reduction to ethene.

To accomplish the second objective, I will maintain the MBfR at the maximum TCE loading (above) for 6 months, a sufficiently long time to assess potential nutrient limitations that may arise during sustained reduction without addition of fermentable substrates. If at this maximum TCE loading, reductive

dechlorination stalls at intermediate products, potentially indicating a nutrient limitation, I will assess whether addition of lactate allows for restored activity.

I will analyze the biofilm and liquid microbial communities at various steady-state conditions and wil carry out molecular analyses as described in Chapter 5.

Study 3: Controlling H_2 delivery to promote efficient electron utilization and decrease acetate production in the MBfR reducing TCE.

In the MBfR in Chapter 5, homoacetogenesis was strongly favored over methanogenesis by suppressing methanogens during start-up. Since homoacetogenic activity was not suppressed, these organisms were able to out-compete methanogens for bicarbonate during continuous flow operation. This strategy relied on 100% utilization of the bicarbonate, which introduces two significant disadvantages. First, since bicarbonate is the primary buffer in groundwater, unless the pH increase due to H^+ and acetate production from homoacetogenesis exactly equals the H^+ production from reductive dechlorination, this strategy could be detrimental to the pH and in turn to reductive dechlorination. Addition of a phosphate buffer is cost-prohibitive for scale-up. Second, the acetate and biomass produced in homoacetogenesis contribute to the biochemical oxygen demand (BOD) that must be removed with downstream treatment.

On the other hand, some amount of homoacetogenesis is valuable for reductive dechlorination as it provides acetate and other co-factors, described in Chapter 5. In addition, no reports in the literature have shown that

Dehalococcoides are able to form a biofilm, so continued homoacetogenesis may be essential for sustaining a biofilm matrix for *Dehalocococides*. Thus, it would be beneficial to allow for just enough homoacetogeneis to benefit sustained reductive dechlorination.

An optimal strategy to control hydrogenotrophic methanogens and homoacetogens may be to rely on a H_2 limitation; this is the usual strategy for MBfR operation with other contaminants (Rittmann 2007; Ziv-El and Rittmann, 2009, 2010). Creating a H_2 limitation is also the primary strategy employed for *in-situ* bioremediation, described in Chapter 1. With the Mitsubishi Rayon fibers used in Chapter 5, I could not adequately control the H_2 pressure to create a H_2 limitation.

The objectives of this study are to operate the MBfR with a H_2 limitation in order to: (1) Manage competition from homoacetogens and methanogens. (2) Determine the lower limit for homoacetogenic activity that allows for sustained reductive dechlorination to ethene.

I propose two separate strategies to minimize H_2 availability and accomplish these objectives. The first strategy is to use fibers with a lower diffusion rate than that of the Mitsubishi Rayon membranes. Polypropylene fibers are now used in full-scale MBfRs, and their H_2 diffusion coefficient is about 10 times smaller than for the Mitsubishi-Rayon fibers (Tang et al., submitted). The second strategy is to decrease the partial pressure of H_2 in the fibers while maintaining a realistic gas pressure by using a gas mix of N_2 and H_2. If methanogenesis proves to be problematic in initial tests of these strategies, then it

would be valuable to assess other strategies to minimize or inhibit methanogens, including the idea proposed in Study 7.

I will carry out the startup according to Chapter 5, but with a 5 fold higher H_2 pressure to accommodate for the lower diffusion rate in the new fiber type. The HRT will be that determined from Study 1 and the maximum concentration from Study 2. To investigate both objectives with the first strategy, I will incrementally decrease the H_2 pressure and chemically assess whether an electron donor limitation can be reached to minimize acetate production, but still allow for sustained reduction of TCE to ethene. To assess the second strategy, I will incrementally change the ratio of H_2 to N_2 from 100:0 to 5:95, and possibly an even lower ratio, while keeping the total pressure in the fibers constant. As in the other studies, biofilm and liquid samples will be taken at various steady-states in order to carry out molecular analyses described in Chapter 5.

Study 4: Co-reduction of sulfate, bicarbonate, and TCE in the MBfR.

Sulfate is a common constituent in groundwater and can interfere with reductive dechlorination in several ways described in Chapter 1. Most notably, sulfate reducing bacteria (SRB) can compete with dechlorinators for H_2, acetate, and other growth factors. Additionally, incomplete sulfate reduction can produce sulfite, which is toxic to some dechlorinators (Hägblomm and Bossert, 2003). Sulfate reduction consumes alkalinity according to (Rittmann and McCarty, 2001; Tang et al., submitted):

$$SO_4^{2-} + 4.2H_2 + 0.015NO_3^- + 0.075CO_2 + 1.515H^+ = 0.5H_2S + 0.5HS^- + 4.17H_2O + 0.015C_5H_7O_2N \quad (1)$$

It is therefore of primary interest to assess the role of sulfate in reductive dechlorination in the MBfR.

The literature shows conflicting results about the necessity of removing sulfate in order to obtain complete reductive dechlorinaton (Hägblomm and Bossert 2003). Past MBfR research also shows conflicting results. In Chung et al. (2008a,b), TCE reduction to ethene was nearly complete in both reactors that had sulfate and nitrate in the influent medium; no sulfate reduction was observed in one MBfR and complete reduction occurred in the other MBfR. Zhang et al. (2010) hypothesized that the reason for complete sulfate reduction in Chung et al. (2008b) was the presence of the co-reduction products from chloroform and trichloroethane. They suggest that since sulfate-reducing bacteria can respire these chlorinated compounds, this provided additional selective pressure for sulfate reduction. I note that a second distinguishing feature between these two studies was the ratio of nitrate to sulfate loading. In Chung et al. (2008a), the ratio was 0.14:0.074 = 1.9 (e^- eq/m^2-day for both) and it was 0.33:1.18 = 0.28 in Chung et al. (2008b). Thus, sulfate reduction observed in the latter study may also have been favored by the lower nitrate loading relative to sulfate.

The objective of this study would be to assess whether complete reductive dechlorination of TCE is possible in the presence of sulfate, or if sulfate must be fully reduced. To accomplish this objective, I would do the following. The start-up would resemble that in Chapter 5, but with the modifications to the feed and medium from Study 1 and sulfate would be present in the medium. If the fiber used in Study 3 was successful, then it should be used here. I would transition the

reactor to use the HRT and the corresponding maximum TCE loading from Study 2. I would then incrementally vary the H_2 pressure or the ratio of N_2 to H_2, whichever worked best in Study 3, to create a H_2 limitation, maintaining a constant concentration of sulfate, and assess any changes to the reactor effluent. I would operate a second reactor where the sulfate loading would be changed and the H_2 availability would be held constant, based on the results from Study 3. The liquid and biofilm microbial community would be assessed at various steady-state conditions, using the molecular techniques described in Chapter 5.

Study 5: Spatial distribution of Dehalococcoides in MBfR biofilms.

The spatial distribution of *Dehalococcoides* in biofilms has been studied sparingly, as described in the Chapter 1. In Chapter 5, I used scanning electron microscopy (SEM) to look at the MBfR biofilm. In any of the above-proposed studies, it would be of great value to use Laser Scanning Confocal Microscopy (LSCM or CLSM) to: (1) Measure the biofilm thickness along the length of the fibers using a LIVE/DEAD stain. (2) Look at the spatial distribution of *Dehalococcoides*, *Acetobacterium*, and *Proteobacteria* in the biofilm by combining with Fluorescence *In-Situ* Hybridization (FISH).

Study 6: Comparison of Proteobacteria in the MBfR with unclear metabolic functions.

A surprising aspect of the liquid and biofilm microbial communities presented in Chapter 5 was the predominance of *Proteobacterial* organisms whose metabolic niche remains unclear. A hypothesis for the presence of *Wolinella* is that this organism was using H_2 as the electron donor, a polysulfide

as the electron acceptor, and acetate as the carbon source (Simon et al. 2006). Because *Pseudomonas* are either aerobic or denitrifiers, it is surprising that they were so abundant, considering that the influent media was anaerobic and did not contain nitrate. It is remotely possible that the MBfR had a constant, slow O_2 leak, but this seems unlikely as *Dehalococcoides* are irreversibly inhibited at very low O_2 concentrations, (Maymó-Gatell et al. 1995, Amos et. al. 2008). *Comamonas* and *Pseudomonas* have been shown to biodegrade polyurethane (Howard 2002), a component of the MBfR fibers; so, one possibility is that this compound was slowly leaching from the fibers and served as the electron donor and carbon source and O_2 as the electron acceptor. Similarly, *Azoarcus* use aromatic compounds as the electron donor and O_2 as the electron acceptor, and in this way may have also consumed polyurethane if O_2 were leaking into the MBfR.

In studies 1-4 above I suggest using pyrosequencing to assess the bacterial communities in the liquid and fiber biofilm. It is possible that the community in Chapter 5 was obscured by the relatively long HRT. If these organisms continue to show up in the studies that I propose here then it will be of value to determine their metabolism. How to progress should be determined based on a comparison of the communities from the various studies.

Study 7: Methanogen inhibition using CO. All currently identified organisms able to metabolize CO, termed carboxidotrophs, are facultative, and all use the carbon monoxide dehydrogenase (CODH) for CO uptake. The stoichiometry of this process is:

$$CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^- \quad (2)$$

Carboxidotrophs couple the electrons generated in the CO reaction to the reduction of the following: protons to form H_2, carbon dioxide (CO_2) to form acetate or methane (CH_4), or sulfate to form hydrogen sulfide (H_2S). An in-depth review on anaerobic carboxidotrophs can be found in Oelgeschläger and Rother (2008). Here I primarily focus on the impact of CO to the predominant organisms in DehaloR^2 grown with direct addition of H_2. As described in the previous chapters, the main organisms in DehaloR^2 are the homoacetogen *Acetobacterium*, the methanogen *Methanobacteriales*, and the dechlorinators *Dehalococcoides* and *Geobacter*.

Direct uptake of CO is widespread in homoacetogens, as this compound is part of their central metabolic pathway; CO oxidation through the CODH enzyme catalyzes the synthesis of Acetyl Coenzyme A (AcetylCoA), which is necessary for acetate and biomass production. This reaction occurs primarily through the following stoichiometry (Diekert and Thauer 1978):

$$4CO + 2H_2O \rightarrow CH_3COO^- + 2CO_2 + H^+ \quad (3)$$

Thus, these organisms should tolerate high headspace concentrations of CO. Following an adaption period, Kerby et al. (1983) demonstrated that *Acetobacterium* was able to grow on a headspace CO concentration of 100% and with a doubling time of 13 hours (Genthner and Bryant 1986).

In contrast, only select species within three methanogenic genera have been shown to be capable of growth on CO according to (O'Brien et al. 1984, Sipma et al. 2003, Rother and Metcalf 2004)

$$4CO + 2H_2O \rightarrow CH_4 + 3CO_2 \quad (4)$$

CO oxidation is not part of the typical metabolism of methanogenic species. The first CO oxidizer, *Methanothermobacter thermoautotrophicus,* is an obligate, hydrogenotrophic methanogen, but this organism is not of significant concern in our systems because it is thermophilic. The other two are facultative methanogens. *Methanosarcina barkeri* is a mesophilic mixotroph capable of acetotrophic, methylotrophic, and hydrogentrophic growth. O'Brien et al. (1984) showed that it grows on CO, but with a long doubling time of 65 hours, compared to growth on acetate (48 hours) or methanol (12 hours). Additionally, CO concentrations above 20% were inhibitory to its methanogenic pathway; at this concentration, the metabolism shifted to production of H_2 as the primary end product, still through the CODH enzyme. The third, *Methanosarcina acetivorans,* with a doubling time of ~24 hours when utilizing CO, is capable of acetotrophic and methylotrophic growth, but shifted to increased acetogenesis beginning at a CO concentration of ~40%, with sole production of acetate at a CO concentration of ~100% (Rother and Metcalf 2004). The slower growth and greater toxicity to CO of methanogens compared to homoacetogens was further supported by a study of Sipma et al. (2003), who reported that, in an anaerobic bioreactor grown with a headspace CO concentration of 90-100%, the overwhelming end product was acetate, with negligible methane. Only M*ethanobacteriales* has been thus far detected in DehaloR^2; to the best of my knowledge, this organism has not been studied in the literature.

Since toxicity of CO to dechlorinators has not been studied, the focus of this work is to assess this toxicity and determine whether methanogenesis can be

inhibited without halting dechlorination. A promising point for *Dehalococcoides* and is that their genomes includes the genes that encode for the CODH enzyme, but it is not known whether this enzyme can be expressed (Islam et al. 2010). The genome of *Geobacter sulfurreducens* also includes these genes (Methé et al. 2003), so it is possible that dechlorinating *Geobacter,* e.g. *G. lovleyi,* contain this gene as well.

The goal of this work would be to do a screening study to assess whether CO inhibits reductive dechlorination of TCE to ethene in DehaloR^2. The study variables are summarized in Table 1. All experiments would use 160-mL serum bottles with 100 mL of media, as described in Chapters 2 and 3, except for the following modifications. First, the buffer would be 10 mM phosphate and 7.5 mM bicarbonate, the combination that I have been using successfully in MBfR operation described in Chapter 5. Second, 10 mL H_2 (0.41 mmoles, 0.82 me- eq.) would be added directly instead of through fermentation of lactate and methanol. The initial experiment would involve duplicate bottles of the following concentrations of CO in the headspace, with a N_2 balance: 0%, 10%, 25%, 50%, and 100%.

Table 6.1

Experimental design of screening study to assess the impact of CO on 'Dehalococcoides.'

Duplicate Bottles	**a**	**b**	**C**	**d**	**e**
CO	0%	10%	25%	50%	100%
Electron Donor	10 mL H_2 = 0.41 mmoles = 0.82 me- eq.				
Electron Acceptor	35 µmoles TCE = 0.21 me- eq.				
Buffer	10 mM phosphate at pH = 7.3 and 2.5 mM bicarbonate				

Figure 1 shows the potential reactions in DehaloR^2. The dashed lines representing the methanogenic reactions that I hope to inhibit. Reductive dechlorination products and methane will be analyzed regularly with headspace samples using a GC-FID and acetate using HPLC, both protocol were described in Chapter 2. The next steps would be determined based on the outcome of this experiment. These could include: (1) Assessing lower CO concentrations, if 10% CO is toxic. (2) Acclimating the culture to increasing concentrations of CO. (3) Assessing whether *Dehalococcoides* can grow with CO, but without direct addition of H_2, as in Figure 2. (4) Assessing the growth of the key microorganisms using qPCR to track the 16S rRNA genes of *Dehalococcoides* and *Geobacteracaea,* as well as functional genes FTHFS of homoacetogens, *mcrA* of methanogens, and *bvcA*, *vcrA*, and *tceA* of *Dehalococcoides*. The change in the microbial community after several transfers could be assessed with pyrosequencing. (5) If CO is not able to inhibit the methanogens, then it may be of value to assess which groups are inhibited and which are not being inhibited,

following the qPCR protocols to track the methanogenic orders, as in Yu et al. (2005).

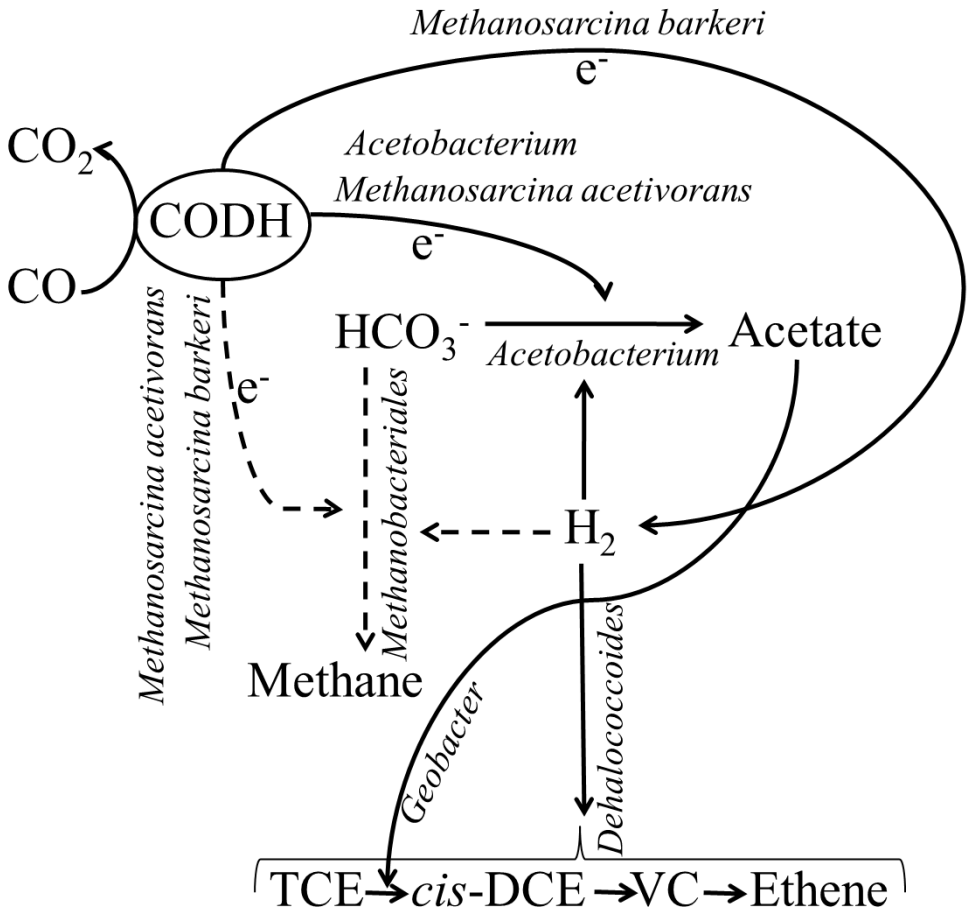


Figure 6.1. Schematic of possible microbial processes in DehaloR^2 grown with CO and H_2. Dashed lines represent the processes I hope to inhibit by addition of CO. Solid lines are reactions that I hope will not be significantly inhibited by CO since they either carry out the reductive dechlorination or provide growth factors to the dechlorinators.

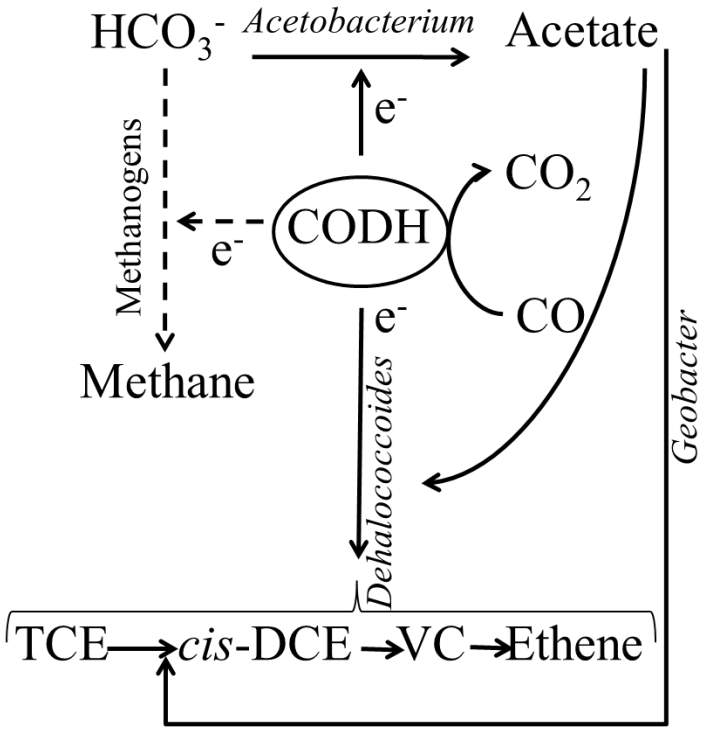


Figure 6.2. Schematic of potential microbial processes in DehaloR^2 grown with CO, but no H_2. Acetate is the C-source for *Dehalococcoides* and *Geobacter* and can also serve as the electron donor for *Geobacter*.

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