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SPATIAL AND TEMPORAL EVALUATION OF DECHLORINATING POPULATIONS DURING CRAB SHELL ENHANCED BIOREMEDIATION

A Dissertation in

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by

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ABSTRACT

Due to its dense, hydrophobic, and toxic nature, the restoration of trichloroethene (TCE) contaminated aquifers presents a significant challenge to both government and industry. Remediation of contaminated sites typically falls into two categories: the traditional pump and treat, which is costly and generally inefficient at removing dense non-aqueous phase liquids (DNAPLs) like TCE; and bioremediation with native or bioaugmented halorespiring organisms. Hydraulic fracturing ("fracking") of substrates and proppants into TCE contaminanted soils is a developing, but understudied, practice of stimulating *in situ* bioremediation. Laboratory studies combining chemical and molecular analyses to evaluate reductive dechlorination stimulated through the addition of a crab shell and sand mixture (substrate used in fracking processes) may lend insight into the assessment and monitoring of contaminated field locations.

This study evaluates the spatial and temporal distribution of dechlorinating populations during crab shell enhanced bioremediation. Laboratory tests were conducted using a commercially available mixed dechlorinating culture (Bio-Dechlor INOCULUM) containing *Dehalococcoides* species, capable of complete dechlorination of TCE to ethene, and crab shell (SC-20, SC-40, or SC-80) as a slowly fermentable substrate. Semi-continuous flow columns tests were conducted to evaluate the ability of different grades of commercially available crab shell to enhance degradation of chlorinated solvents at various concentrations. Continuous flow column tests were conducted to further assess the distribution of dechlorinating populations throughout the course of anaerobic reductive dechlorination.

In the semi-continuous flow column study, the SC-20 grade of crab shell provided the greatest variety of fermentation products, supported the greatest ethene production, and maintained a more stable pH than the other grades of crab shell for all test parameters. The less processed, less costly SC-20 has a higher proportion of protein which contributed to the increased variety of fermentation products, supplying an increased source of reducing equivalents and supporting greater conversion of TCE to ethene. A circum-neutral pH is also important for the efficient conversion of TCE to ethene, and the natural buffering capacity of the $CaCO_3$ in the SC-20 maintained pH in the columns within the optimal range for dechlorination. Due to the findings of this study, all subsequent tests were conducted using the SC-20 grade of crab shell.

In the continuous flow column tests, *Dehalococcoides* cell numbers were observed to increase from <1% of the total bacterial population in the influent end of the columns to 9-40% in the second half of the columns. The individual reductive dehalogenase (RDase) genes only accounted for a portion of the total *Dehalococcoides* 16S rRNA gene copies suggesting that there may be unidentified RDases in the BDI inoculum. T-RFLP fingerprinting analysis showed the greatest richness and diversity in communities associated with the solid-phase column materials in the beginning of the experiment (<~10 pore volumes) and in communities associated with pore water at the end of the experiment, suggesting Statistical analysis of the T-RFLP data indicated that neither the solid-phase nor the pore water sampling effectively captured all the dominant populations in the continuous flow columns. This has implications for field assessments of subsurface microbiology which are traditionally conducted through groundwater sampling.

Fluorescent in situ hybridization (FISH) processing of intact biofilm extracted from the columns showed archaeal cells in close proximity to *Dehalococcoides* cells, indicating that competition, if it exists, is not creating spatial stratification, and suggests the possibility of a synergistic relationship. The close proximity of potentially competing hydrogenotrophs also indicates that the crab shell substrate is capable of supporting multiple hydrogen utilizing microbes.

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I attribute all that I have accomplished and all that I will accomplish to my parents. It is through their unconditional love and unyielding support that I have become the person that I am today. The example they have set for me has taught me the importance of hard work and perseverance. *Mom and Dad, I dedicate this to you.*

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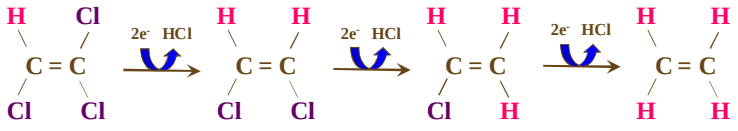
CHAPTER 1

INTRODUCTION

1.1 Introduction

Trichloroethene (TCE, C_2HCl_3), a persistent groundwater pollutant, is currently one of the most prevalent contaminants found in industrialized countries (Tandoi et al., 1994; Wu et al., 1998; He et al., 2003; Stroo et al., 2003; McKnight et al., 2010). The restoration of TCE contaminated aquifers presents a significant challenge to government and industry, due to its dense, hydrophobic, and toxic nature. Remediation of contaminated sites typically falls into two categories: the traditional pump and treat, which is costly and generally inefficient at removing dense non-aqueous phase liquids (DNAPLs) like TCE (He et al., 2003; Quinn et al., 2005); and bioremediation with native or bioaugmented halorespiring organisms. The bioremediation of TCE proceeds under anaerobic conditions through the well-known reductive dechlorination pathway of TCE to cis-1,2-dichloroethene (DCE) to vinyl chloride (VC), and finally to ethene:



TCE **_cis_-DCE** **VC** **Ethene**

One limitation to natural attenuation in the subsurface environment is the unavailability of appropriate electron donors (He et al., 2002), and the addition of

electron donor substrates can constitute a large portion of the costs associated with bioremediation. Soluble electron donors require continuous or semi-continuous addition to the subsurface, increasing operation and maintenance expenditures as well as material costs. As a result, interest has increased in the application of slow-release electron donors which release a sustained, continuous source of fermentation products including volatile fatty acids (VFAs) and hydrogen (H_2) to the contaminated zone without active maintenance. One slow-release electron donor which has been applied for passive bioremediation is chitin (Harkness et al., 2003).

Chitin, derived from the shell material of crustaceans such as crab and shrimp, is the second most abundant biopolymer on earth after cellulose (Beaney et al., 2005). With a chemical formula of $(C_8H_{13}NO_5)n$ (6-7% nitrogen), chitin has a nearly ideal carbon to nitrogen ratio for bacterial growth (Harkness et al., 2003). In its natural form, the crustacean shell is a porous solid consisting mainly of calcium carbonate ($CaCO_3$), protein, and chitin. Dechlorinating microorganisms have a low H_2 threshold (0.3 nM, Löffler et al., 1999) making the slow steady release of acetate and H_2 from the fermentation of the shell material ideal for sustaining these microorganisms. The natural buffering capacity of chitin-associated $CaCO_3$ also makes it an attractive substrate due to its ability to counteract the low pH caused by the generation of the dechlorination byproduct hydrochloric acid (HCl). In practice, crab shell particles are mixed with sand as a proppant to maintain hydraulic conductivity following hydraulic fracturing (or "fracking") of the mixture into contaminated subsurface soils. Although crab shell chitin has already been shown to be an effective substrate for the bioremediation of a variety of chlorinated volatile organic compounds (Jacob et al., 2005; Brennan et al., 2006; Price,

2007; Buser et al., 2010), the response of dechlorinating organisms to chitin purity and proppant loading has not yet been evaluated.

Although several microbial species are capable of degrading chlorinated compounds, *Dehalococcoides* spp. are the only microbes identified to date capable of the complete dechlorination of TCE to ethene (C_2H_4). Halorespiring bacteria, like *Dehalococcoides ethenogenes* (Fennell et al., 2004), are able to couple the energy released during reductive dehalogenation with growth (Löffler et al., 1999) by using a chlorinated ethene as a terminal electron acceptor (Major et al., 2002) and hydrogen (H_2) as an electron donor in a strictly anaerobic environment. *Dehalococcoides* populations grow robustly in mixed cultures, but their essential growth factors, which are likely from other members of the consortia, have yet to be defined (Maymo-Gatell et al., 1997; Holmes et al., 2006; Rowe et al., 2008). While *Dehalococcoides* spp. are naturally present at many contaminated sites, other locations either have no native *Dehalococcoides* spp. or are presumably missing trace nutrients or key strains resulting in accumulation of cDCE or the more toxic VC. This phenomenon is known as cDCE- or VC-stall, and the exact reasons why it occurs have yet to be determined.

To date, there have been several *Dehalococcoides* strains identified, all of which exhibit a high degree of 16S rRNA gene sequence conservation with each other (>98% similarity). Since not all *Dehalococcoides* species have the ability to transform DCE and VC, the key to achieving complete dechlorination of TCE to ethene is the presence of a combination of reductive dehalogenase (RDase) genes from one or more organisms which can encode for reductase enzymes which support the entire dechlorination pathway. The use of quantitative polymerase chain reaction (qPCR) analysis targeting

the RDase genes provides a distinct advantage over quantification analyses targeting signature regions of the 16S rRNA gene due to the high degree of *Dehalococcoides* 16S rRNA gene similarity. Although previous studies have applied quantification of these RDase genes to evaluate the physiology of *Dehalococcoides* spp. in samples from contaminated sites, laboratory cultures, and environmental enrichments (Ritahlati et al., 2006; Holmes et al., 2006), little is known about the distribution of these functional genes in engineered treatment systems. A deliberate examination of the critical transition periods in the dechlorination pathway would enable more targeted strategies to improve bioremediation performance.

Competition for hydrogen between methanogens and dechlorinating microorganisms, and more recently, the possibility of a synergistic relationship existing between these two functional groups, has been speculated and debated (Heimann et al., 2006). Applying the molecular technique of fluorescence in situ hybridization (FISH) to an intact biofilm from an actively dechlorinating system would allow visualization of the *in situ* architecture and may provide information about the ecological relationships involved in the remediation of chlorinated compounds. To date, no one has used an intact *in situ* biofilm to evaluate the distribution of microorganisms within a dechlorinating community.

The aim of this work was to assess the commercially available grades of crab shell chitin, and based on the results, simulate a reductively dechlorinating system using the selected grade of chitin to stimulate a mixed dechlorinating consortium. Chemical and molecular analysis of microcosm, semi-continuous flow, and continuous flow column tests were conducted to evaluate the different substrates and to capture “snap

shots" of the halorespiring community throughout the complete reductive dechlorination of TCE to ethene.

1.2 Hypothesis and Objectives

It was hypothesized that the use of a complex material such as raw crab shell chitin for the remediation of TCE would not only be effective in the biostimulation of dechlorinating species, but also play an important role in water chemistry due to the natural buffering capacity of the chitin-associated $CaCO_3$. Chemical and molecular analyses were combined to lend insight into the assessment and monitoring of TCE contaminanted sites throughout the course of anaerobic reductive dechlorination.

The following are the objectives that guided this study.

- Examine the effect of chitin purity, substrate loading, and contaminant feed on the rate and extent of halorespiration through chemical and molecular analyses;
- Examine the critical transition periods during the reductive dechlorination of TCE to ethene with a fermentable substrate by correlating the relative abundance of RDase genes with water quality data over space and time;
- Evaluate the microbial community through fingerprint (T-RFLP) analysis to determine if DNA extracted from planktonic species in effluent pore water samples is representative of the biofilm attached to up-gradient sediments;
- Collect an intact biofilm from an actively dechlorinating system, and maintain the *in situ* architecture of that biofilm throughout the application of multiplex-FISH

and confocal scanning laser microscopy (CSLM), allowing the spatial community dynamics to be visually inspected.

1.3 Dissertation Layout

This dissertation is composed of three manuscripts which discuss the performance of reductive dechlorination systems enhanced with crab shell chitin and bioaugmented with a mixed dechlorinating consortium for the remediation of TCE. The dissertation is divided into the following chapters:

- CHAPTER 2: "Effects of Chitin Purity and Proppant Loading on the Bioremediation of Chloroethenes"

  Material presented in this chapter is under revision for publication in the *Journal of Environmental Engineering*.
- CHAPTER 3: "Spatial and Temporal Abundance of *Dehalococcoides* spp. and Reductive Dehalogenases During Trichloroethene Degradation"
- CHAPTER 4: "Visualization of Spatial Distribution of *Dehalococcoides* spp. and Archaea During Trichloroethene Degradation using Multiplex-FISH"

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CHAPTER 2

Effects of Chitin Purity and Proppant Loading on the Bioremediation of Chloroethenes

2.1 Abstract

Hydraulic fracturing (or "fracking") of substrates and proppants into contaminated soils is a developing, but understudied, practice of stimulating *in situ* bioremediation. In this work, three different purities of the substrate crab shell chitin (SC-20, SC-40, and SC-80), two proppant loadings (sand:chitin mass ratios of 5:1 and 15:1), and three chloroethene concentrations (1 and 10 mg/L trichloroethene, and 1.5 mg/L cis-1,2-dichloroethene) were experimentally and statistically examined to determine their effects on halorespiration. The least refined crab shell, SC-20, produced the greatest variety of electron donors, converted the highest percentage of contaminant mass to ethene, and supported a significantly greater *Dehalococcoides* population than the other substrates. Although influent chloroethene concentration and proppant loading did not significantly affect halorespiration (p-values > 0.079), decreasing the proppant loading from 15:1 to 5:1 increased the longevity of electron donor production. These results indicate that funds need not be expended for purification of crab shell substrates, and that SC-20 should be used with proppant loadings of 5:1 or lower to increase the longevity of treatment.

2.2 Introduction

Trichloroethene (TCE), a persistent groundwater pollutant, is currently one of the most prevalent contaminants found in industrialized countries (Tandoi et al., 1994; Wu et al., 1998; He et al., 2003; Stroo et al., 2003; Guilbeault et al., 2003; McKnight et al., 2010). According to the Agency for Toxic Substances and Disease Registry (ATSDR), TCE and its chlorinated ethene daughter products cis-1,2-dichloroethene (cDCE) and vinyl chloride (VC), have been detected at 60%, 10%, and 37% of the National Priority List (NPL) sites, respectively (Griffin et al., 2004). Traditional remediation strategies, such as pump and treat, are not only costly and generally inefficient at removing dense non-aqueous phase liquids (DNAPLs) like TCE (He et al., 2003; Quinn et al., 2005), but also tend to transfer the contaminant from one phase to another versus complete destruction (Freedman and Gossett, 1989).

One potential alternative to the conventional treatment of chlorinated ethenes is *in situ* bioremediation using halorespiring bacteria, like *Dehalococcoides ethenogenes* (Fennell et al., 2004), which couple the energy released during reductive dehalogenation with growth (Löffler et al., 1999) by using a chlorinated ethene as a terminal electron acceptor (Major et al., 2002). Although the potential for complete destruction of the contaminant makes *in situ* bioremediation an attractive alternative, the unavailability of appropriate electron donors generally limits natural attenuation in subsurface environments (He et al., 2002). Soluble electron donors such as molasses (Kao et al., 2003), glucose (He et al., 2002), ethanol (Fennell and Gossett, 1997), and lactate (de Bruin et al., 1992) have been applied to contaminated aquifers to overcome electron

donor limitations and effectively enhance anaerobic reductive dechlorination (ARD), but these substances degrade quickly and require continuous or semi-continuous addition to the subsurface, increasing operation and maintenance expenditures as well as material costs. As a result, interest has increased in the application of slow-release electron donors such as tetrabutoxysilane (TBOS) (Seungho and Semprini, 2009; Yu and Semprini, 2002), emulsified soybean oil (Long and Borden, 2006), olive oil (Yang and McCarty, 2002), vegetable oil (Bell et al., 2001; Wiedemeier et al., 2001), oleate and cell biomass (Yang and McCarty, 2000). Slow-release substrates provide a sustained, continuous source of electron donors (like volatile fatty acids (VFAs) and hydrogen (H_2)) to the contaminated zone without active maintenance, which can increase the effectiveness and reduce the overall cost of remediation.

Another slow-release electron donor which has recently been applied for passive bioremediation is chitin (Harkness et al., 2003). Chitin, derived from the shell material of crustaceans such as crab and shrimp, is the second most abundant biopolymer on earth after cellulose (Beaney et al., 2005). With a chemical formula of $(C_8H_{13}NO_5)_n$ (6-7% nitrogen), chitin has a nearly ideal carbon to nitrogen ratio for bacterial growth (Harkness et al., 2003). Due to the abundance of this material as a waste product of the shellfish industry, availability is not limited and costs are low. Different grades (i.e., purities) of chitin are available, and the associated cost increases with the extent to which the raw shell material is refined. In its natural form, the crustacean shell is a porous solid consisting mainly of calcium carbonate ($CaCO_3$), protein, and chitin. The least refined and least expensive grade of chitin is crushed and dried crustacean shell and contains all three components ($CaCO_3$, protein, and approximately 20% chitin = SC-20). The next

refined grade of chitin is produced through a caustic wash to remove the protein (leaving $CaCO_3$ and ~40% chitin = SC-40), and the most refined grade is produced by applying a strong acid to remove the $CaCO_3$ (leaving >80% chitin = SC-80). With each refinement, the cost of chitin increases from $1.00-1.25/lb, to $2.50-3.00/lb, and finally to $4.00-6.00/lb for SC-20, SC-40, and SC-80, respectively (JRW Bioremediation, personal communication, 2010).

Successful results have been obtained using crab shell chitin for anaerobic dechlorination of TCE (Jacob et al., 2005), tetrachloroethene (PCE) (Brennan et al., 2006), chlorinated volatile organic compounds (VOC) (Price, 2007), PCE and 1,1,1-trichloroethane (1,1,1-TCA) (Buser et al., 2010), as well as for denitrification (Robinson-Lora and Brennan, 2009a), and acid mine drainage remediation (Daubert and Brennan, 2007; Venot et al., 2008; Robinson-Lora and Brennan, 2009b; Newcombe and Brennan, 2010; Robinson-Lora and Brennan, 2010). In all of these studies, SC-20 rather than SC-40 or SC-80, was used to keep material costs low, but the potential benefit of using a higher purity chitin was not examined. In addition, previous studies mixed the crab shell particles with sand as a proppant to maintain hydraulic conductivity following hydraulic fracturing (or "fracking") of the mixture into contaminated soils, but an evaluation of the effect of the proppant loading (sand:chitin ratio) on dechlorination has yet to be performed. While the distribution of *Dehalococcoides* in laboratory columns receiving a continuous feed of chitin fermentation products and 3.7 mg/L (22 uM) PCE was evaluated (Brennan, 2003; Brennan et al., 2006), the response of the organisms to chitin purity, proppant loading, and other chloroethene types and concentrations was not

examined. To assist practicing engineers with future application of this substrate, further study of these variables is warranted.

To fill these knowledge gaps and develop the commercialization potential of crab shell chitin as a substrate for the remediation of sites contaminated with chlorinated solvents, Phase I and Phase II Small Business Innovation Research (SBIR) grants were obtained from the National Science Foundation (NSF) in collaboration with North Wind, Inc. In the Phase I grant, SC-20 was mixed in a sand-water slurry and emplaced into low permeability, variably saturated sediments at the Distler Brickyard Superfund site (Hardin County, KY) in July 2001 using a pilot test of the Bio-Frac^{TM} hydraulic fracturing process (Martin et al., 2002; Sorenson et al., 2002). The groundwater at this site was contaminated with relatively low levels of cDCE (~400 ug/L, ~4 uM) and TCE (< 15 ug/L, < 0.11 uM); however, both were at concentrations in excess of federal Maximum Contaminant Levels (70 and 5 ug/L, respectively), despite almost a decade of pump and treat remedial activities. The predominance of biogenic cis-DCE indicated the presence of indigenous dechlorinating bacteria at the site, presumably inhibited by a lack of suitable electron donor (Sorenson et al., 2002). After the Phase I injection of SC-20, chitin fermentation products and halorespiration of cDCE to ethene were observed within 1 month, proving the potential of the substrate and the injection method (Sorenson et al., 2002). A follow-up Phase II study was funded to evaluate and improve the commercial viability of the Bio-Frac^{TM} technology by generating remediation performance data. During Phase II, different grades of crab shell chitin (SC-20, SC-40, and SC-80) were injected at different locations throughout the site (Lebow and Starr, 2005). After the Phase II injection of SC-20, increases in VFA and ethene concentrations were observed

within just one week, and were sustained for 8 – 12 months (Martin et al., 2004; Lebow and Starr, 2005). To fully meet the objectives of the Phase II study, the laboratory studies described herein were also conducted to evaluate different process configurations including chitin purity, proppant loading, and type and concentration of contaminant.

The purpose of this work was to examine the effect of chitin purity, proppant loading, and contaminant feed on the rate and extent of halorespiration through chemical and molecular analyses. The concentrations of chlorinated solvents and chitin fermentation products were monitored over time, and *Dehalococcoides* spp numbers were quantified at the conclusion of the experiment to evaluate the effect of the treatment on bioremediation. Due to its more complex composition, it was hypothesized that the cheaper, less refined SC-20 would provide a more diverse suite of electron donors and support an equivalent or higher number of *Dehalococcoides* spp. with greater dechlorination activity than the more refined and costly SC-40 and SC-80. If true, then the use of SC-20 would be justified, and electron donor costs using chitinous materials could be significantly decreased in future field-scale applications. It was also hypothesized that decreasing the proppant loading would extend the longevity of treatment, and the use of crab shell substrate would effectively enhance dechlorination of both TCE and cDCE at various concentrations.

2.3 Materials and Methods

2.3.1 Chemicals and Substrates

TCE and cis-1,2-DCE were purchased from VWR International, Inc. (West Chester, PA). VC and ethene gases were purchased from Alltech Associates, Inc. (State College, PA). Volatile fatty acid standards were created from a 10 mM volatile acid standard mix obtained from Supelco (Bellefonte, PA). The chitin sources were derived from Dungeness crab shells, and used as received from the distributors: SC-20 and SC-40 were donated by JRW Bioremediation, LLC (Lenexa, KS), and SC-80 was provided by Vanson, Inc. (Redmond, WA). Limestone (0.35-1.0 mm chips, 49.6% $CaCO_3$, donated by Prairie Central, IL) was added to all treatments to maintain a baseline buffering capacity, regardless of the $CaCO_3$ content of the substrate, to help avoid extreme pH changes created by fermentation (VFAs) and halorespiration (HCl). Silica sand (16-20 mesh, donated by Badger Mining Corp., Berlin, WI) was used as an inert proppant in the column study to maintain hydraulic conductivity. All other chemicals used were reaction grade or higher quality.

2.3.2 Medium Preparation

A reduced anaerobic basal salts medium was prepared for use in this study (Brennan and Sanford, 2002). The reduced medium consisted of (per liter of distilled deionized water): 10 mL mineral salts solution (per liter: 100g NaCl; 50g $MgCl_2 \cdot 6H_2O$; 20g KH_2PO_4; 30g NH_4Cl; 30g KCl; and 0.015g $CaCl_2 \cdot 2H_2O$); 1 mL trace element

solution (per liter: 10 mL HCl (25% solution, w/w); 1.5g $FeCl_2 \cdot 4H_2O$; 0.19g $CoCl_2 \cdot 6H_2O$; 0.1g $MnCl_2 \cdot 4H_2O$; 70mg $ZnCl_2$; 6mg H_3BO_3; 36mg $Na_2MoO_4 \cdot 2H_2O$; 24mg $NiCl_2 \cdot 6H_2O$; and 2mg $CuCl_2 \cdot 2H_2O$); 1 mL selenium-tungsten solution (per liter: 6mg $Na_2SeO_3 \cdot 5H_2O$; 8mg $Na_2WO_4 \cdot 2H_2O$; and 0.5g NaOH); 0.25 mL resazurin (0.1 % solution); 0.031g L-cysteine; 0.016g Na_2S; 2.52g $NaHCO_3$; 0.007g Na_2SO_4. The medium was prepared by adding the first four components to 1-L of distilled deionized water, boiling, cooling under a nitrogen gas purge, and then adding the remaining components. A 20% CO_2, 80% N_2 purge was used to adjust the pH to approximately 7.2. The prepared medium was then transferred to N_2/CO_2 purged, 160 mL serum bottles, and sterilized in an autoclave.

2.3.3 Dechlorinating Cultures

A mixed *Dehalococcoides* culture (BDI culture) capable of complete dechlorination of TCE to ethene (kindly provided by Dr. Frank Löffler, then at the Georgia Institute of Technology), was used in all experiments. The BDI culture is a commercially available dechlorinating microbial consortium that contains *Dehalococcoides* spp. strains FL2 (He et al., 2003a), BAV1 (He et al., 2003b), and GT (Ritalahti et al., 2006). Strain FL2 is physiologically similar to *Dehalococcoides ethenogenes* except it is not able to convert PCE to TCE at high rates (Löffler et al., 2000; Smidt and de Vos, 2004). Strain BAV1 has been identified to utilize cDCE and VC metabolically as electron acceptors for growth (Cupples et al., 2003; He et al., 2003a; He et al., 2003b; Smidt and de Vos, 2004). The more recently identified GT strain is

capable of metabolically degrading TCE to ethene (Sung et al., 2006; Ritalahti et al., 2006). The mixed BDI culture was maintained in an enriched basal salts medium containing 0.3 mM TCE and 5 mM lactate and was periodically transferred as needed.

2.3.4 Microcosm Study

A microcosm study was conducted to rapidly evaluate the effectiveness of three different grades of crab shell chitin at supporting the halorespiration of 10 mg/L (76 uM) TCE. Duplicate microcosms consisted of 160 mL glass serum bottles containing 100 mL of enriched basal salts medium in an N_2/CO_2 headspace sealed with butyl rubber stoppers and aluminum crimp caps. Each serum bottle received 0.05 g substrate (SC-20, SC-40, or SC-80), 0.01 g limestone, and was inoculated (5% v/v) with the BDI culture. TCE was periodically replenished as levels became exhausted by spiking the medium with 0.75 uL neat TCE, to give an aqueous concentration of ~ 76 uM. This was later discontinued to eliminate preferential use of TCE over daughter products as the electron acceptor. Initially, liquid samples were withdrawn every other day and replaced with an equal volume of nitrogen, removing a total volume of 80 mL over the course of 31 days. After day 31, the dechlorination process was allowed to continue undisturbed in the microcosms, until day 86, at which time one final liquid sample (5mL) was withdrawn.

2.3.5 Semi-continuous Column Study

A semi-continuous column study was conducted to evaluate three different grades of crab shell chitin (SC-20, SC-40, SC-80) at two different proppant loadings (sand:chitin mass ratios of 5:1 and 15:1). The columns were treated with basal salts medium containing one of the following chlorothenes: TCE at 7 uM (0.9 mg/L), TCE at 100 uM (13 mg/L), or cDCE at 16 uM (1.6 mg/L). These concentrations were chosen to be representative of chloroethene concentrations commonly found in groundwater at contaminated sites (Lee et al, 2004; Mayer et al., 2001; Puls et al., 1999). Control columns, which were not inoculated with the BDI culture, and a control column which did not contain chitin but was inoculated, were also evaluated. The full experimental design matrix is presented in Table 2.1.

The experiment was performed in 25 cm-long by 1 cm-diameter stainless steel columns connected in pairs with stainless steel tubing as shown in Figure 2.1. The first, front columns (i.e., chitin columns) were wet packed with a mixture of silica (fracking) sand, chitin, and limestone chips as indicated in Table 2.1, to represent the substrate injection zone. The second, back columns were similarly packed with approximately 20 g of silica sand only to represent downgradient aquifer materials. After packing, all columns were flushed with approximately three pore volumes of reduced basal salts medium to remove fines and insure anaerobic conditions. The back columns were then inoculated with approximately 1.5 pore volumes of the BDI culture and allowed to incubate statically for three to four days prior to sampling. The sampling procedure consisted of pumping TCE- or cDCE-amended, basal salts medium at a flow rate of 2.0

mL/min (8.33 cm min^{-1}) through the column pairs (front and back) every two days to exchange one pore volume (approximately 6 mL) using a Masterflex® L/S™ model 7519-06 cartridge pump (Cole-Parmer) equipped with Viton tubing. The effluent sample was collected in a 20 mL Perfektum® glass syringe (Popper & Sons, Inc.) from the end of the second (back) column. After collection, aqueous samples were analyzed for pH, fatty acids, and chlorinated ethenes. The columns were evaluated over a period of 172 days. For the first 47 days, sampling was conducted every other day. Incubation times were then extended to five, ten, twenty, and finally thirty days in an effort to increase ethene production. In addition, columns that did not exhibit any significant dechlorination activity by 33 days were allowed to incubate for one month before sampling was initiated again. At the conclusion of the experiment, the columns were preserved at -80°C for future DNA extraction.

2.3.6 Analytical Methods

Chlorinated ethenes, ethene, and methane were measured with an Agilent model 6890N headspace gas chromatograph (GC) equipped with a GS-Q column (30 m length, 0.53 mm diameter, J&W Scientific) and a flame ionization detector (FID). Aqueous samples (1 mL) were injected into 20 mL autosampler vials containing 0.002 g copper sulfate to inhibit microbial activity, and capped with Teflon-lined septa. The vials were equilibrated for 15 minutes at 70 ºC prior to automated injection by an Agilent G1888 Network Headspace Autosampler. Target analytes were separated using the following temperature program: initial hold at 35ºC for 2 minutes, increase to 180ºC at a rate of

45ºC per min, hold for 2 minutes, increase at a rate of 45ºC per min to 200ºC, hold for 5.34 minutes. Ultra-high purity helium was used as the carrier gas at a flow rate of 22.6 mL/min.

VFA analysis was performed by a Waters 2695 high-performance liquid chromatograph (HPLC) equipped with an Aminex HPX-87H ion exclusion column and a photodiode array detector. Aqueous samples were placed in 2 mL eppendorf tubes, and stored at -20ºC until analysis. Prior to analysis, the samples were thawed, centrifuged to remove any solids, and the supernatant (1.5 mL) added to 0.2 mL of 2.0 N H_2SO_4 in 2 mL autosampler vials.

The pH of each sample was measured using a Fisher Accumet AB15 pH meter equipped with an Orion Thermo Electron combination pH electrode.

2.3.7 DNA Extraction

At the conclusion of the semi-continuous column experiment, DNA samples were extracted from the column packing materials to determine the relative abundance of *Dehalococcoides* species. DNA was extracted from 1-2 grams of wet column material collected aseptically from the influent and effluent ends of each inoculated (back) column using a MoBio Ultraclean Soil DNA Isolation Kit (MoBio, Solana Beach, California) following the alternative protocol for maximum yields.

2.3.8 PCR

The presence of *Dehalococcoides* 16S rRNA genes in column samples was initially determined using a *Dehalococcoides* 16S rRNA gene-specific primer pair. The following oligonucleotides were used: 5'-GATGAACGCTAGCGGCG-3' (forward primer; Dhc1F) and 5'-GGTTGGCACATCGACTTCAA-3' (reverse primer; Dhc1377R) (Hendrickson et al., 2002). Amplification reactions were performed in a total volume of 50 uL containing 5 uL of 10X buffer (USB, Cleveland, Ohio), 2 mM $MgCl_2$ (USB), 0.2 mM of each deoxynucleoside triphosphate (TaKaRa, Otsu, Japan), 1.25 units Hot Start IT Taq polymerase (USB), 6.5 mg bovine serum albumin (Roche, Indianapolis, IN), 0.8 uM of each primer (Integrated DNA Technologies, Coralville, IA), and 10 uL of template DNA. Positive controls for the PCR reactions consisted of heat-lysed *E. coli* clones with 16S rRNA gene inserts from *Dehalococcoides* sp. strain FL2 as the template DNA. Negative controls had no added template DNA. PCR was performed in a Flexigene (Techne, Burlington, N.J.) thermocycler under the following conditions: 94˚C for 2 min (1 cycle); 94˚C for 1 min, 58˚C for 1 min, 72˚C for 2 min 10 s (30 cycles); 72˚C for 7 min (1 cycle). PCR products (5 uL) were imaged by agarose gel electrophoresis to confirm successful amplification.

2.3.9 q-PCR

Real-time PCR was conducted using a linear hybridization probe (TaqMan) approach and oligonucleotides targeting 16S rRNA gene sequences of members of the *Dehalococcoides* group as described by He et al. (2003a) and Ritalahti et al. (2006). The

following oligonucleotides were used: 5'-CTGGAGCTAATCCCCAAAGCT-3' (forward primer; Dhc1200F), 5'-CAACTTCATGCAGGCGGG-3' (reverse primer; Dhc1271R), and 5'-TCCTCAGTTCGGATTGCAGGCTGAA-3' (probe; Dhc1240Probe) (He et al., 2003a; Ritalahti et al., 2006). The reporter fluorochrome on the 5' end of the probe was 6-carboxy-fluorescein (FAM) and the quencher on the 3' end was N,N,N',N'-tetramethyl-6-carboxy-rhodamine (TAMRA). Each well of a 96 well plate (VWR International) contained a total reaction volume of 30 uL containing 15 uL 1X iQ Supermix (BioRad, Hercules, California), 300 nM of each primer and probe (SIGMA GENOSYS, the Woodlands, Texas), and 1 uL of template DNA. PCR was conducted using a spectrofluorimetric thermal cycler (iCycler, BioRad) with cycle parameters as described by He et al. (2003a). The target DNA used for q-PCR standard curves was plasmid TOPO TA pCR4 (Invitrogen, Carlsbad, California) inserts containing cloned 16S rRNA gene fragments from *Dehalococcoides* specific PCR products inserted into chemically competent *Escherichia coli* cells. A QIAprep Spin Miniprep Kit (QIAGEN, Valencia, California) was used to extract vectors from the *E. coli* host. Plasmids were extracted into 50 uL of Tris buffer (10mM Tris•Cl, pH 8.5) and quantified spectrophotometrically at 260 nm. Calculation of DNA concentration was determined using the formula DNA (ng/uL) = optical density at 260 nm × 50 × dilution factor (Ausubel et al., 1997). Plasmid insert sequences were verified by DNA sequencing (Nucleic Acid Facility, University Park, Pennsylvania). Calibration curves (arbitrarily set cycle threshold value [C_t] versus log gene copy number per uL) were created using serial dilutions of plasmids carrying a single, cloned *Dehalococcoides* target gene. The number of gene copies in a known amount of DNA and the number of target genes per gram of

wet column materials were calculated using equations described by Ritalahti et al. (2006). Amplifications were conducted in triplicate along with negative controls (no template DNA, and template DNA from *Desulfuromonas michiganensis* strain BB1).

2.3.10 Statistics

Statistical analyses of the data were performed using Minitab software with one-way analysis of variance (ANOVA) and Tukey multiple comparison testing. A 95% confidence interval was selected for all analyses establishing a critical p-value of 0.05. Statistical analysis of chitin grade and proppant loading as well as influent feed and concentration were conducted to assess whether the means of any of the compared groups were statistically different. If a significant difference (p-value <0.05) was reported for the one-way ANOVA analysis, a multiple comparison using the Tukey test was conducted to reveal which means were significantly statistically different.

2.4 Results and Discussion

2.4.1 Microcosm Study

2.4.1.1 Dechlorination

In the microcosm experiment, crab shell fermentation products promoted dechlorination of TCE ultimately to ethene in all of the bottles (Table 2.2). Higher ethene concentrations were observed at later times indicating that dechlorination activity,

or at least the extent of dechlorination, increased in the bottles with time. The greatest ethene production was observed in the SC-20 microcosms (20.98±0.25 umol/bottle; average±one standard deviation) followed by SC-80 (9.80±8.05 umol/bottle), and finally SC-40 microcosms (6.99±3.18 umol/bottle). 2.4.1.2 Fermentation Products

Some methane production (0.6 – 1.0 uM) was observed in all of the bottles, indicating that redox conditions were strongly reducing and favorable for reductive dechlorination. This level of methane production is consistent with that observed previously (Brennan et al., 2006), and does not represent a major loss of electrons toward methanogenesis (< 0.02% of total electron equivalents provided by the substrates). Fatty acid concentrations were observed to increase in all bottles by the second day. The type and magnitude of fatty acids produced was dependent on the chitin grade (Table 2.2). In the SC-20 microcosms, caproate, acetate, and propionate were consistently observed, with average concentrations over the experiment of 1.52±0.23, 0.65±0.02, and 0.11±0.005 mM, respectively. In the SC-40 and SC-80 microcosms, acetate dominated with average concentrations of 0.28±0.01 and 1.50±0.65 mM, respectively. Propionate was also detected in these microcosms, but at lower concentrations (≤ 0.2 mM).

2.4.1.3 pH

pH values for all grades of chitin in the microcosm experiment remained within the circum-neutral range of 7.3 – 7.8, with SC-20 and SC-40 remaining fairly stable and SC-80 showing the greatest drop in pH over the course of the experiment (Table 2.2). Despite the addition of limestone, the drop in pH observed for SC-80 was likely due to

the lack of biogenic $CaCO_3$ in the substrate. Others in our lab have determined that the surface area and alkalinity production of raw crab shells (SC-20) is over an order of magnitude higher than that of powdered limestone (Robinson-Lora and Brennan, 2009b), which explains the exceptional pH stability observed with SC-20 and SC-40 here. Typically, TCE dechlorination is most favorable near neutral conditions of pH 6 – 8 (Gerritse et al., 1999), and all of the bottles in the microcosm experiment still remained within this range. Table 2.2 summarizes the performance of the different grades of chitin tested in the microcosm experiment in terms of average pH, VFAs, and ethene concentrations.

2.4.2 Semi-continuous Column Study

2.4.2.1 Dechlorination

Dechlorination activity was observed in all of the semi-continuous columns, indicating that chitin should support dechlorination in the field for the range of contaminant concentrations tested (Figure 2.2). While neither crab shell purity and proppant loading (p-value = 0.079) nor contaminant feed (p-value = 0.091) appeared to significantly affect the magnitude of dechlorination products produced, the percentage of the contaminant in the influent converted to ethene was affected by contaminant feed concentration (p-value = 0.033). Comparison using the Tukey test revealed that the columns fed 7 uM TCE had significantly different ethene production (normalized as percentage of the influent concentration) than the columns fed 100 uM TCE. The

columns that were fed16 uM cDCE were not statistically different from either the 100 uM TCE or the 7 uM TCE fed columns. Comparing all columns tested in this study, the greatest total ethene production was observed in the 5:1 columns fed 100 uM TCE and loaded with SC-20 (106.44 uM), followed by SC-40 (88.2 uM), and finally SC-80 (18.2 uM) (Appendix A). Although most of the columns exhibited some dechlorination activity of TCE to DCE and VC in the first 47 days of the experiment, significant ethene production was generally not observed until the incubation time was increased to at least 10 days. With longer incubation times, greater conversion to ethene was observed. Indeed, other studies have shown a delay in VC degradation to ethene until all other chloroethene compounds have been depleted (Maymó-Gatell et al., 1995), and studies using the BDI culture in particular have noted a lag time in VC dechlorination of at least 10 days (He et al., 2003). Incubation time, rather than the grade of chitin, seemed to be a more accurate predictor of the onset of dechlorination activity: inoculated SC-20 columns generally had the shortest lag period before measurable ethene was produced (69±10 days), whereas slightly longer, but statistically insignificant, lag phases were observed for the SC-40 and SC-80 columns (92±15 days, and 85±25 days, respectively). The longer lag phase in the SC-40 and SC-80 columns may be due, in part, to the lower pH in these columns (section 3.2.4).

The total chloroethene mass balance was generally greater than 83% for all columns. Lack of complete mass balance between chloroethene parent compounds and daughter products has also been observed at many field locations, leading to the suspicion that an anaerobic oxidizer of VC or cDCE must exist, although it has yet to be isolated (Gossett, 2010). Studies by Bradley and Chapelle (1999a; 1999b; 2000) reported the

anaerobic biodegradation of VC to methane and carbon dioxide through acetotrophic methanogenesis, and predicted acetate to be a significant intermediate of the VC mineralization. These studies present a mechanism for anaerobic VC oxidation and may also explain the secondary increase in acetate (Figure 2.3) observed in this study. In addition to potential VC oxidation, the low apparent production of ethene observed in the columns may also be due to sample loss as a result of the sampling procedure. Despite efforts to capture all the effluent from the columns, gases formed within the column were undoubtedly lost through the process of removing the end cap and attaching the glass collection syringe. It is also possible that low levels of oxygen inadvertently intruded into the columns during this sampling procedure. A study by Amos et al. (2008) found oxygen levels (even $\leq$ 4 mg/L) significantly impacted the dechlorinating ability of *Dehalococcoides* organisms in the BDI consortium. Nevertheless, trends in the dechlorination pathway and ethene production are still apparent between the different substrates.

2.4.2.2 Fatty Acid Production

As in the microcosm experiment, fatty acid concentrations were observed to increase in all of the semi-continuous columns by the second sampling event (total elapsed time of 4 to 5 days since chitin emplacement). Examples of typical fatty acid profiles for columns containing the three different grades of chitin are shown in Figure 2.3. Propionate concentrations were similar for the three grades of chitin throughout the semi-continuous column experiment with an average concentration of 2.22±0.19 mM.

The average concentration of butyrate was greatest in the SC-20 columns (7.99±3.27 mM) followed by the SC-80 (2.99±0.56 mM) and finally the SC-40 columns (2.06±1.20 mM). In addition, methyl-butyrate and formate were also produced at early times (< 15 days), but at much lower concentrations (average concentration over the experiment generally < 1.5 mM). Methyl-propionate was also present, but was produced at the greatest concentration (~2.0 mM) in the control column, which did not contain chitin, but was inoculated with the BDI culture which had been maintained on lactate as an electron donor. In the columns with higher loadings of chitin (sand:chitin ratio of 5:1), an increase in fatty acid concentrations was observed (Table 2.3) when incubation times were increased from 2 to 10 days (Figure 2.3), indicating an excess of electron donor and a build-up of unutilized VFAs. Although the average acetate concentrations were similar for the three grades of chitin in the first 33 days of the experiment (14±9 mM), acetate acid dominated towards the end of the experiment in all columns as the other VFAs were depleted.

The type, profile shape, and magnitude of fatty acids generated were mainly dependent on chitin grade and less dependent on proppant loading. For example, caproate was the dominant fatty acid in the SC-20 columns (average concentration = 47.17±6.19 mM), but exhibited significantly lower average concentrations in the SC-40 columns (10.78±1.69 mM), and was extremely low in the SC-80 columns (0.29±0.11 mM). These results indicate that the production of caproate in the SC-20 and SC-40 columns is not from chitin, but rather from residual protein that is removed during

purification of chitin. Average methane concentrations in the columns ranged from 0.5 – 1.5 μM, in the aqueous phase (data not shown).

2.4.2.3 Carbon Balance

A carbon balance was performed for each grade of chitin by comparing the total theoretical amount of carbon donated from chitin to the measured total amount of carbon associated with the fermentation products (Table 2.4; Appendix A). To determine the theoretical amount of carbon from chitin, column contents were weighed, ashed (heated in a muffle furnace at 600˚C for 24 h), rinsed with 1 + 1 hydrochloric acid, dried, and reweighed to determine the mass of chitin lost due to fermentation during the experiment. This information was used to calculate the theoretical mass of carbon released from chitin using equation 2.1.

$$C\ released = (\text{g chitinous material fermented})(\%\ pure\ chitin)\left(\frac{mol\ chitin}{203.22 g\ chitin}\right)\left(\frac{96.08 g\ C}{mole\ chitin}\right) \quad (2.1)$$

Approximately 48±5.7, 31±2.9, and 100±1.4 percent of the original chitinous material was fermented in the SC-20, 40, and 80 columns, respectively. Statistical analyses verify that the amount of fermentation was significantly dependant on the grades of chitin and proppant loading (p-value < 0.001). Multiple comparisons with the Tukey test revealed that differences exist between the following groupings: SC-20 (5:1) and SC-80 (5:1); SC-20 (control columns, 15:1) and SC-40 (5:1); and SC-20 (15:1) and SC-40 (15:1). The analysis shows that more chitinous material was fermented with lower proppant loading

(i.e., lower sand:chitin ratio): SC-20 fermentation was higher in (5:1) than (15:1), and SC-40 fermentation was higher in (5:1) than in (15:1).

Released carbon calculations based on effluent VFA concentrations were found to be dependent on the grade of chitin, but independent of proppant loading. The amount of carbon calculated from effluent fermentation products was found to be in excess of what could be donated by just chitin in SC-20 and SC-40 columns, but less than what was available from chitin in the SC-80 columns (Table 2.4). These results indicate that the majority of fermentation products released from SC-20 and some of the fermentation products released from SC-40 were likely derived from protein, and that only a fraction of the available carbon from SC-80 was released in the effluent as VFAs over the course of the semi-continuous column experiment. Using equation 2, the theoretical amount of fermented protein was calculated from the amount of carbon in excess of what could be donated by chitin.

$$\text{g fermented protein} = (\text{g excess carbon})\left(\frac{mol\ C}{12.01 g\ C}\right)\left(\frac{mol\ protein}{16\ mol\ C}\right)\left(\frac{352.2 g\ protein}{mol\ protein}\right) \quad (2)$$

The theoretical amount of fermented protein was calculated to be approximately 26.1±3.2 and 1.2±1.5 percent of the raw material for SC-20 and SC-40, respectively (Table 2.4). These numbers are in agreement with information given by the manufacturer, who reports that SC-20 and SC-40 consist of 5-50% and 1-5%, respectively, of protein by weight (JRW Bioremediation, personal communication, 2010).

The wide variety of fermentation products released by the anaerobic degradation of chitinous materials have the potential to be excellent electron donors for

halorespiration. The primary sources of electrons for dehalogenating populations are acetate and hydrogen (H_2) (Smidt and de Vos, 2004). More complex VFAs, like propionate, butyrate, and caprionate also have the potential to be excellent electron donors after they are further fermented to acetate and H_2. Slowly degraded substrates such as butyrate and propionate, which only degrade in environments with low H_2 partial pressures, may give reductive dechlorinators an advantage over competing methanogens/acetogens by providing a slow and steady release of H_2 (Fennell and Gossett, 1997; He et al., 2002; Smidt and de Vos, 2004). While not directly measured in this study, previous work with crab shell chitin as a substrate has only been able to quantify low levels of detectable H_2 (< 100 nM), presumably due to rapid utilization within the system (Brennan et al., 2006). Acetate is also a good electron donor for reductive dechlorination, and its oxidation has been shown to support the complete dechlorination of PCE to ethene in the presence of syntrophic microorganisms (He et al., 2002). In previous experiments with chitin, acetate appeared to be the chitin fermentation product primarily responsible for observed dechlorination activity (Brennan et al., 2006). The research conducted by He et al. (2002) indicated that applying methods that increase the flux of both acetate and H_2 may be the most promising approach for supporting complete dechlorination at certain field locations (He et al., 2002).

2.4.2.4 pH

Likely due to the presence of $CaCO_3$ in the shell material, the pH in columns containing SC-20 always remained within a tight range between 7.2 – 7.7 throughout the experiment (Figure 2.4). Although more variable due to chemical treatment during protein-stripping, the columns containing SC-40 generally remained between 6.7 to 8, whereas the pH in columns with SC-80 approached 5.7 before stabilizing at approximately 6.1 by the end of the experiment (Figure 2.4). While the drop in pH observed in the SC-80 columns was not great enough to stop dechlorination activity, it most likely impacted complete degradation, as the SC-80 columns displayed the lowest ethene production.

2.4.2.5 q-PCR

Dehalococcoides species in the influent and effluent ends of each back column were quantified using real-time PCR on DNA extracted from column materials (Figure 2.5; Appendix A). Results indicate that the number of *Dehalococcoides* cells (assuming that one 16S rRNA gene copy = 1 cell) is affected to a greater extent by chitin purity than by the type of contaminant feed or proppant loading. The greatest number of *Dehalococcoides* (cells/g) in each of the substrate types was 2.00E+07 (the influent end of SC-20 column #1), 3.66E+06 (effluent end of SC-40 column 4), and 2.85E+06 (effluent end of SC-80 column #12). Statistical analysis showed no significant differences in the number of *Dehalococcoides* in the effluent ends of the columns based

on chitin purity and proppant loading (p-value = 0.756) or contaminant feed and concentration (p-value = 0.179). Similarly, the number of *Dehalococcoides* in the influent ends of the columns was not affected by contaminant feed and concentration (p-value = 0.852); however, it was affected by chitin purity (p-value < 0.001). The mean number of *Dehalococcoides* cells in the influent end of columns with SC-80 grade chitin was significantly different from columns containing either SC-20 or SC-40. The average number of *Dehalococcoides* in the influent and effluent ends of all columns (±one standard deviation) is organized by substrate grade in Table 2.2.

Some spatial trends in *Dehalococcoides* populations were also observed. The control column which did not contain chitin showed detectable numbers of *Dehalococcoides* in the influent end of the inoculated column. This indicates that the microorganisms were most likely deposited in this location during inoculation in all the columns. At the end of the experiment, greater numbers of *Dehalococcoides* were observed clustered near the influent of the SC-20 columns, whereas greater numbers were found at the effluent end of SC-80 columns, and a fairly uniform distribution was found in the influent and effluent sediments of the SC-40 columns (Figure 2.5). This could be due to differences in the production of VFAs and pH associated with the different grades of chitin. The VFA and pH data represented here were measured in the effluent of the columns. Since the columns behave like plug flow reactors, electron donor concentrations in the influent end (where the inoculum was initially deposited) of the columns would be greater than the corresponding concentrations measured in the effluent (Rittmann and McCarty, 2001). Following this line of thought, the localized pH in the influent end of the columns was most likely more acidic than the pH documented in the

effluent of the SC-20 (7.05), SC-40 (6.74), and SC-80 (5.84) columns. With the optimum pH range for the growth of *Dehalococcoides* being between 6.8 and 7.5, the non-optimal conditions within the SC-40 and SC-80 columns could have caused all of the detectable *Dehalococcoides* cells in the SC-80 columns and some of the cells in the SC-40 columns to detach and be carried with the flow toward the effluent ends of these columns. Table 2.2 summarizes the performance of the different chitin grades tested in the semi-continuous experiment including average *Dehalococcoides* 16S rRNA copy numbers.

2.5 Conclusions

Results from this laboratory study demonstrated key differences in performance between the commercially available grades of crab shell chitin. The following key conclusions are noted:

1. The three grades of chitin tested all supported dechlorination activity in both batch microcosm and semi-continuous column experiments. The number of *Dehalococcoides* 16S rRNA gene copies and the magnitude of ethene production were highest with SC-20, followed by SC-40, and SC-80.

2. Increasing the fraction of chitin (i.e., decreasing the sand:chitin ratio from 15:1 to 5:1) did not significantly affect chloroethene degradation in the column experiment, but did increase the longevity of VFA production; therefore, proppant loadings of 5:1 or lower should be used in the field to increase the longevity of treatment.

3. The type and concentration of VFAs appeared to be controlled predominantly by the grade of chitin used, with SC-20 producing the greatest variety of fermentation products. The protein component associated with the unrefined shell material (SC-20) is likely responsible for caproate production and may have ultimately contributed to the production of acetate and hydrogen, which are believed to fuel dechlorination reactions.

4. The pH in systems containing SC-20 and SC-40 was more stable than with SC-80 due to the presence of chitin-associated $CaCO_3$ which has been shown to be a more effective buffer than limestone. For this reason, SC-80 is not recommended as an electron donor source in low-alkalinity waters, where detrimental drops in pH could inhibit dechlorination activity.

5. Increasing commercialization of this "green technology" would have several benefits, including the reduction of a waste stream generated by the seafood industry, the potential for complete destruction of chlorinated ethenes in contaminated aquifers, and a reduction in costs compared to conventional approaches for groundwater remediation.

Since this study was conducted, the use of crab shell chitin to enhance bioremediation of chlorinated ethenes has been used at several field locations including the Distler Brickyard Superfund site. In November of 2004, a pilot-test, which placed

4,000 lbs of unrefined (SC-20) crab shell into 16 augered borings, was conducted at a former aerospace test site located near Seattle Washington (Jacob et al., 2005). At Cresticon Twin City Tool, Olathe, Kansas, crab shell injection activities were initiated in July 2007 in order to address offsite VOC groundwater contamination (Price, 2007). Most recently, in Ormond Beach, Florida, crab shell was applied to the base of excavations at an active electrical components manufacturing facility to remediate soil and groundwater impacted with chlorinated solvents (Buser et al., 2010).

2.6 Acknowledgments

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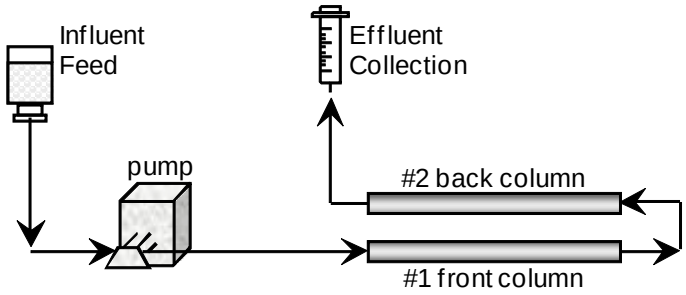

Figure 2.1. Semi-continuous column study experimental set up. #1, front column containing chitin, sand, and limestone; #2, back column containing sand, limestone, and inoculum.

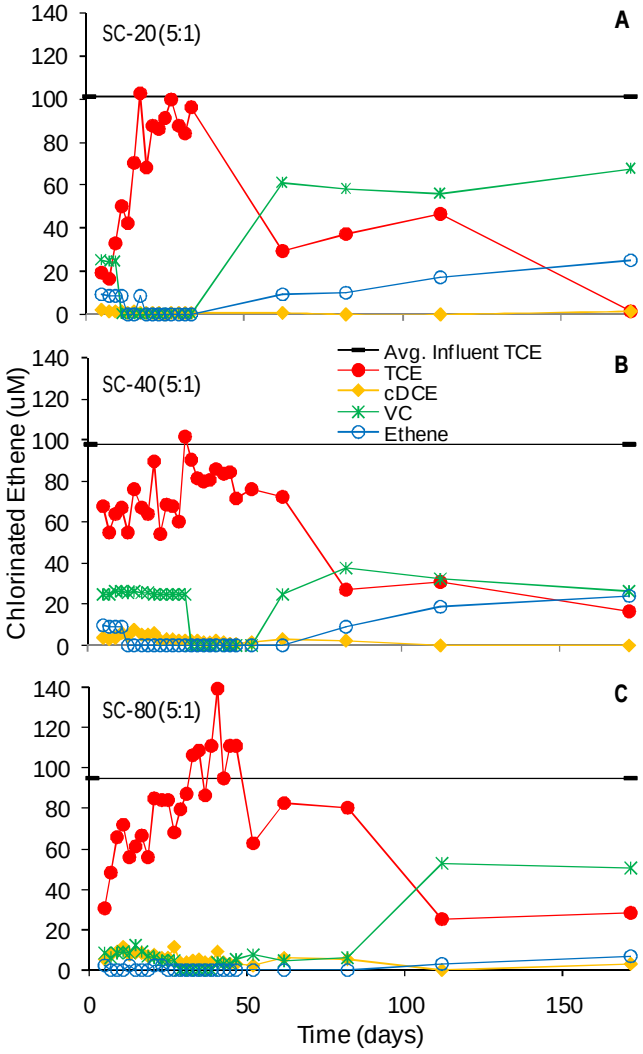

Figure 2.2. Typical chlorinated ethene profiles observed in the effluent of semi-continuous columns given 100 uM TCE, treated with three different grades of chitin. A) SC-20, column 1; B) SC-40, column 3; C) SC-80, column 5. A complete list of column conditions is provided in Table 2.1.

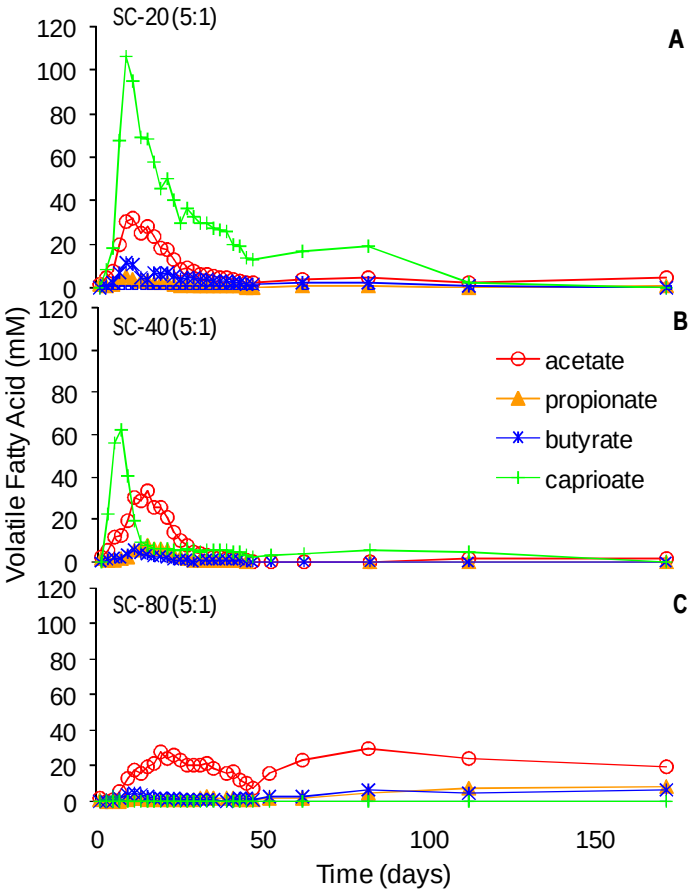

Figure 2.3. Typical fatty acids observed in the effluents of semi-continuous columns given 7 uM TCE, containing three different grades of chitin. A) SC-20, column 8; B) SC-40, column 10; C) SC-80, column 12. A complete list of column conditions is provided in Table 2.1.

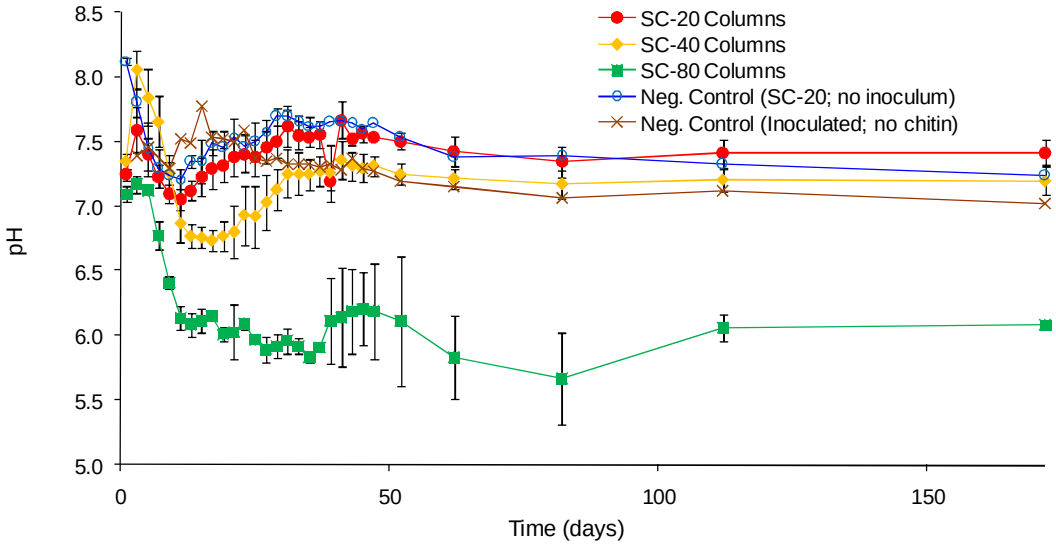

Figure 2.4. Average pH in the effluent of the semi-continuous columns for the three different grades of chitin. Error bars represent one standard deviation.

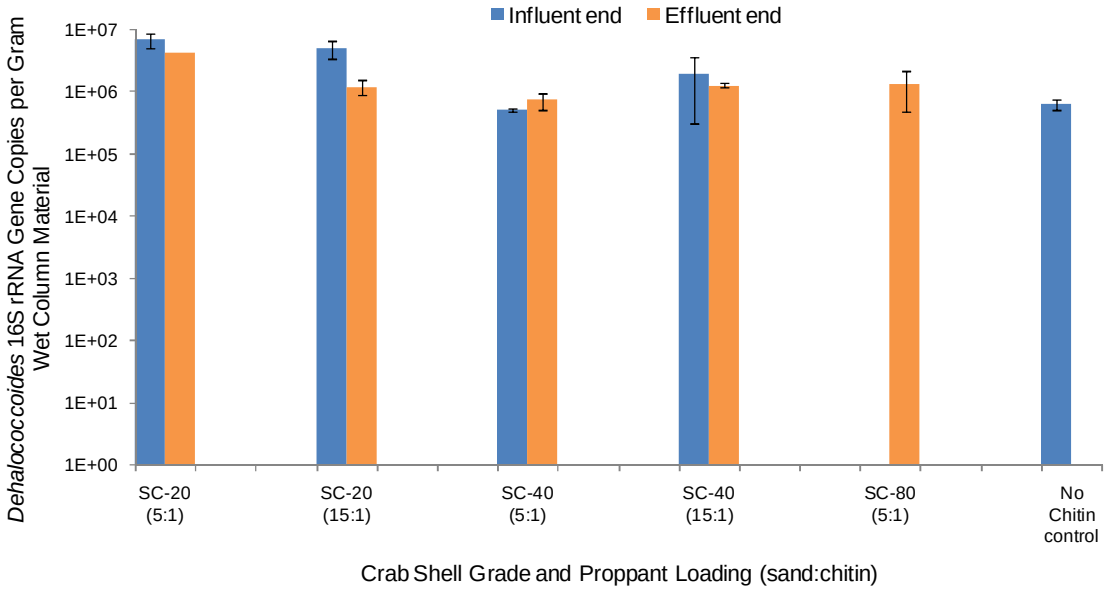

Figure 2.5. q-PCR analysis of the semi-continuous flow columns. Each bar represents the average number of *Dehalococcoides* 16S rRNA gene copies from three columns for each grade of chitin and proppant loading (n = 3). Each q-PCR analysis was quantified in triplicate. Error bars represent one standard deviation. Note: *Dehalococcoides was not detected in the influent of the SC-80 columns nor the effluent of the no chitin control column.*

Table 2.1. Experimental design matrix for the semi-continuous column experiments. The masses shown are for the #1, front (upgradient) columns only (see Figure 2.1).

Column ID	Chloroethene name	Chloroethene feed (uM)	Chitin Grade	Sand (g)	Substrate (g)	Limestone (g)	BDI Culture Inoculated *
1	TCE	100	SC-20	13.5	2.7	0.54	Yes
2	TCE	100	SC-20	18.0	1.2	0.24	Yes
3	TCE	100	SC-40	13.5	2.7	0.54	Yes
4	TCE	100	SC-40	18.0	1.2	0.24	Yes
5	TCE	100	SC-80	5.5	1.1	0.22	Yes
6	TCE	100	SC-20	18.0	1.2	0.24	No
7	TCE	100	n/a	20.0	0.0	0.24	Yes
8	TCE	7	SC-20	13.5	2.7	0.54	Yes
9	TCE	7	SC-20	18.0	1.2	0.24	Yes
10	TCE	7	SC-40	13.5	2.7	0.54	Yes
11	TCE	7	SC-40	18.0	1.2	0.24	Yes
12	TCE	7	SC-80	5.5	1.1	0.22	Yes
13	TCE	7	SC-20	18.0	1.2	0.24	No
14	cDCE	16	SC-20	13.5	2.7	0.54	Yes
15	cDCE	16	SC-20	18.0	1.2	0.24	Yes
16	cDCE	16	SC-40	13.5	2.7	0.54	Yes
17	cDCE	16	SC-40	18.0	1.2	0.24	Yes
18	cDCE	16	SC-80	5.5	1.1	0.22	Yes
19	cDCE	16	SC-20	17.9	1.2	0.24	No

**Inoculated into #2, back (downgradient) column only.*

Table 2.2. Comparative performance summary of the different chitin grades in the batch microcosm and semi-continuous column experiments.

	Microcosm experiment			Semi-continuous column experiment		
	SC-20	**SC-40**	**SC-80**	**SC-20**	**SC-40**	**SC-80**
Average pH	7.69±0.03	7.57±0.04	7.48±0.17	7.40±0.16	7.19±0.31	6.18±0.38
Average acetate (mM)	0.65±0.02	0.28±0.01	1.50±0.65	15.16±2.08	11.08±0.66	22.19±1.81
Average propionate (mM)	0.11±0.005	0.02±0.002	0.09±0.02	2.11±0.13	2.12±0.32	2.44±0.49
Average caprioate (mM)	1.52±0.23	0.06±0.07	0.19±0.0	47.17±6.19	10.78±1.69	0.29±0.11
Average final ethene*	16.42±0.20	3.47±0.35	7.67±6.29	106.4	88.20	18.18
Average *Dehalococcoides* influent column end (cells/g)	N/A	N/A	N/A	7.06±1.99E06	5.31±0.29E05	ND
Average *Dehalococcoides* effluent column end (cells/g)	N/A	N/A	N/A	4.28±0.05E06	7.49±2.3E05	1.34±0.84E06

Microcosm values are duplicate averages (±one standard deviation); column values are averages (±one standard deviation) observed within all columns for that substrate type (described in Table 1), with the exception of ethene values which are for the 100 uM TCE columns only to enable comparison with the microcosms (76 uM TCE).

**Average final ethene concentrations for the microcosm experiment are umol/ bottle, and for the semi-continuous experiment are umol/L measured in one pore volume (6 ml) of the liquid effluent.*

N/A = not applicable. ND = not detected.

Table 2.3. Comparison of cummulative fermentation products for SC-20 and SC-40 grade crab shell at proppant loadings (sand:chitin) of 5:1 and 15:1.

		Cumulative VFA (mmol)			
		acetate	propionate	butyrate	caproate
SC-20	5:1	1.7±0.1	0.2±0.02	0.9±0.2	5.2±0.8
	15:1	0.6±0.1	0.1±0.02	0.5±0.1	2.4±0.2
SC-40	5:1	1.4±0.2	0.3±0.1	0.2±0.1	1.5±0.4
	15:1	0.6±0.1	0.1±0.01	0.1±0.03	0.5±0.4

Table 2.4. Carbon balance calculations for each of the semi-continuous columns.

Column ID	Chitin grade	Proppant Loading (sand:substrate)	Measured Substrate Loaded (g)	Measured Substrate Fermented (g)	Calculated C from Chitin (g)	Calculated C from VFAs (g)	Calculated Excess Carbon (VFA C - Chitin C) (g)	Theoretical Protein (g)	Theoretical Protein (%)
1	SC-20	5:1	2.70	1.25	0.12	0.42	0.30	0.55	20.216
8	SC-20	5:1	2.70	1.49	0.14	0.55	0.40	0.74	27.485
14	SC-20	5:1	2.70	1.24	0.12	0.52	0.41	0.74	27.495
2	SC-20	15:1	1.20	0.48	0.05	0.21	0.17	0.30	25.260
9	SC-20	15:1	1.20	0.61	0.06	0.25	0.19	0.35	29.514
15	SC-20	15:1	1.20	0.54	0.05	0.23	0.17	0.32	26.560
3	SC-40	5:1	2.70	0.81	0.15	0.18	0.02	0.04	1.520
10	SC-40	5:1	2.70	0.96	0.18	0.21	0.03	0.06	2.101
16	SC-40	5:1	2.70	0.81	0.15	0.13	-0.02	0.00	0.000
4	SC-40	15:1	1.20	0.41	0.08	0.10	0.02	0.04	3.676
11	SC-40	15:1	1.21	0.28	0.05	0.05	0.00	0.00	0.000
17	SC-40	15:1	1.21	0.36	0.07	0.04	-0.02	0.00	0.000
5	SC-80	5:1	1.10	1.09	0.41	0.12	-0.29	0.00	0.000
12	SC-80	5:1	1.11	1.11	0.42	0.10	-0.32	0.00	0.000
18	SC-80	5:1	1.10	1.13	0.43	0.10	-0.33	0.00	0.000
6	SC-20*	15:1	1.20	0.52	0.05	0.26	0.21	0.38	31.804
13	SC-20*	15:1	1.20	0.41	0.04	0.49	0.45	0.82	68.453
19	SC-20*	15:1	1.20	0.78	0.07	0.18	0.11	0.20	16.336
7	N/A**	1:0	0.00	0.00	0.00	0.00	0.00	0.00	0.000

* Neg. Control (SC-20; no inoculum). **Neg. Control (Inoculated; no chitin) N/A=not applicable

CHAPTER 3

Spatial and Temporal Abundance of *Dehalococcoides* spp. and Reductive Dehalogenases During Trichloroethene-Degradation

3.1 Abstract

The critical transition periods during the reductive dechlorination of trichloroethene (TCE) to ethene were evaluated by correlating the relative abundance of RDase genes with water quality data in a set of replicate, sacrificial continuous-flow columns. Biostimulation with crab shell chitin provided a suite of electron donors that ultimately led to the complete dechlorination of TCE to ethene. VC-stall in one column was overcome through the temporary (~1 Pore Volume (PV)) addition of a nutrient rich medium, which was needed to "jump start" ethene production, but was not needed to sustain it. After the addition of the medium, an increase in the abundance of the bvcA gene was observed, indicating that strains with the bvcA gene (eg., strain BAV1) may have a specialized nutritional requirement for metabolizing VC. In general, *Dehalococcoides* cell numbers were observed to increase with increasing distance from the influent (<1% of the total bacterial population in the influent area of the columns to 9-40% in the second half of the columns), but to decrease with time in most columns. The majority of the column samples had sums of the RDase genes that accounted for only a portion (8 - 87%) of the total *Dehalococcoides* 16S rRNA gene copies, suggesting that there may be *Dehalococcoides* spp. that do not contain any of the known RDases. T-

RFLP community analysis suggested an increased importance of planktonic species over the course of remediation, and indicated similar membership but different community structure in ethene-producing columns that did and did not experience VC stall. This manuscript is in progress. All blank sections represent uncompiled work.

3.2 Introduction

Trichloroethene (TCE, C_2HCl_3), a persistent groundwater pollutant, is currently one of the most prevalent contaminants found in industrialized countries (Tandoi et al., 1994; Wu et al., 1998; He et al., 2003; Stroo et al., 2003; McKnight et al., 2010). The restoration of TCE contaminated aquifers presents a significant challenge to the government and industry, due to its dense, hydrophobic, and toxic nature. As our understanding of the microbiology involved in reductive dechlorination of chlorinated compounds has rapidly increased over the last fifteen years, the remedial approach of *in-situ* bioremediation using halorespiring bacteria has become standard practice. While there are several microbial species capable of degrading chlorinated compounds, the only halorespiring microbes identified to date as capable of the complete dechlorination of TCE to ethene (C_2H_4) belong to the genus *Dehalococcoides*.

While *Dehalococcoides* spp. are naturally present at many contaminated locations and can support the complete reductive dechlorination of TCE to ethene, other locations have either no native *Dehalococcoides* spp., inadequate buffering capacity to maintain the pH within the circumneutral range that these organisms require, lack of suitable trace nutrients, or are missing key strains that have the proper reductive dehalogenase (RDase)

genes, any of which can result in cis-dichloroethene (cDCE)/vinyl chloride (VC) stall. In pure culture, *Dehalococcoides* spp. require a complex medium for growth (Freeborn et al., 2005), and it has been shown that long-term dechlorination activity of enrichment cultures can be maintained through the addition of filtered culture supernatant from more diverse systems (DiStefano et al., 1992). This led many researchers to experiment with nutritional amendments, such as yeast extract, in hopes of enhancing the activity of halorespiring populations (Kengen et al., 1999; Long and Borden, 2006). While the "key nutrient" for *Dehalococcoides* spp. has not been definitively identified, their nutritional dependency on the activities of other organisms has led to the generally accepted theory that they require the presence of a larger microbial community to sustain dechlorination activity (Distefano et al., 1992; He et al., 2007). Provided that nutritional requirements are met, bioaugmentation with one of several commercially available, mixed microbial cultures has led to successful reduction of TCE to ethene at many field locations that were lacking a suitable indigenous population (Major et al., 2001; Ritalahti et al., 2005; Amos et al., 2008; Hood et al., 2008; Peale et al., 2010).

Since not all *Dehalococcoides* species have the ability to transform DCE and VC, the key to achieving complete dechlorination of TCE to ethene is the expression of a combination of RDase genes from one or more organisms which can support the entire dechlorination pathway. To date, several RDase genes involved in the dechlorination of TCE have been characterized based on their function, including tceA, vcrA, and bvcA. The tceA RDase gene, which encodes for a protein that catalyzes the metabolic transformation of TCE to cDCE and cDCE to VC, and the cometabolic reduction of VC to ethene, was cloned and sequenced from *Dehalococcoides ethenenogenes* strain 195

(DET0079; GenBank accession no. NC_002936, Magnuson et al., 2000) and from *Dehalococcoides* strain FL2 (GenBank accession no. AY165309, He et al., 2005). One of the most recently discovered chloroethene RDases, vcrA, catalyzes the reduction of cDCE to VC and VC to ethene, and was cloned from *Dehalococcoides* strain GT (GenBank accession no. AY914178, Youlboong et al., 2006). The expression of the bvcA gene is associated with the dechlorination of VC to ethene only, and was cloned from *Dehalococcoides* strain BAV1 (DehaBAV1_0847, GenBank accession no. NC_009455, Krajmalnik-Brown et al., 2004). Of the *Dehalococcoides* spp. that have been characterized to date, the majority are believed to contain only one RDase gene; however, there are several (like strains 195 and VS) that are known to contain at least two (Behrens et al., 2008).

When analyzing halorespiring communities, the use of quantitative polymerase chain reaction (qPCR) analysis targeting the RDase genes provides a distinct advantage over quantification analyses targeting signature regions of the 16S rRNA gene due to the high degree of *Dehalococcoides* 16S rRNA gene similarity (>98% identity). Although previous studies have applied quantification of these RDase genes to evaluate the physiology of *Dehalococcoides* spp. in samples from contaminated sites, laboratory cultures, and environmental enrichments (Ritahlati et al., 2006; Holmes et al., 2006), little is known about the distribution of these functional genes in engineered treatment systems.

To better understand how RDase genes affects dechlorination activity during enhanced bioremediation, studies linking microbial quantification and chemical data have recently become more prevalent in the literature. Several laboratory studies (Behrens et

al., 2008; Amos et al., 2009) have used continuous flow columns to assess the relationship between reductive dechlorination and the microbial community by extracting DNA from solid-phase samples collected at the conclusion of the experiment. In Behrens et al. (2008), qPCR analysis revealed the highest expression of RDase genes near the influent, and higher bacterial clone sequence diversity near the effluent, of columns inoculated with a mixed culture and fed tetrachloroethene (PCE) and lactate. Amos et al. (2009) performed a similar lactate-fed column study, but with dense nonaqeous phase liquid (DNAPL) PCE, and found that the number of *Dehalococcoides* cells in liquid samples increased by 1 to 2 orders of magnitude over the length of the column, but only represented 12-77% of the total *Dehalococcoides* cells associated with sediments in the column. Amos et al. therefore cautioned that the form of the environmental sample (i.e., ground water versus aquifer solids) may impact the interpretation of bioremediation site assessment and monitoring. While these laboratory studies provided insights into microbial distribution and activity during reductive dechlorination, they were conducted with a continuous feed of aqueous electron donor, a practice which is becoming less common as more slowly fermentable substrates are used (ex., chitin, tetrabutoxysilane, powdered whey, and various vegetable oils). The use of fermentable substrates not only requires lower operation and maintenance costs, but also provides a suite of electron donors that can promote the activity of indigenous microorganisms, which may be an important factor for sustaining the long-term activity of halorespiring organisms (Bell et al., 2001; Wiedemeier et al., 2001; Yang and McCarty, 2002; Brennan et al., 2006; Long and Borden, 2006; Lee et al., 2008; Seungho and Semprini, 2009). With this in mind, a field study using a fermentable powdered whey substrate evaluated the *Dehalococcoides*

population in TCE-contaminated groundwater through the use of qPCR and reverse transcription qPCR (RT-qPCR) (Lee et al., 2008). They found an overall increase of more than 3 orders of magnitude in the total *Dehalococcoides* cell numbers over a 1-year period of bioaugmentation and biostimulation, and found that cells containing the tceA gene made up less than 5% of the total *Dehalococcoides* population, whereas vcrA- and bvcA-containing cells represented the dominant fractions and were the most physiologically active. They also discovered two RDase genes that were not previously monitored with RT-PCR, one of which appeared to be co-located in genomes containing the bvcA gene. This type of evaluation of changes in gene expression over time is critical to improving the efficiency of remediation systems, but a deliberate examination of the critical transition periods in the dechlorination pathway would enable more targeted strategies to improve bioremediation performance.

The aim of this study was to examine the critical transition periods during the reductive dechlorination of TCE to ethene with a fermentable substrate by correlating the relative abundance of RDase genes with water quality data over space and time. This was done using a series of replicate, continuous flow columns, which were sequentially sacrificed at each of the major chemical transitions during the reduction of TCE to ethene (i.e., TCE to cDCE, cDCE to VC, and VC to ethene). The use of replicate columns enabled sampling of solid-phase column materials for DNA extraction throughout the experiment without disturbing the ongoing activity in other columns. In all columns, crab shell was used as the substrate to provide both a long-lived, slowly fermentable electron donor source (chitin), as well as natural buffer (calcium carbonate) to help regulate potential pH fluctuations that can be encountered in practice due to the formation

of reductive dechlorination byproducts (i.e., HCl). To assess how dechlorination activity may be affected by the development and dynamics of the general microbial community, terminal fragment length polymorphism (T-RFLP) was also used to evaluate microbial community profiles generated from planktonic pore water samples collected from the effluent of the system, as well as from the biofilm formed on upstream column sediments over time.

3.3 Materials and Methods

3.3.1 Chemicals

TCE and cis-1,2-DCE were purchased from VWR International, LLC (West Chester, PA). VC and ethene gases were purchased from Alltech Associates, Inc. (State College, PA). Volatile fatty acid standards were created from a 10 mM volatile acid standard mix obtained from Supelco (Bellefonte, PA). The commercial crab shell product (SC-20), derived from Dungeness crab shells, was used as received from the distributors (JRW Bioremediation, LLC, Lenexa, KS). This material has a reported composition of 10-30% chitin, 5-50% protein, and 20-60% calcium carbonate(JRW Bioremediation, personal communication, 2010), and a measured bulk density of 0.45 g mL^{-1}. Silica sand (16-20 mesh, donated by Badger Mining Corp., Berlin, WI) was used as an inert proppant in the column study to maintain hydraulic conductivity, and had a bulk density of 0.81 g mL^{-1}. Aliquots of crab shell and sand were sterilized by gamma irradiation (Radiation Science and Engineering Center Breazeale Nuclear Reactor,

Pennsylvania State University, University Park, PA) for use in the sterile control column. All other chemicals used were reaction grade or higher quality.

3.3.2 Inoculum

The commercially available dechlorinating microbial consortium, Bio-Dechlor INOCULUM (BDI), was donated by the Löffler group (then at Georgia Institute of Technology). BDI is a mixed *Dehalococcoides* culture capable of complete dechlorination of TCE to ethene, and has been successfully applied via bioaugmentation at many field locations (Amos et al., 2008; Ritalahti et al., 2005). BDI is known to contain at least three *Dehalococcoides* spp. strains: FL2 (He et al., 2003a); BAV1 (He et al., 2003b); and GT (Ritalahti et al., 2006); as well as the PCE to cDCE dechlorinating *Dehalobacter spp.* (Amos, 2007). Strain FL2 metabolically dechlorinates TCE to VC and contains the tceA gene, strain BAV1 metabolically dechlorinates DCEs to ethene and contains the bvcA gene, and strain GT metabolically dechlorinates TCE to ethene and contains the vcrA gene (Fletcher et al., 2011; Sung et al., 2006; He et al., 2003). Each of the known strains in the BDI culture is believed to contain only one RDase gene. The mixed BDI culture was maintained in a mineral salt medium prepared as previously described (Löffler et al., 1996; Sung et al., 2003) amended with 5 mM lactate and 0.33 mM TCE, and was periodically transferred as needed.

3.3.3 Groundwater Source

Natural groundwater was obtained from a well that draws from the medium to coarse-grained Bald Eagle sandstone formation located in the Seven Mountains, Potter Township, Centre County, Pennsylvania (40N 46' 7"; 77W 35' 44.85"). Prior to amendment with TCE, the groundwater was filter sterilized (0.2 um), degassed with Argon, and maintained under an Argon headspace in a 50 L carboy wrapped in opaque black plastic. Groundwater aliquots were transferred with a sterile 60 mL pipet to degassed, autoclaved, 160 mL serum bottles, fitted with Teflon-lined stoppers and aluminum crimp caps. A TCE stock solution was added to each bottle for a final concentration of approximately 13 mg TCE L^{-1} (100 uM). The bottles were allowed to equilibrate for 24 hours prior to transfer to 100 mL gas tight syringes.

3.3.4 Continuous Flow Columns

Glass columns (69 cm length x 4.5 cm i.d.) equipped with glass end caps and kovar tips were retrofitted with 5 lateral (borosilicate glass vials) side ports spaced at approximately 10 cm intervals along the length of each column to facilitate sample collection (Pennsylvania State University Glass Shop, University Park, PA). The side ports were sequentially numbered from 1 (influent end) to 5 (effluent end). All column components were cleaned in alconox and water and sterilized by autoclave prior to packing. The influent end of each column, except the two control columns, was wet packed in degassed groundwater with a "substrate plug" (30 g chitin mixed with 150 g sand) extending approximately 15 cm into the column, and the remaining length was wet

packed with silica sand only (Figure 3.1). The Sterile Control column was packed using gamma irradiated crab shell and silica sand, and the No Chitin Control column was wet packed with silica sand only. Following packing, the columns were flushed with approximately three pore volumes of degassed groundwater, inoculated, except for the un-inoculated and sterile control columns, with 150 mL of the BDI culture, and allowed to incubate overnight. The total mass of TCE, cDCE, VC, and ethene in the inoculum for each column averaged 634.1±185.5 umol. Prior to inoculation, an aliquot of the BDI consortium was preserved for subsequent DNA extraction and qPCR analyses. Following incubation, the columns were fed a continuous flow (0.5 mL hr^{-1}) of natural groundwater amended with approximately 13 mg L^{-1} (100 uM) of TCE using a model PHD 22/2000 syringe pump (Harvard Apparatus, Holliston, MA) equipped with 100 mL gastight syringes (SGE Inc., Austin, TX). The gastight syringes were attached to the columns with sterilized stainless steel (SS) fittings and tubing. Due to the slow flow rate, effluent sampling loops (1.5 mL volume) constructed from 1/16 inch SS tubing (Alltech Associates Inc., Deerfield, IL) were attached to the exit of each column by a three-way Swagelok valve (Pittsburgh Valve and Fitting Company, Pittsburgh, PA) to enable more efficient sample collection. Breakthrough curves with a conservative chloride tracer test indicated a hydraulic residence time in the packed columns of 20 days, corresponding to a total pore volume (PV) of 240 mL. The entire experiment was conducted over a period of 369 days (~18.5 PV), which was the time required to observe the complete dechlorination of TCE to ethene.

Throughout the experiment the columns were periodically sacrificed to capture a “snapshot” of the microbial community at major transitions throughout the reductive

dechlorination pathway (i.e.: TCE to cDCE; cDCE to VC; and VC to ethene; Figure 3.2). Changes observed in the longitudinal chlorinated ethene profiles indicated when each column should be sacrificed, thereby preserving the microbial community during the initial dominance of TCE (t=20), the conversion of TCE to cDCE (t=55), the transition of cDCE to VC (t=114), and ultimately ethene production (t=369). Sacrificing a column consisted of removing the column from the continuous flow set-up, placing the column in a laminar flow hood, removing end caps and lateral side port stoppers, and retrieving internal column materials using a flame sterilized metal spatula. Collected column materials were placed into sterile 50 mL conical vials and stored at -80º C until analysis. In addition to serving as a source of microbial DNA, stored column materials were also later used to determine the utilization of the solid-phase substrate (crab shell) over time.

3.3.5 Analytical Methods

Chlorinated ethenes and ethene concentrations in aqueous samples were determined using gas chromatography as previsously described (Chapter 2, Section 2.3.6). Briefly, 100 uL of aqueous sample was injected into a 20 mL autosampler vial, heated in a Agilent G1888 Network Headspace Autosampler to volatilize the sample into the headspace, and 1-mL of the headspace injected into an Agilent model 6890N headspace gas chromatograph (GC) equipped with a GS-Q column (30 m length, 0.53 mm diameter, J&W Scientific) and a flame ionization detector (FID).

Volatile fatty acid (VFA) analysis was performed by a Waters 2695 high-performance liquid chromatograph (HPLC) equipped with an Aminex HPX-87H ion

exclusion column (Bio-Rad Laboratories, Inc.) and a photodiode array detector. Aqueous samples were placed in 2 mL eppendorf tubes, and stored at -20 º C until analysis. Prior to analysis, the samples were thawed, filtered to remove any suspended solids, and the supernatant (diluted with deionized water) added to 0.2 mL of 2.0 N H_2SO_4 in 2.0 mL autosampler vials.

The pH of each sample was measured using a Fisher Accumet AB15 pH meter equipped with a SympHony pH electrode (VWR International, West Chester, PA). Moisture content was determined gravimetrically according to the procedure described in Standard Methods (APHA, 2005).

3.3.6 DNA extraction

Total community, chromosomal DNA was collected from effluent pore water (3.6 mL) and solid phase (1-2 g of wet sand or sand/crab shell mixture) samples. DNA was extracted using an UltraClean™ Soil DNA Kit (MoBio Laboratories, Carlsbad, CA) following the bacterial protocol for maximum yields with modifications as previously described by Ritalahti et al. (2006). The extracted DNA was stored at -20 °C until qPCR analysis.

3.3.7 Quantitative Real-Time PCR (qPCR)

Primers and TaqMan probe sets were used to quantify total numbers of bacterial and *Dehalococcoides* 16S rRNA genes, as well as tceA, bvcA, and vcrA genes, as

previously described (Harms et al., 2003; Ritalahti et al., 2006). Quantification of *Dehalobacter* 16S rRNA genes used primers previously designed by Smits et al. (2004), and a TaqMan probe (Dhb613; 5'-CCAATGCTTTACGGAGTTAAGCTCCG) was specifically designed for this study using NetPrimer (premierbiosoft.com). A table of the primers and probes used in this study is presented in Table 3.1.

qPCR was conducted using a spectrofluorimetric thermal cycler (iCycler, BioRad, Hercules, CA) with cycle parameters as follows: *Dehalococcoides* 16S as described by He et al. (2003a) and Ritalahti et al. (2006); *Dehalococcoides* functional genes as described by Ritalahti et al. (2006); *Dehalobacter* as described by Smits et al., 2004 and Amos et al. (2008); and bacterial 16S as described by Harms et al. (2003). For *Dehalococcoides* sp. 16S and functional genes and *Dehalobacter* sp. 16S qPCR reactions, each well contained a total reaction volume of 30 uL consisting of 15 uL 2X Master Mix (USB, Cleveland, OH), 300 nM of each primer and probe (Biosearch Technologies, Novato, CA), and 3 uL of template DNA. Each well for Bacterial 16S qPCR reactions contained a total reaction volume of 25 uL consisting of 15 uL 2X Master Mix (USB, Cleveland, OH), 300 nM of each primer and probe (Biosearch Technologies, Novato, CA), and 3 uL of template DNA.

Calibration curves (arbitrarily set cycle threshold value [C_t] versus log gene copy number per uL) were created using 10-fold serial dilutions of quantified plasmids carrying a single, cloned target gene. 16s rRNA and functional gene fragment cloning was performed on samples collected from maintained BDI cultures, except the BAV1 bvcA functional gene which was cloned and donated by the Löffler group. Details of standard curve range and qPCR efficiency can be found in Appendix B.

The number of gene copies in a known amount of DNA, the number of target genes per gram of wet column materials, and amplification efficiencies were calculated using equations described by Ritalahti et al. (2006). Amplifications were conducted in triplicate along with negative (no template DNA) control. Randomly chosen qPCR products were sequenced to confirm successful amplification of the desired target gene.

3.3.8 PCR Amplification

DNA extracted from the effluent pore water, as well as from upgradient packing materials retrieved from the influent- and middle-sections of the column was amplified using PCR for T-RFLP analysis. The 16S rRNA gene was amplified using universal bacterial primers 5'-Hex-GTTTGATCCTGGCTCAG (11F) and 5'-ACGGTTACCTTGTTACGACTT (1492R). PCR reaction mixtures contained 1X PCR buffer, 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 uM, each primer at a concentration of 0.1 uM, and 2.5 units of Taq DNA polymerase (USB Corporation, Cleveland, OH) in a final reaction volume of 100 uL. The 11F primer was fluorescently labeled at the 5' end using hexachlorofluorescein (Integrated DNA Technologies, Inc, Skokie, IL). DNA amplification was performed with a thermocycler (iCycler, BioRad, Hercules, CA) by using the following program: 95°C for 15 minutes, followed by 35 cycles consisting of denaturation (30 seconds at 94°C), annealing (45 seconds at 56°C), and extension (2 minutes at 72°C) with a final extension at 72°C for 3 minutes. Replicate PCR reactions were performed for each sample to minimize random PCR artifacts. Amplicons generated in two replicate PCR reactions for each sample were

combined (200 uL total vol.) and purified using a QIAGEN QIAquick PCR Purification Kit following the standard protocol, and eluted in a final volume of 50 uL.

3.3.9 TRFLP

Purified PCR products were digested with the restriction enzymes HhaI, MspI, and RsaI individually as follows. An aliquot of clean PCR products containing approximately 500 ng DNA were added to 2.5 uL reaction buffer and 1 uL restriction enzyme solution (10U) in total reaction volume of 25 uL. The digestions were incubated at 37 °C for 3.5 hours followed by heating at 65 °C for 10 minutes to inactivate the enzyme. To confirm successful restriction digests, 15 uL of each digest was analyzed on 2% agarose gels run at 100V for 40 mins (Ritalahti and Löffler, 2004). Sequencing and T-RFLP analyses were performed at the Penn State Genomics Core Facility (University Park, PA) using an ABI Hitachi 3730XL DNA analyzer (Applied Biosystems, Foster City, CA) using the 1200 LIZ size standard (Applied Biosystems, Foster City, CA). T-RFLP profiles were generated in GeneMapper (Applied Biosystems) with peaks only >100 fluorescent units included in the analysis.

The three T-RFLP profiles (HhaI, MspI, and RsaI) generated in GeneMapper were used to identify bacterial species using the computer program Fragsort (www.oardc.ohio-state.edu/trflpfragsort/). Fragsort compares TRFs generated from processed samples to TRFs from corresponding in silico amplification and digestion of RDP II (Ribosomal Database Project; Maidak et al., 2001) alignments and outputs a list of microorganisms and TRF sizes correlating with the multiple experimentally generated

TRFLP profiles. Using this technique we were able to identify 68-95% (range of all samples) of the total peak area (i.e., all the major peaks were identified). The output includes GenBank accession numbers which were compiled for each sample and entered into Batch Entrez (www.ncbi.nlm.nih.gov/sites/batchentrez) to retrieve FASTA sequences. The FASTA sequences were then screened and aligned using the computer program Mothur (Schloss et al., 2009), followed by alignment to the SILVA bacteria 16S rRNA sequence alignment database (14,956 sequences).

3.3.10 Statistics

Statistical analyses of qPCR data were performed using Minitab software with one-way analysis of variance (ANOVA) and Tukey multiple comparison testing. A 95% confidence interval was selected for all analyses establishing a critical p-value of 0.05. Statistical analysis of the distribution of Dhc 16S rRNA and RDase genes (qPCR quantification) throughout the course of reductive dechlorination, as well as along the length of each column, was conducted to assess whether the means of any of the compared groups were statistically different. If a significant difference (p-value <0.05) was reported for the one-way ANOVA analysis, a multiple comparison using the Tukey test was conducted to reveal which means were significantly statistically different.

The computer program Mothur was used to statistically analyze the T-RFLP data. Mothur quantifies the degree of overlap between the memberships and structures of different microbial communities by assigning sequences to operational taxonomic units (OTUs) based on genetic distance between sequences. Specifically, after the T-RFLP

sequences were aligned, a distance matrix was created by calculating uncorrected pairwise distances among all sequences. Next, based on the distance matrix, the cluster command was used to group sequences in OTUs at different clustering distances using the rule of nearest neighbor and a precision of P=1000. Finally, the total number of OTUs (defined at discrete clustering levels) was estimated and FASTA representative sequences for each OTU were generated. The following specific computations were performed in Mothur: Chao1 richness; Shannon's diversity index; Good's coverage; Jaccard similarity index; and the Yue and Clayton similarity index. The equations for each of these computations are provided in Appendix C. Visualization tools including Venn diagrams and dendrograms were used to screen sequences based on quality, and were viewed using the software programs Inkscape (Free Software Foundation, Inc., Boston, MA) and Geneious (Biomatters, Ltd., Auckland, NewZealand).

3.4 Results and Discussion

3.4.1 Column Performance

Continuous flow columns were sequentially sacrificed as each chlorinated ethene became dominant in the system (i.e., first TCE, then DCE, VC, and finally ethene). The first column (Column 1) was sacrificed at one pore volume (t = 20 days) to preserve the microbial community during the initial stages of reductive dechlorination soon after bioaugmentation. TCE concentrations increased from approximately 100 uM to a maximum of 450 uM in the mid-section of the column, and then decreased to below 100

uM in the effluent. Average concentrations of cDCE (33±15.6 uM), VC (164±16.3 uM), and ethene (<8 uM) were also detected along the length of the column (Figure 3.3 panel A). The high concentration of TCE and the presence of less chlorinated species likely indicate that some of the dechlorination products associated with the inoculum still remained in the column after one pore volume, which is not surprising since these compounds are hydrophobic and exhibit high sorption properties.

Column 2 was sacrificed after the transition from TCE to cDCE was detected (t = 55 days). The transition zone was located 13 cm from the influent (i.e., at port 1) after which the concentrations of both TCE (60 uM) and cDCE (82 uM) remained relatively constant. VC (<15 uM) and ethene (<7 uM) were detected at distances greater than 13 cm from the influent but at lower concentrations (Figure 3.3 panel B).

At day 109, the transition zone from cDCE to VC was detected 13 cm from the influent, and Column 3 was sacrificed shortly thereafter (t = 115 days). TCE concentrations decreased from 33 uM in the influent area of the column to zero by mid-column, while cDCE increased to 56.81 uM by mid-column, and VC was relatively constant (126 uM) throughout the remaining length of the column (Figure 3.3 panel C).

The two remaining sacrificial columns both represent ethene production, but at very different times. The fourth column (Column $Ethene_{115}$; t=115; Figure 3D) produced ethene much earlier than the fifth column (Column $Ethene_{369}$; t=369) which experienced VC stall for 311 days before eventually producing ethene (Figure 3.3 panel E). These columns represent interesting deviations, both involving the ultimate goal of chloroethene bioremediation: ethene production. The $Ethene_{115}$ column produced ethene at the same

time that other columns (Column 3 and the $Ethene_{369}$ column) were still dominated by VC production. Although TCE (40 uM) was present in the influent area of the $Ethene_{115}$ column, it quickly decreased to below detection for the remaining length of the column. VC and cDCE were below detection along the entire length of the column (Figure 3.3 panel D). Ethene concentrations were relatively stable in the $Ethene_{115}$ column, averaging 51±20 uM along the entire length, with concentrations of 65 uM at 13 cm from the influent (port 1), 37 uM mid-column (port 3), and 71 uM at 56 cm from the influent (port 5).

In contrast, the $Ethene_{369}$ column experienced an extended period of VC stall, producing VC throughout the majority of the experiment and only producing ethene after the influent feed was switched from TCE-amended groundwater to TCE-amended BDI media on day 338. Anion analysis of the groundwater and the composition of the BDI media can be found in Appendix D. Eighteen days (approximately 1 PV) after changing the feed to BDI media (t = 356 days), ethene, although not detected in samples collected from the lateral side ports, was detected in the effluent of the column at a concentration of 3.04 uM. At this point, the feed was returned to TCE-amended groundwater, and ethene production continued. At t = 369 days, ethene concentrations had increased and were detected along the entire length of the column (average 38.96 uM ± 10.38, Figure 3.3 panel E), at which point the column was sacrificed and preserved for later microbial community analysis.

In the field, accumulation of the more toxic VC is a serious issue that is not fully understood. One possible explanation as to why some sites experience stall while others

do not is the presence or absence of specific strains of *Dehalococcoides*. It has been hypothesized that the absence of strains possessing RDases involved the final dechlorination step of converting VC to ethene (i.e., bvcA or vcrA) causes dechlorination to stall at VC production (Scheutz et al., 2008). Other factors that may affect the degree of dechlorination include a sufficient supply of electron donor (Cupples et al., 2004), the presence of fermentative microbes to supply hydrogen (Duhamel and Edwards, 2006), and the ability of dechlorinating spp. to outcompete other microorganisms for key nutrients and electron donors (Fennel and Gossett, 1998). However, the incomplete dechlorination in this experiment appears to be linked to a lack of key nutrients since ethene production commenced after the introduction of a nutrient rich media indicating the microbial population was capable of complete dechlorination and the supply of electron donor was not limited. An early study on anaerobic reductive dechlorination using an enrichment culture was only able to sustain dechlorination past 40 days through the addition of a filtered culture supernatant from a more diverse system and concluded that the dechlorinating population had a nutritional dependency on the activities of other organisms (DiStefano et al., 1992). Potentially critical key nutrients present in the BDI media may include vitamin B12. A microcosm study by He et al. (2007) found that the addition of vitamin B12 at a concentration of 0.025 mg L-1 increased the final ethene-to-VC ratio, doubled the rate of TCE dechlorination, and resulted in twice the number of tceA RDase genes (determined through qPCR analysis) versus microcosms receiving 0.001 mg L-1 of the vitamin. Concentrations higher than 0.025 mg L-1 did not have any additional benefit. Although further research would be necessary to determine the critical nutrient(s) required to overcome VC stall, it is clear in this study that it was only

needed to "jump start" ethene production, and was not required continuously. This intermittent, rather than continuous, addition of nutrients has potential cost-saving implications in field settings.

The cumulative molar mass of chlorinated ethenes recovered in the effluent of each column was calculated using Eq. 3.1:

$$Mass\,\mathrm{Recovery}\,(\%) = \frac{\left[Influent\ TCE\ (umol) + \left(TCE + cDCE + VC + ethene\ (umol)\right)_{inoculum}\right]}{\left(TCE + cDCE + VC + ethene\ (umol)\right)_{effluent}} * 100 \quad (3.1)$$

The calculations indicated that a portion of the chlorinated compounds introduced into each column initially accumulated (i.e., sorbed) to the column packing materials, and then were gradually released into the effluent as the experiment progressed. Specifically, the molar mass recoveries of chlorinated compounds were 49, 58, 48, 60, and 97% for Column 1 (TCE; t = 20), Column 2 (TCE to cDCE; t = 55), Column 3 (cDCE to VC; t = 115), Column $Ethene_{115}$, and Column $Ethene_{369}$, respectively. The longest running column, $Ethene_{369}$, reached a mass balance equilibrium by day 290, with the molar concentration of TCE in the influent being within 10% of the sum of chlorinated ethenes in the effluent.

Three control columns were also evaluated as part of this study. The column without crab shell (No Chitin Control) initially demonstrated dechlorination, but gradually tapered off, with all degradation products below detection after ~10 PV. The initial degradation was most likely supported by electron donors and nutrients associated with the inoculum, and without the additional fermentable substrate, dechlorination was

not sustainable. The two remaining uninoculated control columns, containing crab shell (No Inoculum column) and gamma-irradiated crab shell (Sterile Control column), behaved similarly, with no dechlorination products detected. The cumulative mass recoveries of chloroethenes in the No Chitin, No Inoculum, and Sterile Control columns were determined to be 101, 112, and 97%, respectively.

3.4.2 Chitin Utilization

After each column was sacrificed, samples (n=3) of the column material were dried at 104 °C for 4.5 hours, cooled, weighed, ashed at 600 °C in a muffle furnance for 24 hours, and reweighed to determine the average mass of shell material fermented. Throughout the experiment the amount of crab shell material (30 g starting mass) fermented increased from 5.9 g (19.7%) at day 20 to 10.9 g (36.3%) by day 369. The corresponding rate of fermentation over the course of the experiment decreased from 0.30 g day^{-1} to 0.03 g day^{-1}. The change in the rate of fermentation is most likely reflective of the rapid fermentation of the protein portion of the shell material followed by the slower fermentation of the chitin portion. The calculated amount of chitin (eq. 3.2) and protein (eq. 3.3) fermented (based on 10.9 g of fermented shell material) was determined to be 2.2 g and 3.6 g respectively:

$$Chitin = shell\ mat.\ ferm.\left(\frac{0.2g\ chitin}{1g\ chitinous\ mat.}\right) \tag{3.2}$$

$$\mathrm{Pr}otein = shell\ mat.\ ferm.\left(\frac{0.12g\ chitin}{1g\ chitinous\ mat.}\right) \tag{3.3}$$

Correspondingly, the amount of carbon associated with the chitin (eq. 3.4) and protein (eq. 3.5) portion of the fermented shell was calculated to be 13.6 and 85.7 mmol respectively (t=369):

$$Carbon_{chitin} = shell\ \ mat.\ ferm.\left(\frac{0.2g\ chitin}{1g\ chitinous\ mat.}\right)\left(\frac{mol\ chitin}{203.22g\ chitin}\right)\left(\frac{96.08g\ carbon}{mol\ chitin}\right) \quad (3.4)$$

$$Carbon_{protein} = shell\ \ mat.\ ferm.\left(\frac{0.12g\ protein}{1g\ chitinous\ mat.}\right)\left(\frac{mol\ protein}{352.2g\ protein}\right)\left(\frac{192.16g\ carbon}{mol\ protein}\right) \quad (3.5)$$

It should be noted these calculations assume that the loss of crab shell mass is solely due to protein and chitin fermentation and ignores any mass loss associated with potential calcium carbonate dissolution; therefore the resulting values for protein and chitin utilization may be an overestimate. Nevertheless, this substrate utilization rate provides a conservative estimate for practitioners considering the use of crab shell as a substrate in groundwater remediation.

3.4.3 VFAs

VFA concentrations were found to be similar to previous studies using crab shell (Brennan et al., 2006; Robinson-Lora and Brennan, 2009; Chapter 2) with concentrations in the effluent peaking by day 19 (~1 PV) in all columns except the column packed with sterilized shell material (Sterile Control column). The dominant VFAs detected in the active columns were acetate, propionate, and butyrate, with peak concentrations ranging

from 53.5-87.7, 15.5-27.2, and 12.34-25.4 mM, respectively. Acetate production was sustained at an average concentration of approximately 9.3 mM throughout the experiment, with a final concentration of 2.0 mM (t = 369 days). The cumulative mass of acetate, propionate, and butyrate increased over time, with masses totaling 40.10, 7.46, and 10.04 mmol, respectively, in the last active column sacrificed (t = 369 days). VFA concentrations in the No Inoculum and Sterile Control columns were lower than the other columns with the cumulative masses of acetate, propionate, and butyrate totaling 31.8, 5.5, and 6.7 mmol, respectively in the No Inoculum Control and 32.1, 3.7, and 7.5, respectively, in the Sterile Control column. The peak concentrations of acetate, propionate, and butyrate in the No Inoculum Control column were 45.8, 8.1, and 11.7 uM, respectively, and 32.1, 3.7, and 7.5 mM, respectively for the Sterile Control column.. Also the VFA concentrations in the Sterile column did not peak until day 32. The lower VFA concentrations and lag in production observed in the Sterile Control column indicate that the native microbial population associated with the shell material is important in catalyzing fermentation reactions.

3.4.4 Quantifying 16S rRNA and RDase Genes

q-PCR was used to monitor the relative abundance and distribution of the 16S rRNA gene of bacterial and *Dehalobacter* cells, and the 16S rRNA and RDase genes of *Dehalococcoides* cells. Template DNA was extracted from solid-phase (sand or sand-shell material mixture) samples collected from each of the five lateral side ports as well as the influent and effluent areas of each sacrificial column. All target genes were

detected in the inoculated columns and only bacterial cells were detected in the un-inoculated columns.

In order to compare inoculum cell numbers (per mL) to cell numbers associated with solid phase samples (per gram), the cell numbers in solid phase samples are reported here per mass of wet column materials containing a pore volume of 1 mL. To determine the average mass of wet column material associated with a 1 mL pore volume, the moisture content of 32 samples (n_{sand}=16; $n_{sand\text{-}shell\ mix}$=16) extracted from the columns was determined. It was found that a 1 mL pore volume is associated with 6.36 g of wet sand and 4.88 g of wet sand-shell mixture. These values correspond to porosities of 0.19 and 0.26, for the sand and the sand-shell mixture, respectively, which are within the range of course sands.

Initially there was a 2-fold increase in the number of *Dehalococcoides* 16S rRNA gene copies present in the columns, from 2.9 ± 1.2 x 10^8 (cells introduced with the inoculum; average ± one standard deviation) to 6.0 ± 0.54 x 10^8 (Column 1; t = 20 days). After this initial increase, however, *Dehalococcoides* cell numbers began to drop in almost all of the remaining columns. *Dehalococcoides* cell numbers decreased 2.5-fold in Column 2 (t = 55 days), Column 3 decreased 5-fold, (t = 115 days), Column $Ethene_{369}$ decreased 5-fold (t = 369 days), and the No Chitin Control column decreased 12-fold. Conversely, and unlike all the other columns in the experiment, Column $Ethene_{115}$ had a 2.6-fold increase in *Dehalococcoides* cells over the course of 115 days, which is not unexpected given its superior dechlorination activity.

ANOVA analysis of the q-PCR analysis of *Dehalococcoides* 16S rRNA genes indicated a significant difference in the spatial (different sample locations along the

length of each column at each time point; all p-values <0.001) and temporal (same sample location over time; all p-values <0.003) distributions. A general trend of increasing *Dehalococcoides* cell numbers with increasing distance from the influent can be seen in Figure 4. This is consistent with the results of Amos et al. (2009) who observed *Dehalococcoides* cell titers increase by 1 to 2 orders of magnitude over the length of continuous flow columns containing PCE DNAPL fed with lactate, but opposite that of Behrens et al. (2008), who found the highest expression *Dehalococcoides* gene copies and RDase genes near the influent of their PCE- and lactate-fed column. Over time, *Dehalococcoides* cell numbers decreased in most columns (Figure 5), which may be attributed to cell washout (Amos et al., 2009). Even though *Dehalococcoides* numbers generally decreased with time relative to the initial numbers in the inoculum, the lowest number of cells quantified in this study ($1.4 \pm 0.3 \times 10^6$ cells/6.36 grams wet sand-shell mixture) is still greater than the 10^7 *Dehalococcoides* cells/L believed to be required for dechlorination (Ernst, 2009).

The *Dehalococcoides* spp. population accounted for 1.8-6.5% of the total bacterial community in columns containing crab shell, which is similar to the 1-3% found in columns run by Behrens et al. (2008). A distinct spatial trend was observed throughout the experiment with the proportion of the total bacterial community represented by *Dehalococcoides* cells increasing from <1% in the influent area of the columns to 9-40% in the second half of the columns. In contrast, *Dehalococcoides* spp. accounted for 37.1% of the total bacterial community in the No Chitin Control column, where the distribution of cells was relatively constant along the length of the column and ranged between 36-64%. Mathematically, a greater proportion of *Dehalococcoides* in the

columns enhanced with an electron donor could reflect an increase in the *Dehalococcoides* population or a decrease in the number of bacteria. Closer examination of the individual qPCR results indicates that columns enhanced with crab shell material supported a greater total number of bacteria ($10^6 – 10^9$ cells/g) and *Dehalococcoides* ($10^4 – 10^7$ cells/g) than those without substrate ($10^6 – 10^7$ and $10^5 – 10^6$ cells/g, respectively).

Comparison of the *Dehalococcoides* 16S rRNA gene copy number to the sum of the functional genes did not always produce the ideal 1:1 ratio, or 100% recovery (Figure 3.6). A 1:1 ratio is expected because all of the identified *Dehalococcoides* strains in the BDI inoculum are believed to possess one 16S rRNA gene and one functional gene (i.e., FL2 contains tceA, BAV1 contains bvcA, and GT contains vcrA). Although a 1:1 ratio was observed for the total 16S rRNA genes and the sum of the functional genes in inoculum samples within the analytical uncertainty of the qPCR technique, the majority of the column samples had sums of the RDase genes that accounted for only portion (8 - 87%) of the total 16S rRNA gene copies. This is similar to the findings of Behrens et al. (2008), who quantified a 1:1 ratio in their inoculum, but found that the sum of all RDase genes accounted for only 35 – 50% of *Dehalococoides* spp. 16S rRNA gene copies in half of their column sediments. This indicates that either there were *Dehalococcoides* spp. cells that grew to significant numbers in the column which did not contain any of the known RDases (Behrens et al., 2008), or that there were quantitative inaccuracies associated with using different, individual plasmid standards for each target gene (Holmes et al., 2006). In the column that produced the most ethene, recovery exceeded 100% (Figure 3.6 panel C), which may be explained by the selection of *Dehalococcoides*

spp. which contain more than RDase gene, which has been noted previously for *Dehaloccoides* strain 195 and VS (Behrens et al., 2008).

Several trends were observed in the distribution of the different RDase genes in relation to one another over the course of the experiment. In the TCE-grown inoculum, the vcrA gene was the most abundant ($2.47\pm0.38 \times 10^8$ copies/mL) and made up 73% (as determined by the sum of the abundances of all three RDase genes) of the *Dehalococcoides* population in the TCE-grown inoculum. In Column 1, tceA was present in the greatest quantity at all sampling locations (84%). In Column 2 (TCE to cDCE), vcrA dominated (84%), while in Column 4 (cDCE to VC) tceA was again present in the greatest abundance (78%). In the two ethene-producing columns, no single RDase was dominant throughout the entire length of either column, but in general, tceA and vcrA were in the greatest abundance, with lesser quantities of bvcA. In Column $Ethene_{115}$, the proportions of tceA, vcrA, and bvcA were 40, 53, and 6%, respectively, whereas in Column $Ethene_{369}$, the relative percentages of the genes accounted for 34, 42, and 25%, respectively. The greatest numbers of bvcA genes were found in the ethene-producing columns. Interestingly, the only time the bvcA gene was detected in greater amounts than the other RDases was in the sample collected from mid-column in Column $Ethene_{369}$, where it made up 73% of the total abundance of RDase genes. This was the only column that was stimulated through the addition of a nutrient rich medium, which may imply a greater nutrient requirement for cells containing the bvcA gene (i.e., strain BAV1) than the vcrA gene (i.e., strain GT).

Dehalobacter spp. cell numbers (Appendix E) were detected in the lowest abundance ($4.24\pm1.08 \times 10^3$) in the influent end of Column 1 (TCE column), and in the

greatest abundance ($9.58 \pm 1.08 \times 10^6$) in the influent end of the $Ethene_{115}$ column. The *Dehalobacter* spp. population accounted for <1.7% of the total bacterial community in columns containing crab shell, and <7.2% of the total bacterial community in the No Chitin Control column. The low abundance of *Dehalobacter* in all the columns indicates this species was not a major contributor to dechlorination in these communities.

3.4.5 T-RFLP

T-RFLP was used to evaluate changes in microbial diversity due to its ease of use and potential for rapid analysis. DNA templates for T-RFLP analyses were extracted from effluent pore water samples and solid-phase (sand or sand-crab shell mixture) collected from the influent end and port 3 (34 cm from influent, mid-column). Of the three, individual enzymatic digestions performed on each sample, the RsaI digestion produced the most peaks in the mid-column sample collected from Column 2 (TCE to cDCE) and the MspI digestion produced the most peaks in all other samples. The T-RFLP analyses did not indicate the presence of *Dehalococcoides* spp. or *Dehalobacter* spp., which is not unexpected given their relatively small abundance (1.7-6.5%) relative to the rest of the microbial community. To ensure the peaks were not overlooked, T-RFLP profiles were manually checked for the peak sizes predicted for *Dehalococcoides* (200bp, HhaI; 512bp, MspI; 441bp, RsaI) and *Dehalobacter* (230bp, HhaI; 138bp, MspI; 497bp, RsaI) from in silico analyses. Even when peaks between 50-100 fluorescent units (FU) (other analyses typically do not include peaks <100 FU) were included, the

predicted peak sizes for these species were not seen in any of the profiles. Using Fragsort, the three (HhaI, MspI, RsaI) individual T-RFLP profiles generated for each sample were simultaneously evaluated and and matched to DNA sequences from RDP II.

Mothur was used to screen, align, and cluster the sequences identified by Fragsort, and OTU bins were generated using 0.05 and 1.0 distance cutoffs. Based on the 0.05 and 0.10 OTU cutoffs, Good's coverage, Chao1 richness, and Shannon's diversity index were calculated (Table 3.2). The Good's coverage was satisfactory (>75%; Kormas et al., 2009) for the majority of the samples using the 0.10 OTU cutoff, and was used for all subsequent statistical analyses. Richness estimates generally increased over time while the diversity estimates were relatively constant. The greatest richness, diversity, and number of OTUs were from DNA digests from sand samples located in the middle columns sacrificed by day 115, and from pore water samples for the columns with the longest incubation time ($Ethene_{369}$ and No Chitin columns). This indicates an increase in the diversity of planktonic species over time. Since T-RFLP analysis does not disern between live and dead cells, this could be an indication of planktonic species becoming more important over time, or it could be reflective of a greater amount of DNA being captured from the sloughing of dead cells. Statistical analysis describing the similarities in membership and structure in the columns are given in Table 3.3. The Jaccard similarity index showed the greatest membership similarity (0.85) between DNA extracted from the influent end materials and effluent pore water of Column 2. This result is supported by the overlapping membership shown in the Venn diagram for Column 2 which illustrates that 95% of influent community is shared with the pore water community (Figure 3.7). Membership similarity for all other comparisons was <0.55 and

was significantly different than 1.0 (Table 3.3). In comparing structures of the communities in Column 2, the Yue and Clayton similarity index (thetaYC) indicated a high similarity between influent end sediments and effluent pore water communities (0.96), and moderate similarity between mid-column sediments and both effluent pore water and influent end sediments (0.68 and 0.67, respectively). In the remaining inoculated columns containing crab shell, varying levels of dissimilarity (range of 0.03-0.51) were found between pore water and solid-phase samples (Table 3.3). Neither the pore water nor the solid-phase samples effectively captured all the dominant populations in the continuous flow columns, indicating that field assessments which are traditionally done through the extraction of DNA from groundwater samples may give an incomplete picture of the subsurface microbiology at contaminated sites.

Phylogenetic analysis of the T-RFLP based sequences showed that unclassified bacteria were the most abundant sequences analyzed. Other phylogenetic groups identified included *Bacteroidetes*, *Proteobacteria*, *Gammaproteobacteria*, and *Firmicutes*. The sulfate reducing bacteria *Desulfuromusa* and several fermentors (*Clostridium*, *Desulfovibrio*) including the chitin fermentor *Actinobacteria* were also identified. Fermenters such as *Desulfovibrio*, *Eubacterium*, *Acetobacterium*, *Citrobacter*, and *Clostridium* have often been identified in the mixed microbial communities containing *Dehalococcoides* species (Richardson et al., 2002; Ritalahti and Löffler, 2004; He et al., 2007), many of which are similar to those observed in this study.

Repeated attempts to amplify DNA from the archaeal population in this study resulted in PCR products consisting of primer dimers. The concentration of DNA extracted from the archaea may have been below the concentration required for efficient

amplification. If the difficulties in amplification were due to concentration limitations, then molecular analyses such as T-RFLP, that only capture the dominant species, would likely not have been able to identify archaeal species.

3.5 Conclusions

Results from this laboratory column study provide a better understanding of the critical transition periods during the reductive dechlorination of TCE through the correlation of RDase gene abundance with water quality data over space and time. The following key conclusions are noted:

1. The crab shell substrate fermented at a rate of 0.30 g day^{-1} to 0.03 g day^{-1} in this system and provided a suite of electron donors that successfully stimulated complete reductive dechlorination of TCE to ethene. A column with sterile crab shell exhibited a lag in VFA production, indicating that the native microbial population associated with the shell material is important in catalyzing fermentation reactions.

2. VC-stall in one column was overcome through the temporary (~1 PV) addition of a nutrient rich medium, which was needed to "jump start" ethene production, but not needed to sustain it. After the addition of the medium, an increase in the abundance of the bvcA gene was observed, indicating that strains with the bvcA gene (ex., strain BAV1) may have a specialized nutritional requirement for metabolizing VC.

3. In general, *Dehalococcoides* cell numbers were observed to increase with increasing distance from the influent (<1% of the total bacterial population in the influent area of the columns to 9-40% in the second half of the columns), but to decrease with time in most columns.

4. The majority of the column samples had sums of the RDase genes that accounted for only portion (8 - 87%) of the total 16S rRNA gene copies, suggesting that there may be *Dehalococcoides* spp. that do not contain any of the known RDases.

5. Through T-RFLP analysis, the greatest richness, diversity, and number of bacterial OTUs were found from sand samples at the beginning of the experiment (< ~10 PV), and from pore water samples at the end of the experiment, possibly indicating an increased importance of planktonic species over time. Similar membership but different community structure was observed between ethene-producing columns that did and did not experience VC stall.

6. Statistical analysis of T-RFLP data indicated that neither the pore water nor the solid-phase samples effectively captured all the dominant populations in the continuous flow columns, which has implications for field assessments of subsurface microbiology.

The results of this study demonstrate that continuous flow columns are an effective means to evaluate the complexities of reductive dechlorinating systems in a controlled laboratory setting. Molecular and chemical analyses demonstrated that crab

shell is an effective means of biostimulation and influences the distribution of *Dehalococcoides* populations as well as overall bacterial populations. This study also indicated that DNA extracted from pore water represented different community membership and structure than DNA extracted from solid-phase samples. Future work is needed to further characterize the relationships between different functional groups in mixed halorespiring cultures to assess possible syntropic interactions that may drive reductive dechlorination as in the $Ethene_{115}$ column. Also, additional research is needed to better understand the specific nutrient requirements of certain *Dehalococcoides* strains (like BAV1) that can be stimulated to overcome VC stall.

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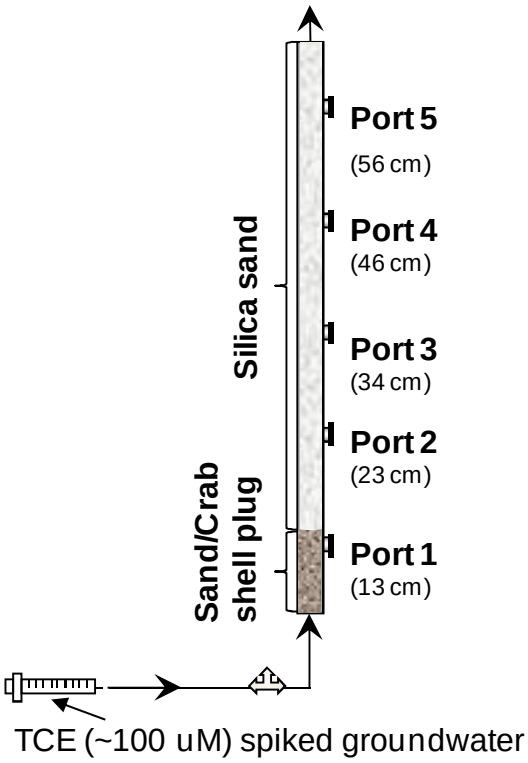


Figure 3.1. Representative column schematic. Note: A total of 8 columns were used in the experiment. The no chitin control column contained silica sand only and the sterile control column contained gamma irradiated crab shell and silica sand. The distance of each sampling port from the influent is listed below the port number.

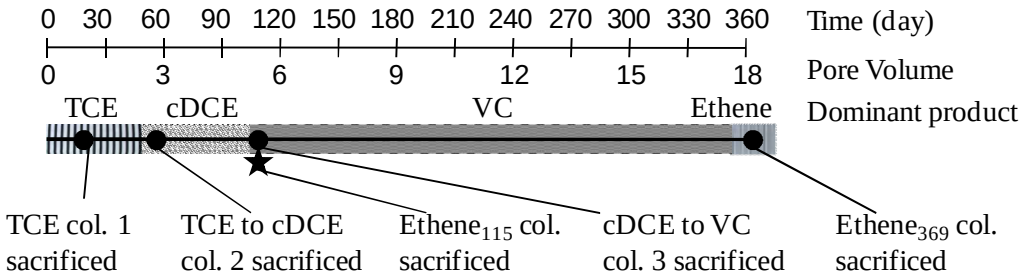


Figure 3.2. Experimental time line representing when each column was sacrificed, the approximate pore volumes, and the dominant chlorinated compound. Note: The un-inoculated control columns containing crab shell packing and sterilized crab shell packing were sacrificed on day 265 and the no chitin control column was sacrificed on day 369.

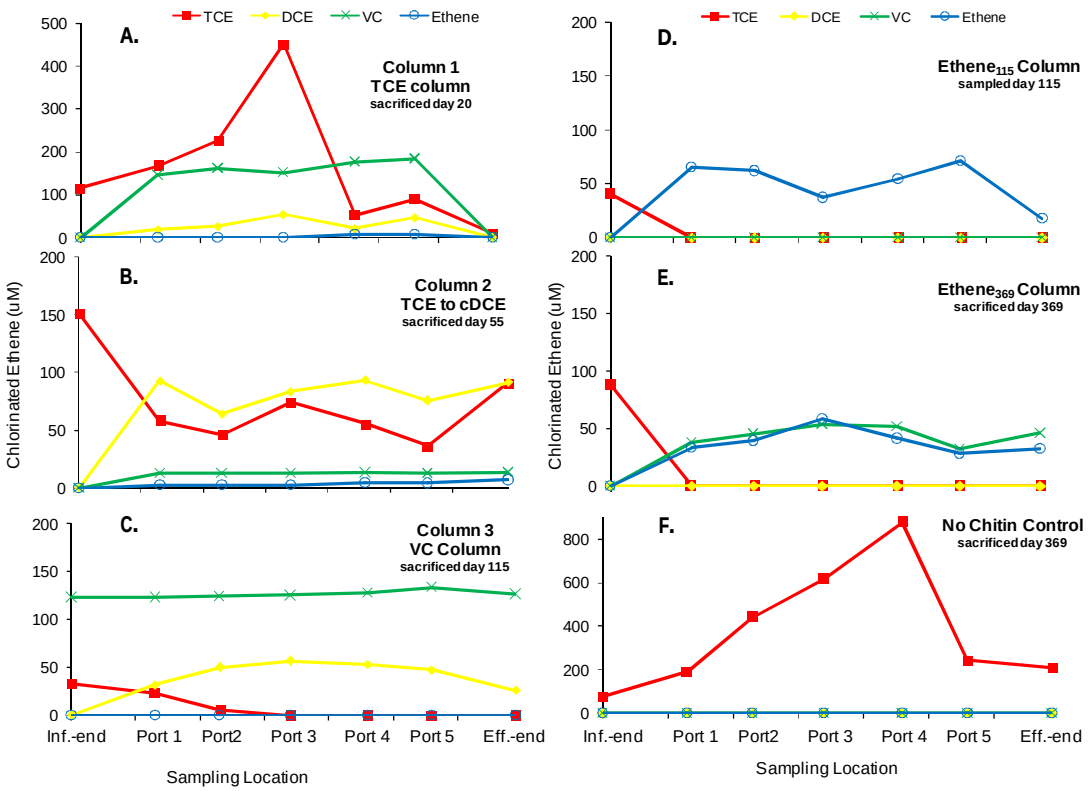


Figure 3.3. Changes in chlorinated ethene profiles observed over time along the length of columns supplied with a continuous source of 100 uM TCE–amended groundwater and containing crab shell substrate (except where noted). Note the different y-axis scale on panels A and F.

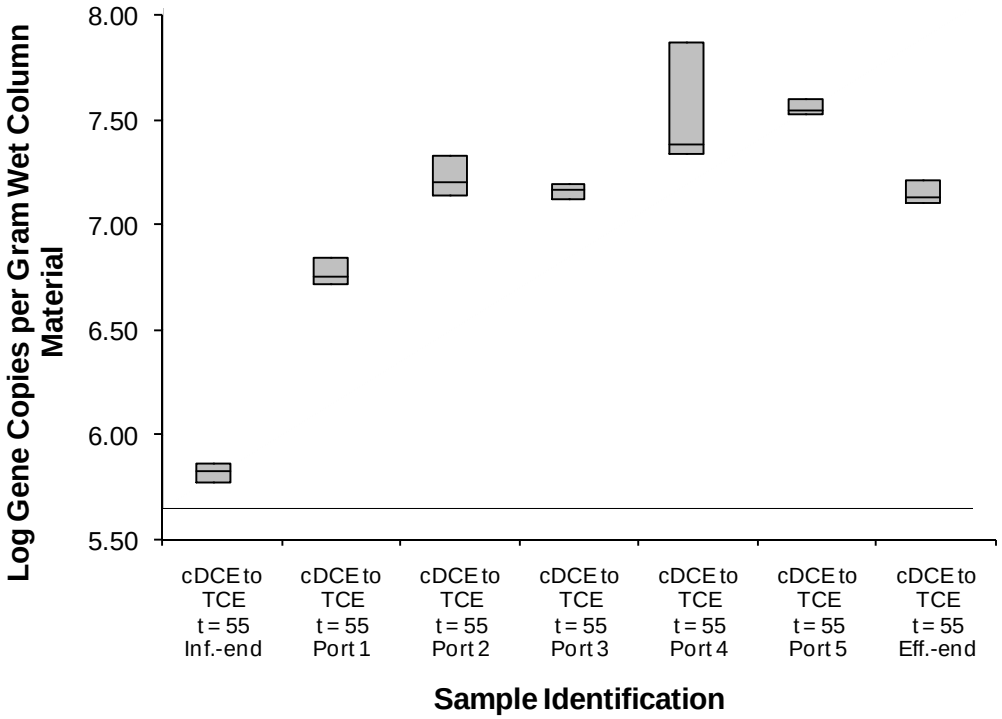


Figure 3.4. Spatial distribution of *Dehalococcoides* cells along the length of Column 2. Data were measured as 16S rRNA copies per gram of wet column, and analyzed using one way ANOVA (95% CI).

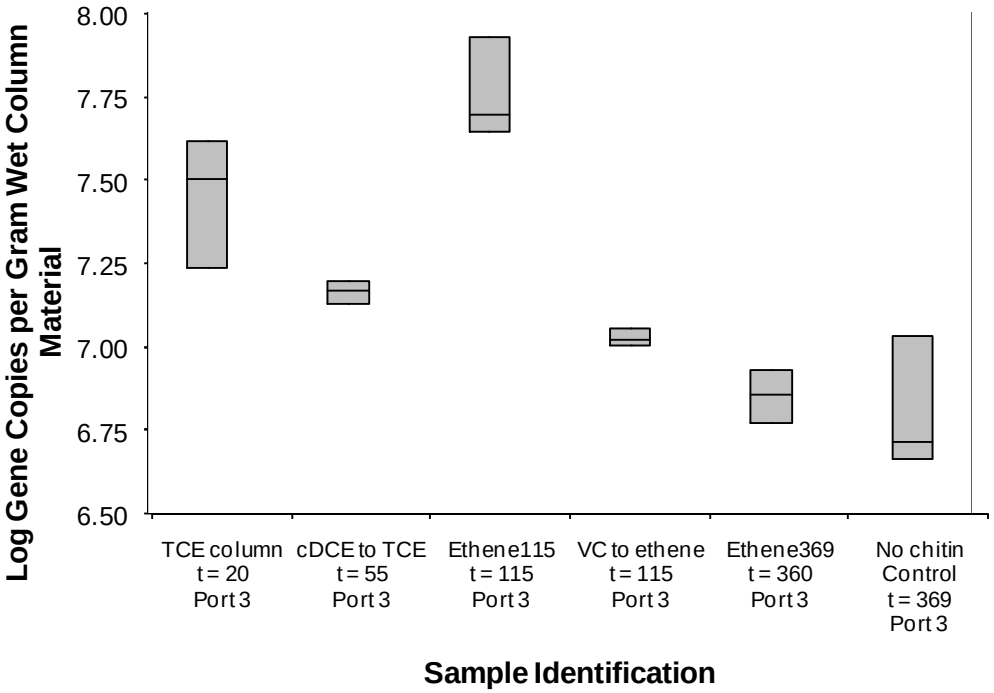


Figure 3.5. Temporal distribution of *Dehalococcoides* cells in the middle of the columns (port 3) over time. Data were measured as 16S rRNA copies per gram of wet column, and analyzed using one way ANOVA (95% CI).

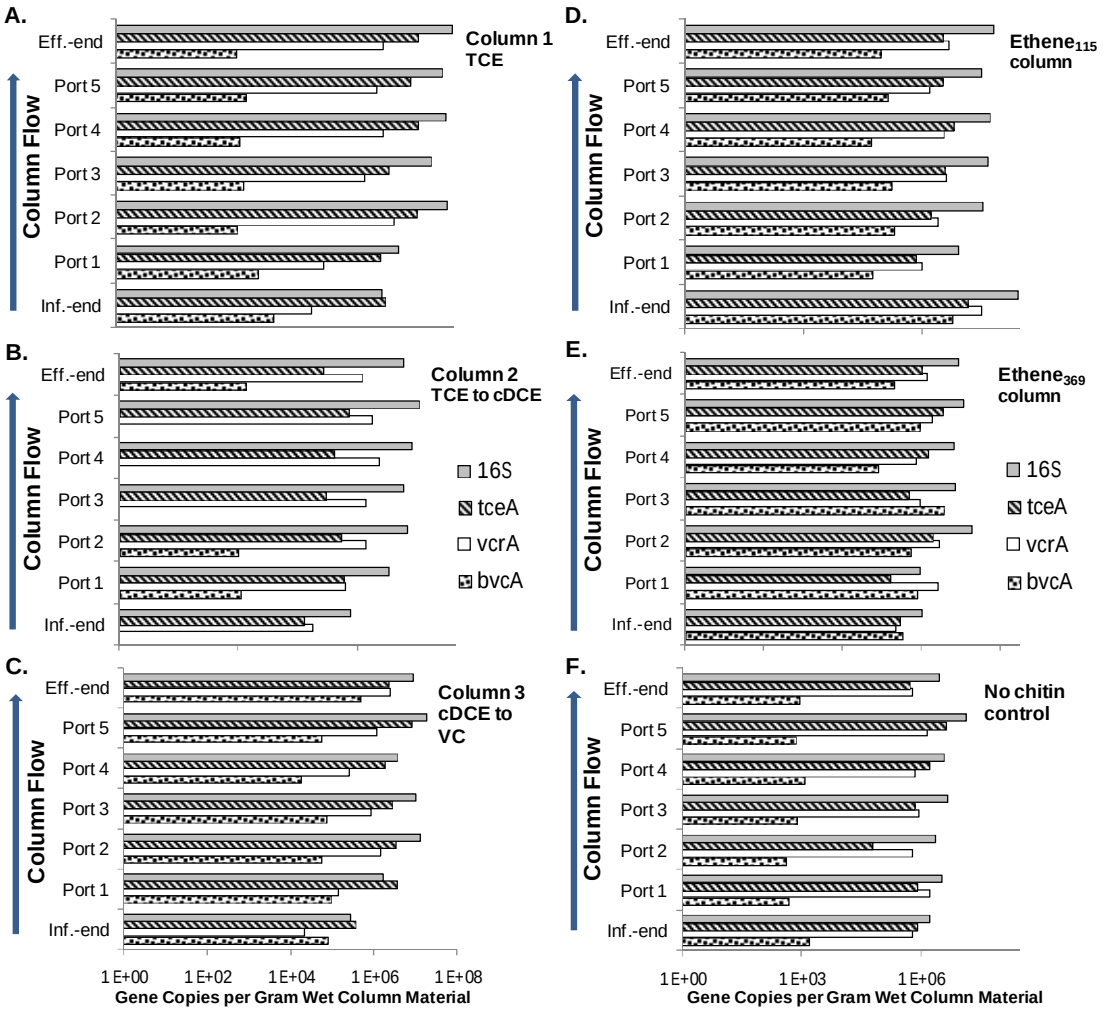


Figure 3.6. Relative abundance of *Dehalococcoides* 16s rRNA and RDase genes in the packing materials of the continuous flow columns over time. Gene copy numbers were determined by qPCR using template DNA extracted from the seven different sections in each column. Each bar represents the average of triplicate qPCR reactions.

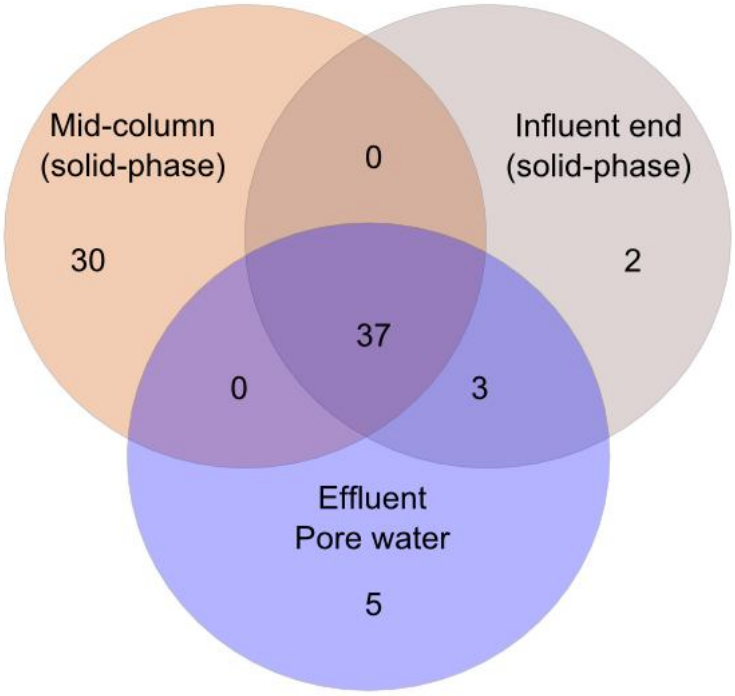


Figure 3.7. Venn diagram comparing the pooled OTU (0.10 distance cutoff) memberships found in one pore water and two solid-phase samples collected from Column 2 (TCE to cDCE; t=55 days). The pore water was collected from the effluent, and the solid-phase samples were collected from the influent-end and mid-column (Port 3).

Table 3.1. TaqMan probes and primers used to quantify Dehalococcoides spp. 16S rRNA and RDase genes, Dehalobacter spp. 16S rRNA, and total Bacterial 16S rRNA genes.

Target Organism	Target gene	Primer/Probe	Sequence	Reference/Source
Dehalococcoides sp.	**16S**	Dhc1240Probe	5'-FAM-TCCTCAGTTCGGATTGCAGGCTGAA-TAMRA	He, et al. 2003
		Dhc1200F	5'-CTGGAGCTAATCCCCAAAGCT	He, et al. 2003
		Dhc1271R	5'-CAACTTCATGCAGGCGGG	He, et al. 2003
Dehalococcoides strain BAV1	**bvcA**	Bvc977Probe	5'-CAL Fluor Red 590-TGGTGGCGACGTGGCTATGTGG-BHQ-2	Ritalahti, et al. 2006
		Bvc925F	5'-AAAAGCACTTGGCTATCAAGGAC	Ritalahti, et al. 2006
		Bvc1017R	5'-CCAAAAGCACCACCAGGTC	Ritalahti, et al. 2006
Dehalococcoides strain FL2	**tceA**	TceA1294Probe	5'-CAL Fluor Orange 560-TGGGCTATGGCGACCGCAGG-BHQ-1	Ritalahti, et al. 2006
		TceA1270F	5'-ATCCAGATTATGACCCTGGTGAA	Ritalahti, et al. 2006
		TceA1336R	5'-GCGGCATATATTAGGGCATCTT	Ritalahti, et al. 2006
Dehalococcoides strain GT	**vcrA**	VcrA1042Probe	5'-FAM-CGCAGTAACTCAACCATTTCCTGGTAGTGG-BHQ-1	Ritalahti, et al. 2006
		VcrA1022F	5'-CGGGCGGATGCACTATTTT	Ritalahti, et al. 2006
		VcrA1093R	5'-GAATAGTCCGTGCCCTTCCTC	Ritalahti, et al. 2006
universal bacteria	**16S**	Bac1115Probe	5'-FAM-CAACGAGCGCAACCC-BHQ-1	Ritalahti, et al. 2006
		Bac1055YF*	5'-ATGGYTGTCGTCAGCT	Ritalahti, et al. 2006
		Bac1392R	5'-ACGGGCGGTGTGTAC	Ritalahti, et al. 2006
Dehalobacter sp.	**16S**	Dhb613Probe	5'-CAL Fluor Orange 560-CCAATGCTTTACGGAGTTAAGCTCCG-BHQ-1	This study
		Dhb441F	5'-GTTAGGGAAGAACGGCATCTGT	Smits, et al. 2004
		Dhb645R	5'-CCTCTCCTGTCCTCAAGCCATA	Smits, et al. 2004

*due to alignment of the 16S rRNA gene sequences revealing a T versus a C in the fifth position from the 5' end in some strains (ie: dehalobacter restrictus, several cetobacterium spp.) the bacterial primer 1055F as described by Harms et al. was modified whereby the residue C was synthesized as a Y (Y=C/T) to account for both nucleotide variations and to include these organisms in the total bacterial count.

Table 3.2. Mothur calculated richness, diversity, and coverage at OTU cutoffs of 0.05 and 0.10 distance units based on the observed OTUs from T-RFLP analysis.

			0.05							0.1						
Sample ID	**No. of reads**	**OTUs**	**Chao[a]**	**Chao lci[b]**	**Chao hci[c]**	**H'[d]**	**H' lci**	**H' hci**	**Good's coverage**	**Chao**	**Chao lci**	**Chao hci**	**H'**	**H' lci**	**H' hci**	**Good's coverage**
Col. 2 Influent	82	42	282	130	720	3.49	3.24	3.73	0.54	123	71	269	3.35	3.12	3.59	0.65
Col. 2 Mid	127	76	236	150	421	4.01	3.82	4.19	0.55	157	107	269	3.87	3.69	4.05	0.65
Col. 2 pore water	86	50	235	118	554	3.53	3.29	3.77	0.55	144	81	315	3.42	3.19	3.65	0.63
Col. 3 Influent	59	23	68	36	180	2.24	1.85	2.64	0.71	37	24	84	2.16	1.79	2.54	0.80
Col. 3 Mid	129	85	370	215	707	4.15	3.97	4.34	0.47	230	137	451	3.91	3.73	4.08	0.63
Col. 3 pore water	160	77	289	169	568	3.83	3.63	4.02	0.66	169	109	311	3.68	3.50	3.87	0.74
$Ethene_{115}$ Influent	51	37	182	85	471	3.47	3.24	3.69	0.41	108	55	278	3.25	3.02	3.48	0.57
$Ethene_{115}$ Mid	195	114	433	277	740	4.35	4.19	4.51	0.55	216	149	359	4.07	3.91	4.22	0.71
$Ethene_{115}$ pore water	114	65	261	146	541	3.82	3.63	4.02	0.57	164	101	319	3.71	3.52	3.90	0.66
$Ethene_{369}$ Influent	87	63	318	165	700	3.99	3.81	4.17	0.41	154	94	303	3.80	3.62	3.98	0.56
$Ethene_{369}$ Mid	135	68	234	141	446	3.67	3.46	3.89	0.61	103	73	176	3.41	3.21	3.62	0.76
$Ethene_{369}$ pore water	280	162	687	452	1113	4.65	4.50	4.79	0.55	376	258	600	4.33	4.19	4.47	0.69
No chitin Influent	125	60	150	99	267	3.55	3.33	3.77	0.66	95	66	170	3.13	2.88	3.37	0.76
No chitin Mid	154	65	155	104	272	3.61	3.42	3.81	0.72	114	71	230	3.08	2.88	3.29	0.81
No chitin pore water	162	86	288	181	514	4.01	3.83	4.20	0.60	228	135	449	3.50	3.29	3.71	0.70

a. Chao1 richness estimator.

b. Chao1 richness lower bound 95% confidence interval.

c. Chao1 richness higher bound 95% confidence interval.

d.Shannon diversity index

Table 3.3. Pairwase comparisons of bacterial communities sampled from the effluent pore water, influent column materials, and mid-column (port 3) materials from each column based on T-RFLP results. Shared OTUs, Jaccard similarity index, and the Yue and Clayton nonparametric maximum likelihood estimator of similarity were calculated with the software program Mothur.

Comparison		shared OTUs	Jaccard abundance	Jaccard index	Yue & Clayton index
Column 2 Mid	Column 2 Influent	37	1.00	0.51	0.67
Column 2 Mid	Column2 Pore water	37	1.00	0.49	0.68
Column 2 Influent	Column2 Pore water	40	1.00	0.85	0.96
Column 3 Mid	Column 3 Influent	14	0.51	0.17	0.03
Column 3 Mid	Column3 Pore water	31	0.61	0.26	0.32
Column 3 Influent	Column3 Pore water	18	0.80	0.24	0.03
$Ethene_{115}$ Mid	$Ethene_{115}$ Influent	29	0.98	0.32	0.22
$Ethene_{115}$ Mid	$Ethene_{115}$ Pore water	52	1.00	0.55	0.51
$Ethene_{115}$ Influent	$Ethene_{115}$ Pore water	27	1.00	0.44	0.33
$Ethene_{369}$ Mid	$Ethene_{369}$ Influent	27	1.00	0.28	0.12
$Ethene_{369}$ Mid	$Ethene_{369}$ Pore water	46	0.65	0.27	0.17
$Ethene_{369}$ Influent	$Ethene_{369}$ Pore water	49	1.00	0.30	0.16
No Chitin Mid	No Chitin Influent	36	1.00	0.45	0.65
No Chitin Mid	No Chitin Pore water	32	1.00	0.30	0.41
No Chitin Influent	No Chitin Pore water	26	0.64	0.23	0.37

CHAPTER 4

Visualization of Spatial Distribution of *Dehalococcoides* spp. and Archaea During Trichloroethene Degradation using Multiplex-FISH

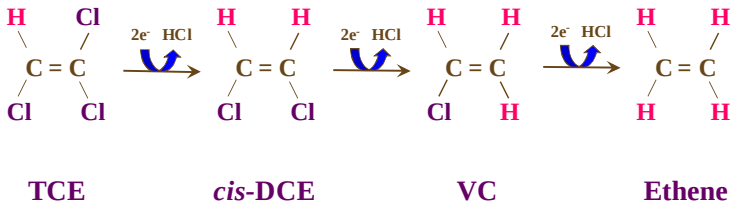
4.1 Abstract

The ecological relationships in reductively dechlorinating communities are complex, and require that the *in situ* biofilm of the system be visualized to confirm speculations regarding localization of species and potential competition for substrates, yet an intact biofilm from an actively dechlorinating system has never been imaged. To accomplish this, a novel biofilm collection device was designed, constructed, and inserted into replicate columns fed a continuous flow of TCE-amended groundwater and enhanced with a slowly fermentable substrate. The static glass packets in the device facilitated establishment of a biofilm and allowed the structural integrity of the biofilm to be maintained throughout the process of sample collection and fluorescence in situ hybridization (FISH). Visualization of the biofilm showed aggregates containing methanogens in close proximity to *Dehalococcoides* cells, indicating that competition, if it exists, is not creating spatial stratification and that the substrate (crab shell) was capable of supporting multiple hydrogenotrophs. Also, the co-location of methanogens and Dhc in biofilm aggregates suggests a synergistic relationship in which non-dechlorinating microbes assist in the dechlorination process either directly through interspecies hydrogen transferor indirectly through the provision of micronutrients. This

technique has the potential to be applicable in other studies to evaluate the architecture of biofilms and the performance remediation systems.

4.2 Introduction

Bioremediation of chloroethene contaminated sites using native or bioaugmented halorespiring bacteria is an effective means of remediation. To date, members of the genus *Dehalococcoides* (Dhc) are the only microorganisms capable of the complete dechlorination of trichloroethene (TCE, C_2HCl_3) to the non-hazardous daughter product ethene (C_2H_4) (Homes et al., 2006; Sung et al., 2006; Cupples et al., 2003; He et al., 2003a; Maymo-Gattell et al., 1997). This remediation process proceeds under anaerobic conditions through the well-known reductive dechlorination pathway of TCE to cis-1,2-dichloroethene (DCE) to vinyl chloride (VC), and finally to ethene:



Dhc spp. are characteristically difficult to isolate, and when isolated, they tend to grow slowly in pure culture and require a complex medium for growth (Freeborn et al., 2005). An early study on anaerobic reductive dechlorination using an enrichment culture was only able to sustain dechlorination past 40 days through the addition of a filtered culture supernatant from a more diverse system and concluded that the dechlorinating population had a nutritional dependency on the activities of other organisms (DiStefano et al., 1992).*Dehalococcoides* populations also grow robustly in mixed cultures, but the

growth factors, which are likely from other members of the consortia, have yet to be defined (Maymo-Gatell et al., 1997; Holmes et al., 2006; Rowe et al., 2008).

Many studies have characterized the mixed communities responsible for reductive dechlorination, identifying major functional groups. The microorganisms, or functional groups, which contribute to biogeochemical processes in anaerobic systems include not only fermenting and terminal electron accepting microorganisms, but also acetate-consuming microorganisms. Fermenting bacteria ultimately transform complex electron donors into hydrogen and acetate, which are then utilized by halorespiring species to reduce chlorinated ethenes. For successful application of bioremediation strategies, the contaminant-degrading organisms must out compete other indigenous hydrogenotrophs, such as methanogens and sulfate reducing bacteria (SRB), for available resources. Competition for hydrogen between methanogens and dechlorinating microorganisms, and more recently, the possibility of a synergistic relationship existing between these two functional groups, has been speculated and debated (Heimann et al., 2006).

While many studies have physiologically and thermodynamically examined possible hydrogen competition in a variety of mixed dechlorinating consortia (Loffler et al, 1999; Smatlak et al., 1996; Yu & Semprini, 2002), only a few studies (Heimann et al., 2006; Duhamel and Edwards, 2007; Rowe et al., 2008) have evaluated ecological relationships within these systems. Duhamel and Edwards (2007)monitored several functional groups in the KB-1 culture though real-time PCR (qPCR), and found that the observed yields versus the thermodynamically predicted yields were in agreement for methanogens and acetogens, but were 5-10 times less for *Dehalococcoides* indicating that they were not as efficient at energy capture. Rowe et al. (2008) used fluorescence in situ

hybridization (FISH) to evaluate theD2 dechlorinating culture in bioflocs extracted from a 9.1 L suspended growth reactor after centrifuging, sonicating, and collecting them on filters. Imaging of the bioflocs consistently showed archaea in close proximity to *Dehalococcoides ethenogenes* strain 195 around black precipitates. Heimann et al. (2006) evaluated the effect of aceticlastic methanogens on VC dechlorinationin suspended growth batch cultures using FISH and radioisotopes. In the presence of a methanogen inhibitor or *Methanosaeta* spp., slow VC dechlorination was observed, but in the presence of acetate-oxidizing, hydrogen (H_2)-producing *Methanosarcina* spp., VC dechlorination rates increased by up to a factor of 7. This was the first evidence of the production of H_2 from acetoclastic methanogens driving reductive dechlorination, and they interpreted the findings as interspecies hydrogen transfer. All of these studies showed that molecular tools could be used effectively to assess community dynamics; however, they were conducted on samples from suspended growth systems. To date, no one has confirmed the ecological relationships of hydrogenotrophs within a dechlorinating community in an *in situ* biofilm. In the past, high-resolution imaging of biofilms was accomplished using electron microscopy, but sample processing often resulted in artifacts and dehydrated samples. The advent of confocal scanning laser microscopy (CSLM) allowed visualization of fully hydrated samples and changed the way biofilms are viewed. If an intact biofilm can be collected from within a halorespiring system, the ideal method to image it and to assess ecological relationships is through the use of CSLM and FISH.

FISH can be used for determining phylogenetic identity, spatial arrangements, morphology, and quantification of microorganisms. The FISH method uses permeablized

whole microbial cells and fluorescently labeled oligonucleotide probes targeting unique regions of 16S rRNA genes. Probes can be designed to be species- or strain-specific. The probe hybridizes to a short, unique region (usually 15-25 bp in length) of the target organism (Hugenholtz et al., 2001), and when exposed to the proper excitation spectrum, the fluorophore emits a fluorescent signal. The availability of spectrally distinct fluorophores enables the simultaneous detection of multiple hybridizations (multiplex-FISH). Through the multiplex-FISH technique and confocal scanning laser microscopy (CSLM), complex biofilms composed of multiple microbial species can be visualized, resulting in a colorful, three-dimensional image of the ecosystem.

Even though FISH can be used to evaluate spatial and temporal community dynamics (Bodelier et al., 2000) there are some drawbacks that must be overcome to enable its utility as a diagnostic tool. The amount of probe applied per hybridization is limited, and background fluorescence, if present, can obscure detection of specific microorganisms (Dubey et al. 2006). The specific detection of Dhc species has been especially difficult. The first FISH probe specifically targeting Dhc was designed by Richardson et al. (2002), but this DhEth probe only produced poor or undetectable signal. It was speculated that the lack of detectable signal was a result of poor membrane permeability, the flattened morphology of the organism, and/or low ribosome concentration (Müller et al., 2002; Aulenta et al., 2004).The first successful application of FISH targeting Dhc was done by Yang and Zeyer (2003)who created two probes targeting Dhc spp. The first probe, Dhe1259c, covers 25% of the known species while Dhe1259t covers the remaining 75% (Panagiotakis et al., 2007). Due to the composition of the enrichment cultures, and because Yang and Zeyer were using a pure culture of *D.*

ethenogenes strain 195 for their experiment, only probe Dhe1259t was used in their hybridizations. The probe Dhe1259t, while only 2 bp upstream of the DhEth probe, produced a strong fluorescent signal and allowed the cells of *D. ethenogenes* strain 195 to be successfully visualized as irregular cocci. Yang and Zeyerspeculated that the weak signal from Richardson's DhEth probe could be due to poor access to the 16S rRNA targeting position of the probe, the small size of the bacteria, or a difference in microscopic set up.

Subsequent studies (Aulenta et al., 2004; Yang et al., 2005; Rowe et al., 2008) have produced FISH images of reductively dechlorinating cultures with various levels of success using the Dhe1259t probe with homogenized samples, but not of *in situ* biofilms. As the studies reviewed here indicate, most of the existing knowledge on dechlorinating communities is based on suspended growth systems even though the majority of biomass in subsurface environments exists in biofilms (Farai et al., 2010). Furthermore, it is generally accepted that the constituent microorganisms in biofilms are responsible for majority of degradation observed in natural systems (Aulenta et al., 2006).

Applying FISH to an intact biofilm from an actively dechlorinating system would allow visualization of the *in situ* architecture and may provide information about the ecological relationships involved in the remediation of chlorinated compounds. The spatial arrangements of microbes in biofilms can be a signpost of syntrophy or other specific interactions (Daims and Wagner, 2007), and recent advancements have enabled researchers to use FISH for examining biofilms collected on solid support structures. Zhang et al. (2005) demonstrated the feasibility of post hoc FISH analysis on frozen biofilm samples from a perchlorate-reducing reactor, and FISH analysis of an intact

biofilm collected from the anode of a microbial fuel cell enabled observations of changes in community structure and distribution of power-producing bacteria (Kiely et al., 2010; Kiely et al., 2011). The experimental nature of these studies allowed biofilm attachment on physical structures that could be easily retrieved from the systems under analysis. Unfortunately, this is typically not the case in laboratory and field evaluations of reductively dechlorinating systems, making the retrieval and maintenance of an intact biofilm a considerable challenge.

In the field, soil sampling, and subsequently biofilm sampling, are rarely done due to the simplicity and lower cost of groundwater sampling. Even though an alternative method of down-well incubation using an *in situ* sampler consisting of a sterilized packet of sediment has been devised to facilitate the sampling of attached microorganisms (Flynn et al., 2008), unavoidable manipulation of the contents during well extraction, as well as subsequent FISH processing of sediment grains, brings the integrity of the biofilm into question. In the laboratory, continuous flow columns are assessable model systems to study dechlorinating populations under environmentally relevant conditions (Behrens et al., 2008). Biofilm is established on the column packing materials which can be sand, soil, or even glass beads. The packing materials can be readily extracted from the column, but handling the loose material presents the same challenges as preserving the integrity of the biofilm from field locations. This study attempts to overcome these challenges through the design of a novel biofilm collection device created using a unique configuration of glass slides.

The establishment of biofilms on glass slides by submersion in beakers containing an inoculum and growth medium is well established (Kawarai et al., 2007; Ristow et al.,

2008; Manijeh et al., 2008). Biofilms have also been successfully established on glass slides placed in natural environments such as rivers (Pohlon et al., 2010) indicating that continuous flow conditions should not inhibit biofilm attachment on slides. Also, since glass slides(optionally Teflon-coated) are traditionally used in FISH analysis, the use of glass microscope slides for biofilm collection minimizes the need to deviate from established hybridization protocols and allows the biofilm to remain intact throughout the hybridization. Limited information on dechlorinating biofilms exists, and to the best of our knowledge an image of an intact biofilm from an actively dechlorinating system has not previously been published.

The objective of the present study was to collect an intact biofilm from an actively dechlorinating system and maintain the *in situ* architecture of that biofilm through the application of multiplex-FISH and CSLM, allowing the spatial community dynamics to be visually inspected. To accomplish this, a novel biofilm collection device was designed, constructed, and inserted into replicate columns fed a continuous flow of TCE-amended groundwater. The contamination was treated using a slowly fermentable substrate (crab shell) to enable development of a diverse microbial community. Over a period of one year, the columns were sequentially sacrificed as different phases of the dechlorination pathway became dominant, and the intact biofilms removed from the columns and preserved. The biofilms were later treated with multiplex-FISH using fluorescent probes targeting *Dehalococcoide*s spp., sulfate reducing spp., and archaea spp., and imaged with CLSM.

4.3 Materials and Methods

4.3.1 Groundwater Source

Natural groundwater was obtained from a well located in the Seven Mountains, Potter Township, Centre County, Pennsylvania (40N 46' 7"; 77W 35' 44.85"). The well draws from the Bald Eagle formation which consists of mainly medium to coarse grained sandstone. Prior to amendment with TCE, the groundwater was filter sterilized (0.2 um), degassed and maintained under an argon headspace in a 50 L carboy. Detectable anions in the groundwater included chloride, phosphate, and sulfate which were measured at concentrations of 1.4, 0.1, and 5.7 mg L^{-1}, respectively, on a Dionex DX-100 ion chromatograph (Dionex, Bannockburn, IL). Nitrite and nitrate were below the detection limit of 0.1 mg L^{-1}. The pH of the degassed groundwater was 8.56. Groundwater aliquots were transferred from the carboy to degassed, 160 mL serum bottles, fitted with Teflon-lined stoppers and aluminum crimp caps, and autoclaved. A TCE stock solution was added to each bottle for a final concentration of approximately 13 mg $TCEL^{-1}$ (100mM). The bottles were allowed to equilibrate for 24 hours prior to being transferred to 100 mL gas tight syringes. The TCE amended groundwater was then continuously fed through the columns as described below.

4.3.2 Chemicals and Substrates

TCE and cis-1,2-DCE were purchased from VWR International, Inc. (West Chester, PA). VC and ethene gases were purchased from Alltech Associates, Inc. (State

College, PA). Volatile fatty acid standards were created from a 10 mM volatile acid standard mix obtained from Supelco (Bellefonte, PA). The chitin source was derived from Dungeness crab shells (SC-20), and used as received from the distributors (donated by JRW Bioremediation, LLC Lenexa, KS). According to the distributors, SC-20 consists of 10-30% chitin ($C_8H_{13}NO_5$), 5-50% protein ($C_{16}H_{24}O_5N_4$), and 20-60% calcium carbonate ($CaCO_3$) by weight (JRW Bioremediation, personal communication, 2010). Silica sand (16-20 mesh, donated by Badger Mining Corp., Berlin, WI) was used as an inert proppant in the column study to maintain hydraulic conductivity. All other chemicals used were reaction grade or higher quality.

4.3.3 Culture

A mixed Dhcculture (Bio-Dechlor INOCULUM (BDI)) capable of complete dechlorination of TCE to ethene (kindly provided by Dr. Frank Löffler, then at the Georgia Institute of Technology), was used in this experiment. The BDI culture, a commercially available dechlorinating microbial consortium, has been successfully applied via bioaugmentation at field locations (Amos et al., 2008; Ritalahti et al., 2005) and is known to contain at least three Dhc spp.: strains FL2 (He et al., 2003a), BAV1 (He et al., 2003b), and GT (Ritalahti et al., 2006), as well as *Dehalobacter spp.*(Amos, 2007). The mixed BDI culture was maintained in mineral salts medium (Cole et al., 1996) containing 0.3 mM TCE and 5 mM lactate and was periodically transferred as needed.

4.3.4 Biofilm Collection Device Design and Construction

Non-frosted, standard microscope slides (78 mm x 26 mm x 1 mm) were used in the construction of static glass packets for the collection of intact biofilm. Each glass slide was scored and snapped in half, producing two smaller glass slides (39 mm x 26 mm x 1 mm). The smaller glass slides were then propped apart using two pieces of 19 gauge stainless steel (SS) wire (OW Houts, State College, PA) and secured together with 24 gauge SS wire (Small Parts, Inc., Miami Lakes, FL). The open ends were covered with SS mesh (80 x 80 mesh; McMaster Carr, Robbinsville, NJ) to prevent clogging by sand grain particles. The constructed slide packets were autoclaved inside aluminum foil and later placed in the influent and effluent ends of each column (Figure 4.1) during the packing procedure.

4.3.5 Continuous Flow Columns

Continuous flow columns were used to mimic subsurface conditions and to create a reductively dechlorinating environment for biofilm formation in the laboratory. Four replicate glass columns (69 cm length x 4.5 cm i.d.)equipped with glass end caps and kovar tips were retrofitted with lateral side ports (borosilicate glass vials, 5 ports total) spaced at approximately 10 cm intervals along the length of each column (The Pennsylvania State University Glass Shop, University Park, PA). All column components were sterilized by autoclave prior to packing. The influent end of each column was packed with a “substrate plug” (30 g crab shell mixed with 150 g sand) extending approximately 15 cm into the column, and the remaining length of the column was filled

with silica sand only (Figure 4.1). Static glass packets were placed in the influent as well as the effluent ends of the columns during packing to enable the collection of biofilm that developed over the course of the experiment. Tracer tests conducted on the packed columns produced an average dispersion number of 0.054 ± 0.008 indicating plug flow conditions. Based on the tracer tests, one pore volume was calculated to be equivalent to 240 mL with a residence time of approximately 20 days (flow rate of 0.5 mL hr^{-1}). The applied flow in this study corresponds to a linear velocity of 0.034 m day^{-1} and is equivalent to the hydraulic conductivity typically encountered in fine sand and silt. Following packing, the columns were flushed with approximately three pore volumes (PV) of degassed groundwater, inoculated with150 mL of the BDI culture, and allowed to incubate overnight. Two control columns were also established: one without crab shell; one without inoculum. Following the incubation period, each column was fed a continuous flow (0.5 mL hr^{-1}) of natural groundwater amended with approximately 13 mg L^{-1} of TCE using a model PHD 22/2000 syringe pump (Harvard Apparatus, Holliston, MA) equipped with100 mL gastight syringes (SGE Inc., Austin, TX). Each gastight syringe was attached to an individual column using sterilized stainless steel fittings and tubing. The column experiment was conducted over a period of 369 days, or approximately 18.5 pore volumes, which was necessary to allow full development of the reductive dechlorination pathway. The columns were sequentially sacrificed as each chlorinated ethene became dominant in the system (i.e., first TCE, then DCE, VC, and finally ethene; Figure 4.2) and the internal contents preserved for future FISH and qPCR analysis.

4.3.6 Slide Packet Fixation and Storage

During destructive sampling, the glass packets with established biofilm were recovered from both the influent and effluent areas of each experimental column. When each column was sacrificed, it was taken to a laminar flow hood, aseptically opened, and ethanol flame-sterilized forceps were used to remove the internal slide packets. Once retrieved from the column, the slide packets were carefully opened, the slides separated and promptly fixed in 4% paraformaldehyde/phosphate- buffered-saline (PBS) solution in sterile 50 mL conical vials for 2 hours at room temperature, rinsed with PBS solution, and stored in 50% ethanol in PBS at -80^{0}C. The PBS solution contained 0.39 M NaCl and 30 mM $Na_2HPO_4 \bullet 7H_2O$ [pH 7.2].

4.3.7 Oligonucleotide Probes and Stains used in FISH

The oliognucleotide probes (Integrated DNA Technologies, Inc. (IDT), Coralville, IA) used in this study are presented in Table 4.1. Dhc spp. detection was accomplished using an equal mix of Cy-3 labeled probes Dhe1259c and Dhe1259t developed by Yang and Zeyer (2003). Archaeal detection was accomplished using Cy-5 labeled probe ARC915 (Stahl and Amann, 1991) and sulfate reducing bacteria (SRB) were targeted using FAM labeled probe DSS658 (Boetius et al., 2000). The DNA-intercalating dye 4'-6-diamidino-2phenylindole (DAPI; Research Organics Inc., Cleveland, OH) was used for counterstaining. No-probe hybridizations and Cy-3 labeled NonEU338 probe hybridizations were applied as negative controls for autofluorescence and non-specific binding, respectively.

4.3.8 Multiplex-FISH Hybridizations

The preserved biofilm was treated using multiplex-FISH with DAPI counterstaining to simultaneously visualize Dhc (strains FL2, GT, and Bav1 in this study), SRB, and archaeal (assumed to be predominantly methanogenic) cells. Hybridizations were performed using biofilm samples that were formed on glass slides *in situ* (i.e., they were not homogenized or otherwise manipulated). Fixed samples were dehydrated in an ethanol series (50, 80, and 95%) for 3 minutes each and allowed to air dry overnight. Since the Dhc probes require an incubation temperature of 42°C and the other probes in this study use an incubation temperature of 46°C, two sequential hybridizations were performed. For both rounds of hybridization, the hybridization buffer (formamide (30% forDhc; 40% for Archaeal and SRB), 0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.01% sodium dodecyl sulfate; 500 uL total vol.) and probe/s (50 ng/uL ea.; 5 ng/uL final concentration) were applied to the slide, covering the majority of the slide surface (slide face area = 1014 mm^2). A silicon gasket (VWR International, LLC., Radnor, PA) was customized to fit each slide, creating a well and maintaining submersion throughout hybridization. After the first hybridization, the slides were placed into pre-warmed (46°C) moisture chambers, and incubated at 46°C for 1.5 hours. Subsequently slides were rinsed with wash buffer (prewarmed at 48°C) followed by immersion in the wash buffer (100 mM NaCl, 20 mM Tris-HCl (pH 8.0), and 0.01% sodium dodecyl sulfate) and incubation at 48°C for 20 minutes. Slides were then rinsed with deionized (DI) water, dried, and the second hybridization buffer applied. The slides were again placed into prewarmed moisture chambers, incubated at 42°C for 1.5 hours,

rinsed with wash buffer, immersed in wash buffer for 20 min. at 48°C, rinsed with DI water, and dried.

4.3.9 Microscopy

Confocal images were collected from each slide packet taken from the columns. Each slide was mounted with SlowFade Gold antifade reagent with DAPI (Invitrogen Inc., Carlsbad, CA) and examined at 1000X magnification (Olympus UPlanFl 100X, 1.4 numerical aperture, in oil) using an inverted Olympus IX-81 microscope, equipped with a Fluoview 1000 confocal scanning unit (Olympus America Inc., Melville, NY). Excitation lasers at 405 nm (Violet, DAPI), 543 nm (Green HeNe, Cy-3), and 633 nm (Red HeNe, Cy-5) were used. DAPI-, Cy3-, and Cy5-labeled images were captured individually from the same examining field using sequential scanning in order to eliminate crosstalk. Biofilm aggregate images were collected as z-series stacks (z-step size of 0.45 um) using sequential scanning. All image combining and processing was performed with the standard software package provided by Olympus.

DAPI stained column material images were taken using a Zeiss Axiophot microscope (Zeiss, West Geremany), and a Zeiss Plan-Apochromat objective (Zeiss, West Germany) at 100X magnification.

4.3.10 DNA Extraction and Quantitative Real-Time PCR (qPCR)

Total community, chromosomal DNA was collected from solid phase (1-2 g of wet sand or sand/crab shell mixture) samples when each column was sacrificed. DNA was extracted using an UltraClean™ Soil DNA Kit (MoBio Laboratories, Carlsbad, CA) following the bacterial protocol for maximum yields with modifications as previously described by Ritalahti et al. (2006). The extracted DNA was stored at -20 °C until qPCR analysis. TaqMan primers and probe sets were used to quantify total numbers of bacterial and *Dehalococcoides* 16S rRNA genes as previously described (Harms et al., 2003; Ritalahti et al., 2006; Chapter 3).

4.3.11 Water Quality Analysis

Chlorinated ethenes and ethene were measured over the course of the experiment with an Agilent model 6890N headspace gas chromatograph (GC) equipped with a GS-Q column (30 m length, 0.53 mm diameter, J&W Scientific) and a flame ionization detector (FID). Aqueous samples were injected into 20 mL autosampler vials capped with teflon lined septa and aluminum crimp tops. The vials were equilibrated for 15 minutes at 70ºC prior to automated injection into the GC by an Agilent G1888 Network Headspace Autosampler. Chlorinated ethenes and ethene were separated using the following temperature program: initial hold at 35ºC for 2 minutes, increase to 180ºC at a rate of 45ºC per min, hold for 2 minutes, increase at a rate of 45ºC per min to 200ºC, hold for 5.34 minutes. Ultra-high purity hydrogen was used as the carrier gas at a flow rate of 22.6 mL/min.

Volatile fatty acid (VFA) analysis was performed using a Waters 2695 high-performance liquid chromatograph (HPLC) equipped with an Aminex HPX-87H ion exclusion column and a photodiode array detector. Aqueous samples were placed in 2 mL eppendorf tubes, and stored at -20ºC until analysis. Prior to analysis, the samples were thawed, filtered (0.45 um) to remove any suspended solids, and the supernatant (diluted with deionized water) added to 0.2 mL of 2.0 N H_2SO_4 in 2.0 mL autosampler vials.

The pH of each sample was measured using a Fisher Accumet AB15 pH meter equipped with a VWR International (West Chester, PA) SympHony pH electrode.

4.4 Results and Discussion

4.4.1 Column performance

A comprehensive discussion and detailed calculations on column performance and qPCR results are reported in a companion paper by McElhoe and Brennan (Chapter 3). Here we provide a brief summary of the results of the previous study. In a 369 day continuous flow study, columns fed TCE-spiked (100 uM) groundwater demonstrated reductive dechlorination through all the steps in the dechlorination pathway, eventually producing ethene. Chlorinated ethene concentrations in samples collected from lateral side ports (Chapter 3, Figure 3.3) indicated that the initial transition zone for each step in the dechlorination pathway occurred within the first 13 cm of the columns with concentrations either decreasing or remaining relatively constant for the remaining

length. A representative plot of chlorinated ethene concentrations observed in the effluent (collected from the VC to ethene column) is presented in Appendix F (Figure F.1).

Fermentation products (VFAs) were detected in the effluent of columns throughout the experiment, and were calculated to provide reducing equivalents in excess of the electrons needed for complete dechlorination of the influent TCE to ethene (Table 4.2). Electron equivalents (e-eq) were calculated using redox half reactions (Table 4.3) and equations 4.1-4.4:

$$e-\ eq._{VFA} = \sum \left[mass\ VFA\ in\ column\ (mmol) \left(\frac{mmol\ e-}{mmol\ VFA} \right) \right] \tag{4.1}$$

Where: VFA electron equivalents (e-eq.$_{VFA}$) is the sum of the electron equivalents calculated for each VFA detected in the continuous flow column effluents.

$$e-eq._{chitin} = shell\,mat.\ ferm.\,(g) \left(\frac{0.2g\ chitin}{1g\ shell\,mat.} \right) \left(\frac{mol\ \ chitin}{203.22g\ chitin} \right) \left(\frac{mol\ \ e-}{0.0312mol\,chitin} \right) \left(\frac{10^3\ mmol}{mol} \right) \tag{4.2}$$

$$e-eq._{protein} = shell\ mat.\ ferm. \left(\frac{0.12g\ \ protein}{1g\ \ shell\ mat.} \right) \left(\frac{mol\ \ protein}{352.2g\ \ protein} \right) \left(\frac{mol\ \ e-}{0.0151\,mol\ \ protein} \right) \left(\frac{10^3\ \ mmol}{mol} \right) \tag{4.3}$$

$$e-eq._{\ TCE \rightarrow ethene} = mol\,TCE \rightarrow eth \left(\frac{mol\,e-}{0.5mol\,TCE \rightarrow cDCE} \right) \left(\frac{mole-}{0.5mol\,cDCE \rightarrow VC} \right) \left(\frac{mole-}{0.5mol\,VC \rightarrow eth} \right) \tag{4.4}$$

The dominant VFAs observed in the effluent of the columns were acetate, propionate, and butyrate (Table 4.2). Acetate dominated towards the end of the

experiment in all columns (Appendix F, Figure F.2), as the other VFAs were depleted by day 163. Assuming a total pore volume of 240 mL per column (determined from tracer tests), the cumulative mass of acetate, propionate, and butyrate produced was 40.1, 7.5, and 10.0 mmol, respectively, at the end of the experiment (t = 369 days; VC to ethene column).

Acetate, the dominant VFA in this experiment, is well known to be a major intermediate in the conversion of organic matter to methane (CH_4) and carbon dioxide (CO_2) (Smith and Mah, 1966; Gujer and Zehnder, 1983), within a pH range of 6 to 8 (Fukuzaki et al., 1990). Some aceticlastic methanogens (i.e., *Methanosarcina* spp.) are also capable of acetate oxidation (Lovley and Ferry, 1985). Acetate oxidation proceeds through the oxidation of the methyl carbon of acetate to produce CO_2 and H_2 (Heimann et al., 2006). The presence of aceticlastic methanogens in dechlorinating systems may provide a source of hydrogen to the dechlorinating population. Although not quantified here, aqueous methane concentrations of 0.6 to 1.0 uM (microcosms) and 0.5 to 1.5 μM (semi-continuous flow columns) were observed in the preceding dechlorination study (Chapter 2, section 2.4.1) using the same culture and substrate sources.

Throughout the experiment, the effluent pH of the individual columns was similar, ranging from 6.79±0.77 to 7.23±0.66, but the average pH along the length of the different columns at the time of sacrifice was quite different, ranging from 6.63±0.07 to 8.24±0.28 (Table 4.2). In general, the pH measurements along the length of each column increased over time, likely due to a decrease in fermentation activity over time as easily degradable portions of the substrate (i.e., protein) were utilized and as alkalinity

continued to be generated by calcite dissolution from the shell (Figure 4.3). The average pH (at ports 1-5) was 6.63±0.07 in the column sacrificed on day 20 (TCE column) and 6.97±0.10 in the column sacrificed on day 55 (TCE to cDCE). In both columns, pH measurements along the length demonstrated similar trends. The pH in Column 1 was 6.57 at port 2 increased to 6.73 (port 4) and then decreased to 6.61 (port 5). In Column 2, the pH at port 1 was 6.82, increased to 7.12 (port 2), and then decreased to 6.93 (port 5). By day 115 (cDCE to VC), the average pH had increased to 7.63±0.19, but continually decreased along the length of the column from 7.95 to 7.45. At the conclusion of the experiment (VC to ethene, t = 369), the average pH was highest at 8.05±0.42, with the pH increasing from7.19 (port 1) to 8.49 (port 3) and then decreasing to 7.68 (port 5). These fluctuations in pH are likely caused by a combination three types of reactions: substrate fermentation at the beginning of the columns; calcite dissolution and subsequent buffering in the middle of the columns; and dechlorination predominantly at the end of the columns.

These differences in pH may have affected the distribution and activity of halorespiring bacteria. q-PCR analysis indicated the percentage of the overall bacterial population represented by Dhc (16S rDNA targeted) in the influent end of the columns was small (0.02-0.23%) and increased to 9.5-26.1% in the effluent end of the columns. Relative quantification of Dhc and bacterial 16S rDNA gene copies for all sampling locations is presented in Figure 4.4. A summary of column performance data, including VFA production, crab shell utilization, chlorinated ethenes, q-PCR data, and pH for each continuous flow column is presented in Table 4.2.

4.4.2 FISH analysis

During optimization of the FISH technique on the glass slide packets, non-specific fluorescence as well as autofluorescence obstacles were encountered. Hybridization with the FAM-labeled SRB targeted probe produced a pattern of punctate fluorescent dots, the majority of which did not correspond to DAPI stained cells. Others have had similar difficulties with fluorescein labeled probes (Heimann et al., 2006) and autofluorescence. Inorganic particles, debris, or even the cells themselves can cause this type of response. The intensity of the non-specific fluorescence was equal to any probe-conferred signal, making it impossible to differentiate the sources with reasonable certainty. For this reason, the results of the SRB functional group are excluded from this report.

The no-probe control treatment produced a high level of autofluorescence on slides retrieved from the influent area of the columns, presumably due to the presence of crab shell material. Many compounds in the environment have fluorescent properties including inorganic particles, coenzyme F420 (Stabnikova et al., 2006), some proteins, and other extracellular materials (Vuoriranta et al., 2003). The inorganic portion of the crab shell substrate (i.e., calcite, $CaCO_3$) or microorganisms responsible for the shell fermentation could be the source of autofluorescence. Since fluorescence was present in all channels and obscured identification of any probe-conferred fluorescence, the processing of influent area slides was abandoned.

Fluorescent hybridizations of slides extracted from the effluent area of the active columns were successful and produced detectable signals for the Cy5-labeled Archaeal

probe and the Cy3-labeled Dhc probe. Imaging randomly selected fields of view showed a patchy biofilm of DAPI stained cells of coccoid and rod shaped morphologies, with signals from Archaeal or Dhc probes rarely detected outside of aggregate clusters. Through careful microscopic examination of the entire surface of the slide (1014 mm^2), several biofilm aggregates with signals from multiple probes were detected.

Biofilm aggregates were identified on slides extracted from the effluent ends of all columns except the control columns (Figure 4.5). Overall, the biofilm aggregates that were imaged increased both in the number identified per slide (3 to 5), as well as the average thickness (3.9 to 8.4 um) over time (Table 4.4). The first sacrificial column was destructively sampled (day 20) after approximately 1 PV had removed most of the residual dechlorination products from the inoculum and TCE was the dominant chlorinated ethene. Three biofilm aggregates with an average thickness of 3.9 ± 0.5 um (± standard deviation) were found on a slide from this column and showed archaeal cells in close proximity (< 1um) to Dhc cells (Figure 4.5 panel A).The second sacrificial column (~3 PV), representing the biofilm during the transition from TCE to cDCE, also showed archaeal and Dhc cells in close proximity and had an average thickness of 5.1± 1.0 um (n=4) (Figure 4.5 panel B). After another 3 PV (6 PV total) the transition from cDCE to VC was observed, and the number of aggregates containing Dhc and archaeal cells on the collected biofilm had again increased by one with an average thickness of 5.7 ± 0.5 um (n=5) (Figure 4.5 panel C). In the cDCE to VC biofilm, a greater number of archaeal cells were observed than in other columns suggesting potential synergism between neighboring cells (Figure 4.5 panel C). The slide extracted from the final sacrificial column (transition from VC to ethene; 18.5 PV) had five biofilm aggregates

with an average thickness of 8.4 ± 2.2 um (Figure 4.5 panel D). The Dhc signal in the biofilm extracted from this final column was particularly strong, and the archaeal signal, while still present, appeared diminished in the cell aggregate (Figure 4.5 panel D). Both control columns showed sparse DAPI staining with few fluorescent signals detected for the Archaeal probe. Dhc cells were identified on slides from the inoculated control column that did not contain crab shell (Figure 4.5 panel E), but were not detected in the uninoculated control column, as would be expected, since only the inoculated columns should contain Dhc cells.

To determine if any biofilm was present on the packing materials extracted from the columns and stored at -80°C, samples were treated with DAPI and imaged (Figure 4.6). These images showed that a biofilm was present on the sand and/or sand + chitin material retrieved from the column. Again, the presence of the biofilm was patchy, but in greater abundance than what was found on the glass slides. FISH attempted on the column materials did not produce a detectable signal. This was most likely due to a lack of preservation of the biofilm on the sediments prior to storage at -80°C and the subsequent loss of rRNA.

The images produced in this study appear to support previous speculation in the literature that dechlorinating microbes live in close proximity with other microbes in aggregates (Aulenta et al., 2006). These results are consistent with the biofloc images from a suspended growth reactor produced by Rowe et al. (2008) which contained archaea in close proximity to *Dehalococcoides ethenogenes* strain 195. Rowe et al. postulated that the distribution and localization of archaeal (methanogenic) and *Dehalococcoides* cells facilitated the transfer of metabolites (from butyrate fermenters

and hydrogenotrophs in their study) based on the concept that spatial orientation is important in syntrophic communities. The orientation of cells in this study indicates that competition is not causing spatial stratification, and supports the recent speculation of syntrophic relationships within halorespiring communities. Considering that the concentration of acetate throughout this study (~2.0 mM even at the end of the experiment) was above the acetate utilization threshold of 0.5 mM for *Methanosarcina* spp. as determined by Heimann et al. (2006), the spatial distribution observed here could be an indication of acetate oxidation and subsequent interspecies transfer of H_2.

The distance between archaeal cells and Dhc cells located within the aggregates imaged in this study ranged from < 1 um up to 12 um. These distances are similar to the distances between syntrophic species observed in other studies. Batstone et al. (2006) used multidimensional modeling to investigate interspecies hydrogen transfer in anaerobic biofilms and found a median distance of 2.0-2.5 um between syntrophic species. In another study by Ishii et al. (2005), the allowable distance for syntrophic propionate oxidation was found to be approximately 2 um, while the distance for syntrophic ethanol and propanol oxidation was 16 and 32 um, respectively.

Additional analysis of in situ biofilms from dechlorinating systems is needed to further investigate the possibility of interspecies hydrogen transfer being a driver of reductive dechlorination and to assess the distribution of other functional groups, such as fermentors, that may be providing a source of nutrients to *Dehalococcoides* spp.

4.5 Conclusions

Results from this laboratory study demonstrated successful fluorescent imaging of an intact, halorespiring biofilm extracted from continuous flow columns treated with a slowly fermentable substrate (crab shell). The following key conclusions are noted:

1. The designed biofilm collection device enabled the successful extraction and FISH processing of an intact biofilm from an actively dechlorinating system.
2. Visualization of the biofilm architecture showed methanogens in close proximity to Dhc, indicating that competition, if it exists, is not creating spatial stratification.
3. The close proximity of potentially competing hydrogenotrophs indicates that fermentation of crab shell material supplies hydrogen in concentrations able to support multiple hydrogen utilizing microbes.

As a result of this study, the fundamental obstacle to the application of FISH in dechlorinating systems has been overcome: extraction of an intact, *in situ* biofilm. The biofilm collection device facilitated establishment of a biofilm on a solid structure placed in a continuous flow system and allowed the structural integrity of the biofilm to be maintained throughout the process of sample collection and the FISH hybridization procedure. Improvements could be made on the method of maintaining submersion during hybridization. The customized silicon gaskets used to create a well on each slide

randomly failed to create a seal, and unless handled with extreme care, the seal often became compromised and the hybridization buffer would be lost during hybridization. Also, the small size of the glass slides (39 mm x 13 mm x 1 mm each) selected to minimize flow disruption in this study, were challenging to manipulate with the stage clip set up on a standard microscope. Even though this difficulty was minimized with the inverted CSLM, there still was no way to secure the slide. If feasible, we would recommend using the full size standard microscope slide in future versions of the device. Although further research is needed to perfect this technique, our main objective was to develop a method to evaluate *in situ* biofilms from dechlorinating systems. Our study demonstrated that FISH can be successfully performed on biofilms formed on glass slides and that this technique might be applicable in other studies to evaluate the architecture of biofilms and remediation performance. Future work must be done to evaluate whether this biofilm collection technique can be applied in field settings.

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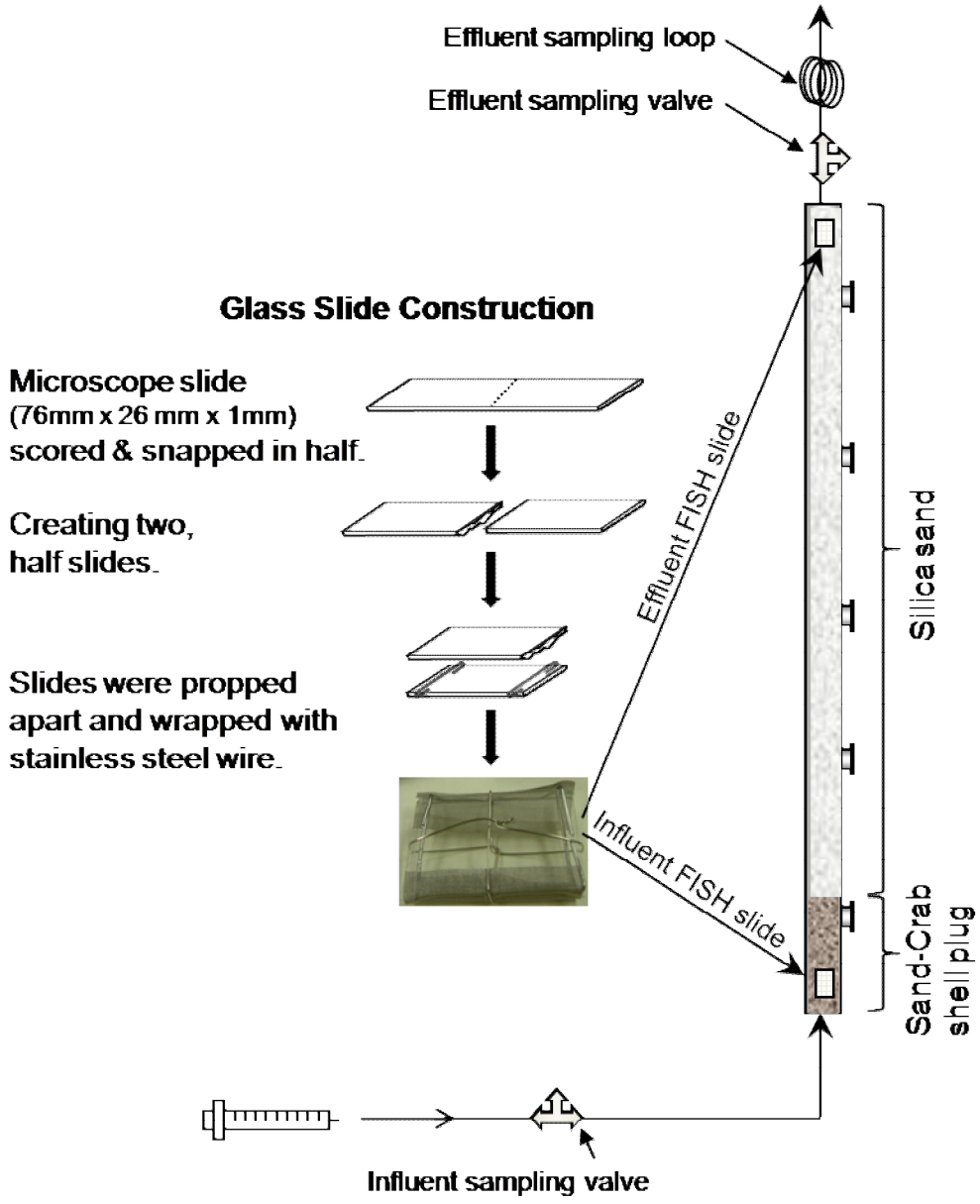

Figure 4.1. Glass packet construction diagram and continuous flow column schematic. The no chitin control column contained silica sand only.

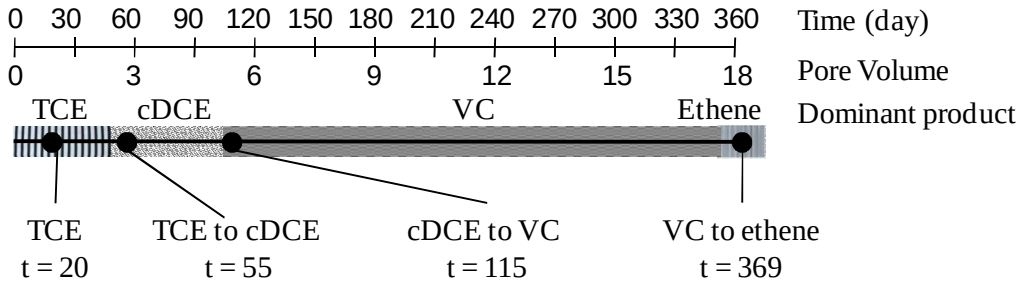

Figure 4.2. Experimental time line showing chlorinated ethene transitions and destructive sampling dates of the continuous flow columns. The no chitin control column was sacrificed on day 369.

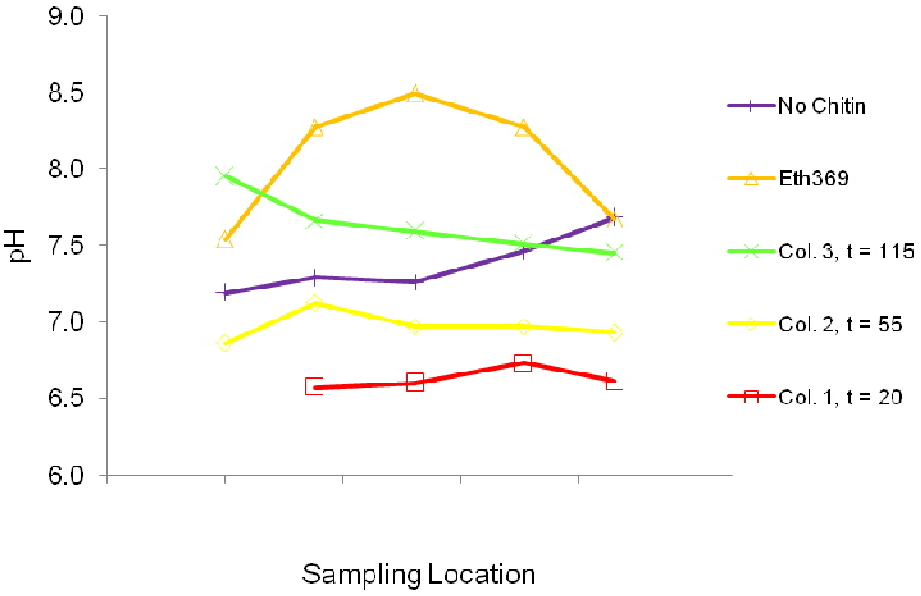

Figure 4.3. pH profiles along the length of continuous flow columns at the time of sacrifice.

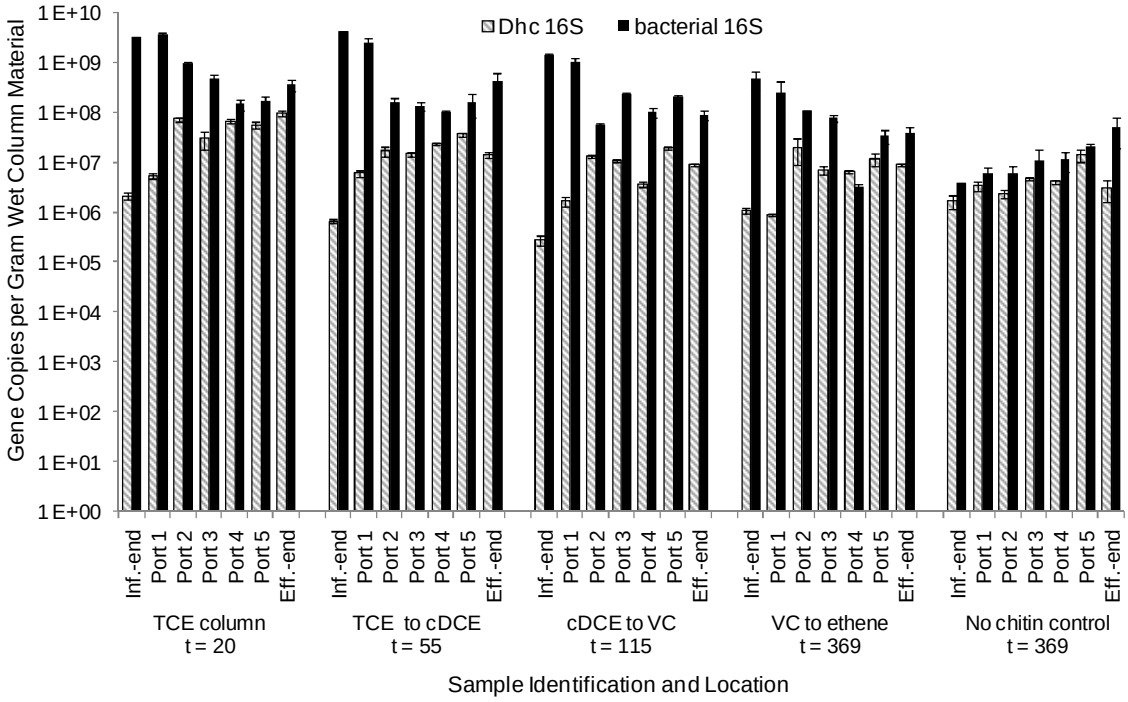

Figure 4.4. Number of *Dehalococcoides* and bacterial 16S rRNA gene copies determined with q-PCR in the continuous flow columns over time. Bars represent triplicate averages; error bars represent one standard deviation. Note: *Dehalococcoides* was not detected in the no inoculum control column so it is not shown here.

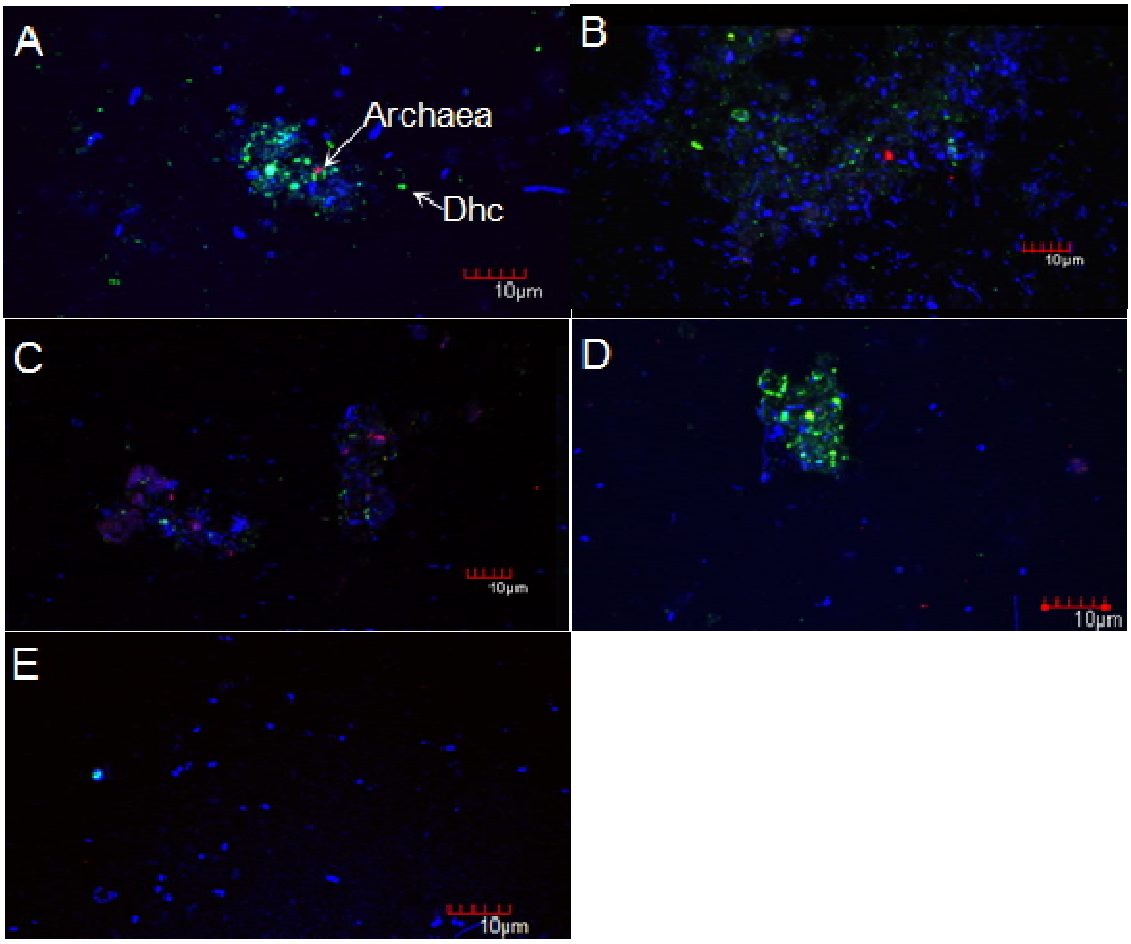

Figure 4.5. Biofilm aggregates extracted from reductively dechlorinating systems imaged using fluorescently labeled rRNA-targeted oligonucleotide probes and confocal laser scanning microscopy. The archaea are shown in red, the Dhc are shown in green, and DAPI stained cells are shown in blue. **A**. TCE dominant column, sacrificed day 20. **B**. TCE to cDCE column, sacrificed day 55. **C**. cDCE to VC column, sacrificed day 115. **D.** VC to ethene column, sacrificed day 369. **E.** No chitin control column, sacrificed day 369. **A., B., C.,** & **D.** are z-series stacks (z-step size of 0.45 um). **E.** is a single xy image.

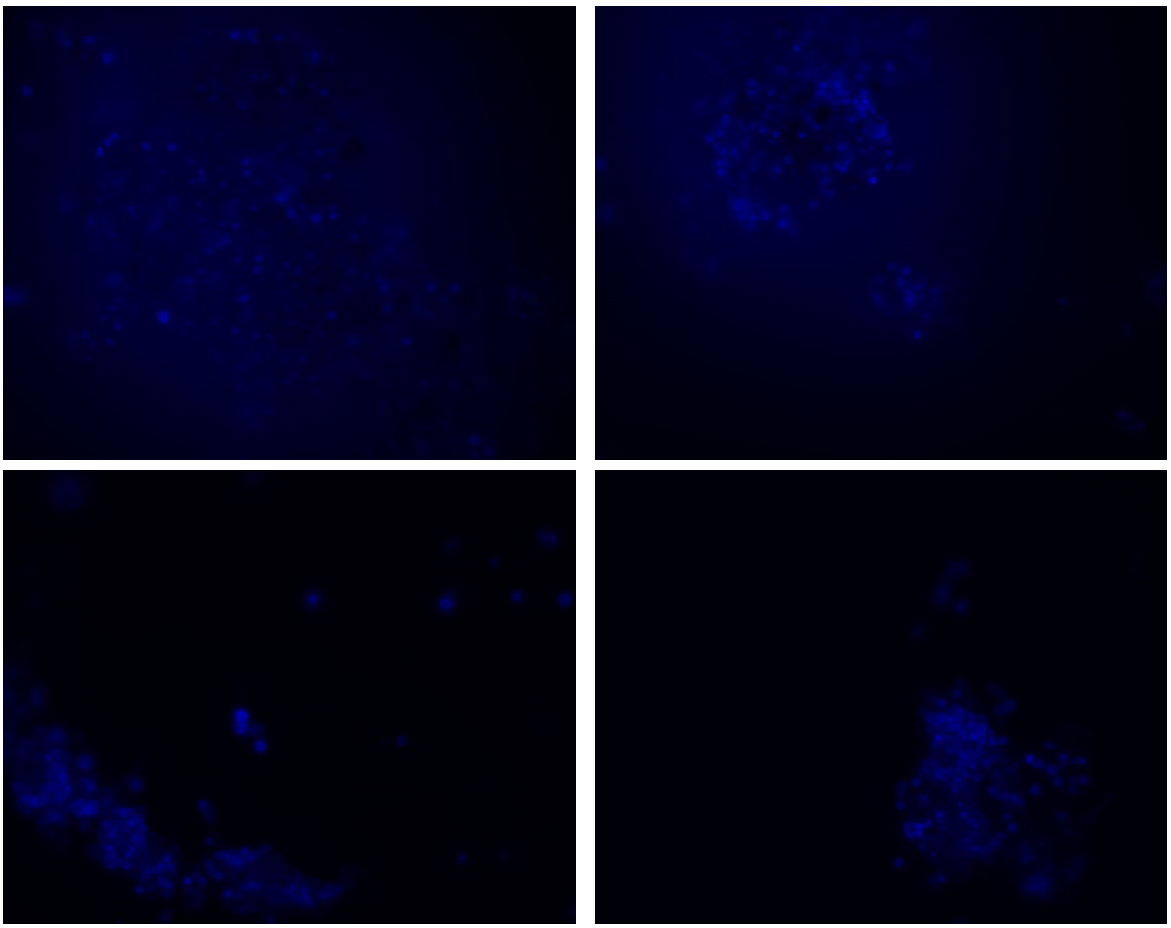

Figure 4.6. Epi-flourescent microscope images showing DAPI stained biofilm located on extracted column material.

Table 4.1. Oligonucleotide probes used with FISH analysis in this study.

Probe	Specificity	Sequence of probe (5'-3')	16S rRNA Target site[a.]	FA (%)[b.]	Reference
ARC915	Archaea	GTGCTCCCCCGCCAATTCCT	915–934	0–50	Stahl & Amann (1991)
Dhe1259t	75% *Dehalococcoides* spp.	AGCTCCAGTTC**A**CACTGTTG	1,259–1,278	0–30	Yang & Zeyer (2003)
Dhe1259c	25% *Dehalococcoides* spp.	AGCTCCAGTTC**G**CACTGTTG	1,259–1,278	0–30	Yang & Zeyer (2003)
DSS658	Sulfate Reducing Bacteria	TCCACTTCCCTCTCCCAT	658-675	40	Boetius et al. (2000)
Non-EU338	negative control	TGAGGATGCCCTCCGTCG	338-355	40	Yang & Zeyer (2003)

[a.] E. coli numbering (Brosius et al., 1981)

[b.] Formamide (vol/vol) contained in hybridization buffer

Table 4.2. Summary of performance data from the continuous flow columns.

Parameter	TCE col. Day 20	TCE to cDCE Day 55	cDCE to VC Day 115	VC to Ethene Day 365	Control Columns No Chitin	Control Columns No Inoculum
VFA data (mmol)[a.]						
acetate	7.51	26.55	12.88	40.10	0.00	31.84
propionate	2.16	6.80	24.51	7.46	0.00	5.50
butyrate	2.23	4.69	3.68	10.04	0.00	6.69
electron equivalents	179.77	495.83	595.53	827.42	0.00	599.78
shell material fermented (g)	5.93	8.24	8.39	10.88	0.00	11.62
electron equivalents (mmol)[b.]	186.73	259.38	264.32	342.77	0.00	366.03
electron equivalents (mmol)[c.]	674.92	674.92	674.92	674.92	0.00	674.92
Chlorinated ethene data (umol)[d.]						
Total influent ethenes	418.44	472.48	858.56	989.16	1093.36	251.53
TCE	53.37	132.02	119.94	127.97	671.87	273.90
cDCE	22.60	59.59	75.79	237.92	202.62	0.00
VC	214.15	84.19	195.41	505.14	153.63	0.00
Ethene	1.65	20.62	22.12	32.54	72.26	0.00
Total effluent ethenes	291.77	296.43	413.26	903.58	1100.38	273.90
Mass balance (out/in*100)	69.73	62.74	48.13	91.35	100.64	108.89
electron equivalents (mmol)[e.]	0.07	0.46	0.67	1.71	2.23	1.51
q-PCR data (cell/column)[f.]						
Dhc 16S rDNA	3.94E+10	1.54E+10	7.67E+09	7.16E+09	4.89E+09	0.00E+00
tceA functional gene	7.26E+09	2.87E+08	3.03E+09	1.15E+09	1.34E+09	0.00E+00
vcrA functional gene	1.35E+09	1.50E+09	7.58E+08	1.42E+09	9.62E+08	0.00E+00
bvcA functional gene	1.35E+06	4.34E+05	8.94E+07	8.67E+08	7.52E+05	0.00E+00
Dehalobacter	2.85E+08	8.91E+08	4.71E+08	1.08E+08	1.48E+08	0.00E+00
Universal Bacteria	1.05E+12	8.35E+11	3.52E+11	1.10E+11	1.32E+10	9.81E+10
pH						
average effluent pH[g.]	6.79±0.77	6.88±0.70	7.09±0.41	7.06±0.40	6.97±0.28	7.23±0.66
average pH along entire length[h.]	6.63±0.07	6.97±0.10	7.63±0.19	8.05±0.42	7.38±0.20	8.24±0.28

a. Represents cumulative VFAs produced.

b. Chitin associated electron equivalents.

c. Protein associated electron equivalents.

d. Represents cumulative chlorinated ethenes and the total influent ethenes accounts for inoculum concentrations.

e. Represents the electron equivalents required to reduce influent TCE to ethene.

f. q-PCR values based on cells per gram of column materials multiplied by total column contents.

g. Values represent pH over entire length of experiment.

h. Values represent pH at time of sacrifice.

Table 4.3. Redox half reactions used to calculate electron equivalents (e- eqs.).

Chemical Species:	Hydrogen donating half reactions:
Chitin	$0.031\,C_8H_{13}O_5N + 0.375\,H_2O \rightarrow 0.219\,CO_2 + 0.031\,NH_4^+ + 0.031\,HCO_3^- + H^+ + e^-$
Protein	$0.015\,C_{16}H_{24}O_5N_4 + 0.470\,H_2O \rightarrow 0.182\,CO_2 + 0.061\,NH_4^+ + 0.061\,HCO_3^- + H^+ + e^-$
Acetate	$0.125\,CH_3COOH + 0.25\,H_2O \rightarrow 0.25\,CO_2 + H^+ + e^-$
Propionate	$0.071\,CH_3CH_2COOH + 0.286\,H_2O \rightarrow 0.214\,CO_2 + H^+ + e^-$
Butyrate	$0.05\,CH_3CH_2CH_2COOH + 0.3\,H_2O \rightarrow 0.2\,CO_2 + H^+ + e^-$
Chemical Species:	Hydrogen consuming half reactions:
TCE to cDCE	$0.5\,C_2HCl_3 + H^+ + e^- \rightarrow 0.5\,C_2H_2Cl_2 + 0.5H^+ + 0.5Cl^-$
cDCE to VC	$0.5\,C_2H_2Cl_2 + H^+ + e^- \rightarrow 0.5\,C_2H_3Cl + 0.5H^+ + 0.5Cl^-$
VC to ethene	$0.5\,C_2H_3Cl + H^+ + e^- \rightarrow 0.5\,C_2H_4 + 0.5H^+ + 0.5Cl^-$

Table 4.4. Number and average thickness of biofilm aggregates imaged per slide (1,014 mm^2 total surface area).

Column	Sampled on day	Pore Volumes	No. Aggregates (per 1014 mm^2)	Average Depth (um)
TCE	20	1.0	3	3.9±0.5
TCE to cDCE	55	3.0	4	5.1±1.0
cDCE to VC	115	6.0	5	5.7±0.5
VC to Ethene	369	18.5	5	8.4±2.2

CONCLUSIONS

The studies included in this dissertation demonstrated key performance differences in commercially available grades of crab shell chitin, evaluated changes in the microbial community throughout the course of TCE remediation with crab shell substrate in sacrificial continuous flow columns, and developed a novel collection device to visually examine *in situ* biofilms.

In evaluating the different grades of chitin, the least refined, least costly grade of shell material (SC-20) was determined to be the best choice for remedial activities for several reasons. The greatest number of *Dehalococcoides* 16S rRNA gene copies, greatest magnitude of ethene production, and greatest variety of VFAs were produced in experiments containing SC-20. Even with the variety of VFAs produced, and accumulation of the dechlorination byproduct HCl, the pH in the SC-20 columns was more stable than in treatments containing the other grades of crab shell. This is probably due to the chitin-associated $CaCO_3$, which has been shown to be a more effective buffer than limestone. The neutralizing capacity of this substrate helps maintain a circumneutral pH near the optimal 6.8 – 7.5 range required for effective halorespiration activity in *Dehalococcoides* spp., making it an attractive candidate for use in remedial activities. It was also demonstrated that a decrease in proppant loading (sand:chitin ratio) from 15:1 to 5:1 did not significantly affect dechlorination, but did increase the longevity of VFA production. Most of the findings in this study, if applied at contaminated field locations, could lower the costs associated of applying biostimulation. Increasing

commercialization of this “green technology” would have several benefits, including the reduction of a waste stream generated by the seafood industry, the potential for complete destruction of chlorinated ethenes in contaminated aquifers, and a reduction in costs compared to conventional approaches for ground water remediation.

A series of replicate, continuous flow columns were sequentially sacrificed to provide insights about the microbial population during critical transition periods during the reductive dechlorination of TCE. The correlation of qPCR results and water quality data over space and time showed the ability of the crab shell substrate to provide a suite of electron donors and stimulate complete reductive dechlorination. A lag in the production of VFAs in the column packed with sterilized crab shell indicated that the native microbial population associated with the shell material is responsible for immediate onset of fermentation reactions. The crab shell also influenced the distribution of *Dehalococcoides* populations as well as overall bacterial populations. In general, *Dehalococcoides* cell numbers were observed to increase with increasing distance from the influent (<1% of the total bacterial population in the influent area of the columns to 9-40% in the second half of the columns), but to decrease with time in most columns. The majority of the column samples had sums of the RDase genes that accounted for only portion (8 - 87%) of the total 16S rRNA gene copies, suggesting that there may be *Dehalococcoides* spp. that do not contain any of the known RDases. VC-stall in one column was overcome through the temporary (~1 PV) addition of a nutrient rich medium, which was needed to “jump start” ethene production, but not needed to sustain it. The finding that a pulse, rather than continuous addition, of nutrients can be used to stimulate

dechlorinating populations has potential cost-saving implications for field locations experiencing VC stall. After the addition of the medium, an increase in the abundance of the bvcA gene was observed, indicating that strains with the bvcA gene (ex., strain BAV1) may have a specialized nutritional requirement for metabolizing VC.

Through T-RFLP analysis of column sediments and effluent pore water, the greatest richness, diversity, and number of bacterial OTUs were found from sand samples at the beginning of the experiment (< ~10 PV), and from pore water samples at the end of the experiment, indicating an increased importance of planktonic species over time. Similar membership but different community structure was observed between ethene-producing columns that did and did not experience VC stall. Neither pore the water nor the solid-phase samples effectively captured all the dominant populations in the continuous flow columns, and the assessment for field locations traditionally done through the extraction of DNA from ground water samples may give an incomplete picture of the microbiology in the subsurface of contaminated sites.

With the development of the biofilm collection device, the fundamental obstacle to the application of FISH in dechlorinating systems has been overcome: extraction of an intact, *in situ* biofilm. The biofilm collection device facilitated establishment of a biofilm on a solid structure placed in a continuous flow system and allowed the structural integrity of the biofilm to be maintained throughout the process of sample collection and the FISH hybridization procedure. Visualization of the biofilm architecture showed methanogens in close proximity to *Dehalococcoides*, indicating that competition, if it exists, is not creating spatial stratification. The distribution and localization of

methanogens and *Dehalococcoides* in biofilm aggregates suggests a synergistic relationship in which non-dechlorinating microbes assist in the dechlorination process either directly through interspecies hydrogen transfer, or indirectly by supplying micronutrients.

Appendix A

Summary of performance data for each of semi-continuous columns

Table A.1. Semi-continuous flow column performance data including VFAs, chloroethenes, and *Dehalococcoides* gene copies.

			VFAs (cumulative)			Chlorinated Ethenes (cumulative)				q-PCR Quantification	
Column ID	Chitin grade	Proppant load (sand:substrate)	Acetate (mM)	Propionate (mM)	Caprioate (mM)	TCE (uM)	cDCE (uM)	VC (uM)	Ethene (uM)	Influent end (cells/g)	Effluent end (cells/g)
1	SC-20	5:1	337.73	43.98	1051.22	1147.92	17.12	315.59	106.37	2.00E+07	1.24E+07
8	SC-20	5:1	347.04	52.85	1090.87	129.51	4.32	66.73	15.03	8.45E+05	3.35E+05
14	SC-20	5:1	330.93	45.71	1019.38	N/A	262.76	66.38	10.26	3.19E+05	1.00E+05
2	SC-20	15:1	113.53	15.55	398.86	1212.46	126.15	284.93	39.81	9.62E+06	7.34E+05
9	SC-20	15:1	129.66	17.31	504.95	79.75	14.93	67.36	14.79	3.58E+06	2.79E+06
15	SC-20	15:1	136.60	22.48	414.53	N/A	244.50	98.61	10.13	2.24E+06	1.52E+05
3	SC-40	5:1	291.69	67.47	265.09	1833.46	74.40	447.12	88.20	1.39E+06	1.37E+06
10	SC-40	5:1	310.48	61.17	355.21	115.41	17.02	47.40	10.20	3.46E+04	2.76E+04
16	SC-40	5:1	234.87	35.60	203.71	N/A	234.66	94.28	14.27	1.67E+05	8.45E+05
4	SC-40	15:1	135.09	26.80	183.35	1868.82	62.23	290.84	64.54	3.54E+06	3.66E+06
11	SC-40	15:1	115.45	21.14	75.31	113.54	15.24	44.76	7.42	1.30E+05	2.00E+05
17	SC-40	15:1	133.42	20.35	50.34	N/A	197.65	143.59	8.07	2.31E+06	2.95E+04
5	SC-80	5:1	647.37	68.17	11.50	2133.64	150.52	222.78	18.18	0.00E+00	2.02E+05
12	SC-80	5:1	542.85	50.90	5.47	128.49	14.11	33.99	8.64	0.00E+00	2.84E+06
18	SC-80	5:1	490.19	61.53	5.75	N/A	285.58	31.46	18.24	0.00E+00	9.71E+05
6	SC-20*	5:1	145.18	20.24	579.71	1925.50	3.64	219.01	22.32	1.11E+06	8.45E+04
13	SC-20*	5:1	170.14	29.43	555.70	150.22	0.33	25.12	2.73	5.20E+05	9.40E+04
19	SC-20*	5:1	105.61	15.43	371.73	N/A	286.47	11.93	0.00	2.76E+06	1.74E+05
7	no chitin	1:0	1.29	1.47	0.00	1247.90	435.31	720.08	27.09	6.43E+05	0.00E+00

* Neg. Control (SC-20; no inoculum). **Neg. Control (Inoculated; no chitin). N/A = not applicable.

Appendix B

Qualitative q-PCR data

Table B.1. Qualitative data for q-PCRreactions including the linear range for standard curves and efficiency.

Target Organism	Target gene	tested conc. range (ng/uL)*	tested conc. Range (copies/reaction)	slope	y-intercept	efficiency	slope average	slope stnd dev	y-intercept average	y-intercept stnd dev	efficiency average	efficiency stnd dev
Dehalococcoides	**bvcA**	10^{-2}—10^{-7}	6.8×10^{1}—6.8×10^{6}	-3.237	40.34	1.0367						
strain BAV1		10^{-2}—10^{-7}	6.8×10^{1}—6.8×10^{6}	-3.3279	39.75	0.9975	-3.28	0.06	40.05	0.42	1.02	0.03
Dehalococcoides	**tceA**	10^{0}—10^{-6}	6.8×10^{2}—6.8×10^{8}	-3.145	39.15	1.0795						
strain FL2		10^{0}—10^{-6}	6.8×10^{2}—6.8×10^{8}	-3.214	40.97	1.04709	-3.18	0.05	40.06	1.29	1.06	0.02
		10^{1}—10^{-6}	6.8×10^{2}—6.8×10^{9}	-3.398	42.44	0.9692						
Dehalococcoides	**vcrA**	10^{0}—10^{-6}	6.8×10^{2}—6.8×10^{8}	-3.099	40.37	1.102						
strain GT		10^{0}—10^{-6}	6.8×10^{2}—6.8×10^{8}	-3.201	41.95	1.053	-3.15	0.07	41.16	1.12	1.08	0.03
		10^{1}—10^{-6}	6.8×10^{2}—6.8×10^{9}	-3.408	42.25	0.9647						
universal bacteria	**16S**	10^{0}—10^{-5}	6.4×10^{3}—6.4×10^{8}	-3.293	39.79	1.0122						
		10^{0}—10^{-5}	6.4×10^{3}—6.4×10^{8}	-3.233	39.81	1.0383	-3.31	0.09	39.54	0.44	1.01	0.04
		10^{0}—10^{-6}	6.4×10^{2}—6.4×10^{8}	-3.409	39.03	0.9647						
Dehalobacter sp.	**16S**	10^{0}—10^{-6}	6.6×10^{2}—6.6×10^{8}	-3.177	39.59	1.0642						
		10^{0}—10^{-6}	6.6×10^{2}—6.6×10^{8}	-3.205	39.19	1.0512	-3.21	0.04	39.72	0.60	1.04	0.03
		10^{0}—10^{-6}	6.6×10^{2}—6.6×10^{8}	-3.258	40.37	1.0112						
Dehalococcoides sp.	**16S**	10^{0}—10^{-6}	6.8×10^{2}—6.8×10^{8}	-3.483	49.46	0.9369						
		10^{0}—10^{-6}	6.8×10^{2}—6.8×10^{8}	-3.281	46.31	1.017	-3.28	0.14	46.31	2.23	1.02	0.06
		10^{1}—10^{-5}	6.8×10^{3}—6.8×10^{9}	-3.369	44.3	0.9807						

*based on spectrophotometric quantification of the DNA at 260nm and 10-fold serial dilutions

Appendix C

Mothur software program equations and additional figures.

Equation used for calculation of the Chao1 richness estimator:

$$S_{chao1} = S_{obs} + \frac{n_1 (n_1 - 1)}{2 (n_2 + 1)}$$

Where:
S_{chao1} = the estimated richness
S_{obs} = the observed number of species
n_1 = the number of OTUs with only one sequence (i.e. "singletons")
n_2 = the number of OTUs with only two sequences (i.e. "doubletons")

Equations used for calculation of the Shannon diversity index:

$$H_{shannon} = -\sum_{i=1}^{S_{obs}} \frac{n_i}{N} ln \frac{n_i}{N}$$

$$var\left(H_{Shannon}\right) = \frac{\sum_{i=1}^{S_{obs}} \frac{n_i}{N} \left(ln \frac{n_i}{N}\right)^2 - H_{shannon}^2}{N} + \frac{S_{obs} - 1}{2N^2}$$

$$LCI_{95\%} = H_{shannon} - 1.96 \sqrt{var\left(H_{shannon}\right)}$$

$$UCI_{95\%} = H_{shannon} + 1.96 \sqrt{var\left(H_{shannon}\right)}$$

Where:
S_{obs} = the number of observed OTUs
n_i = the number of individuals in OTU *i*
N = the total number of individuals in the community

Equation used for calculation of Good's coverage:

$$C = 1 - \frac{n_1}{N}$$

Where:
n_i = the number of OTUs that have been sampled once
N = the total number of individuals in the sample

Equation used for calculation of the Jaccard similarity index:

$$D_{Jaccard} = \frac{S_{AB}}{S_A + S_B - S_{AB}}$$

Where:
S_{AB} = the number of shared OTUs between communities A and B
S_A = the number of OTUs in community A
S_B = the number of OTUs in community B

Equation used for calculation of the Yue and Clayton nonparametric maximum likelihood estimator of similarity:

$$D_{\Theta_{YC}} = 1 - \frac{\sum_{i=1}^{S_T} a_i b_i}{\sum_{i=1}^{S_T} (a_i - b_i)^2 + \sum_{i=1}^{S_T} a_i b_i}$$

Where:
S_T = the total number of OTUs in communities A and B
a_i = the relative abundance of OTU i in community A
b_i = the relative abundance of OTU i in community B

Additional figures generated using the Mothur software program.

A distance tree was generated for all samples to evaluate membership similarity based on OTUs at a cutoff of 0.10 using the Jaccard method (Figure C.1). All the samples from the No Chitin Control column clustered together. This could indicate the substrate associated microorganisms were dominant species in the other columns and/or that without a carbon and electron donor source, the inoculum-associated bacteria became

less dominant. Either way, the presence of substrate clearly played an important role in the development of community members seen in the columns. The effluent pore water from the No Inoculum Control and Sterile Control also formed a cluster. Since neither of these columns was inoculated, this difference seems to indicate that the distribution of crab shell community did not progress past mid-column.

Dendrograms (Figure C.2) were also constructed for the two ethene producing columns using the member-based Jaccard coefficient and the structure-based Yue Clayton similarity coefficient (Θ_{YC}) to further investigate similarity of the microbial populations. Although the generated dendrograms have similar topologies, the terminal branch lengths of the thetaYC tree are longer. This indicates that while the memberships (Jaccard coefficient) are similar, there is a difference in the relative abundances of the shared OTUs. If the reason Column $Ethene_{115}$ produced ethene faster than Column $Ethene_{369}$ is the presence of community members capable of supplying key nutrients to *Dehaloccoides* species, then perhaps these unidentified community members must not only be present but also exist in significant abundance.

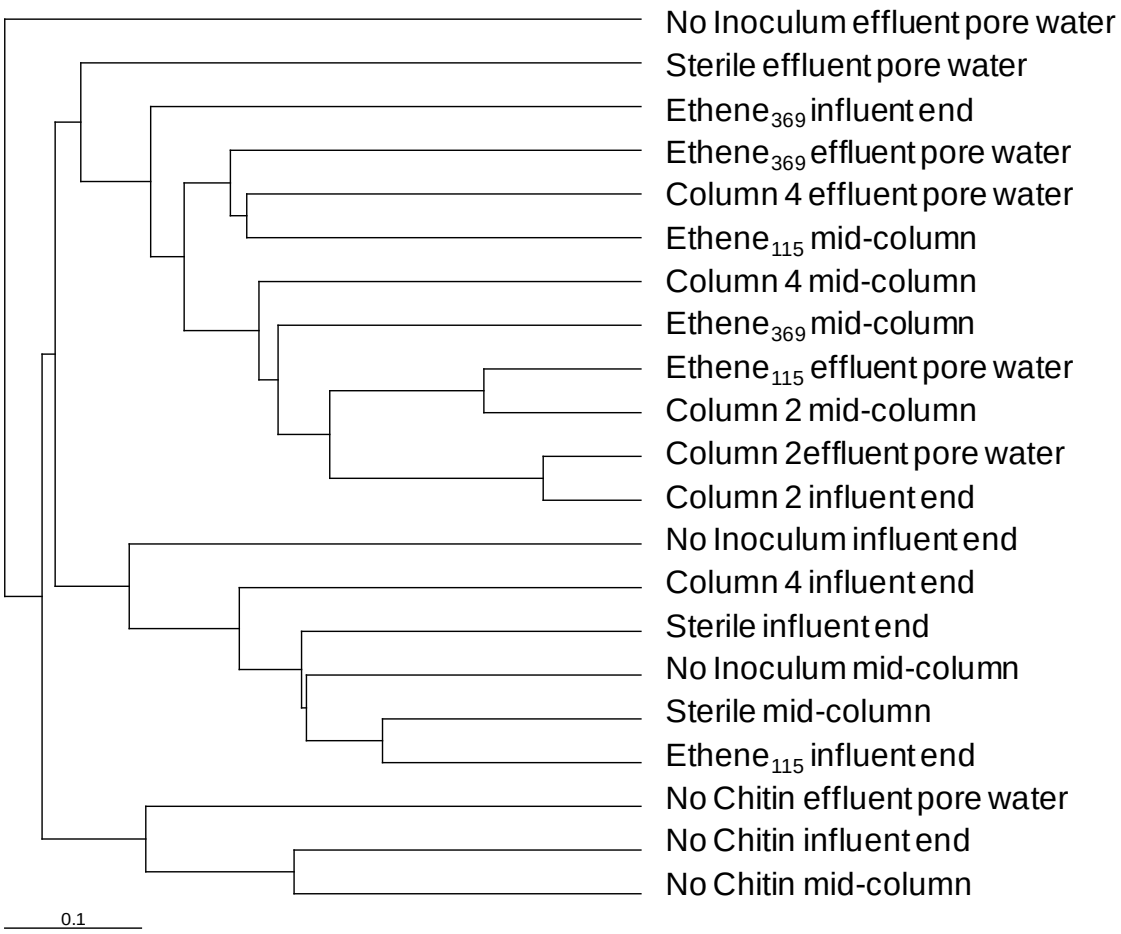

Figure C.1. Distance tree of all pore water and solid-phase samples analyzed by T-RFLP calculated using OTUs bins with a cut off of 0.10 and the membership-based Jaccard coefficient.

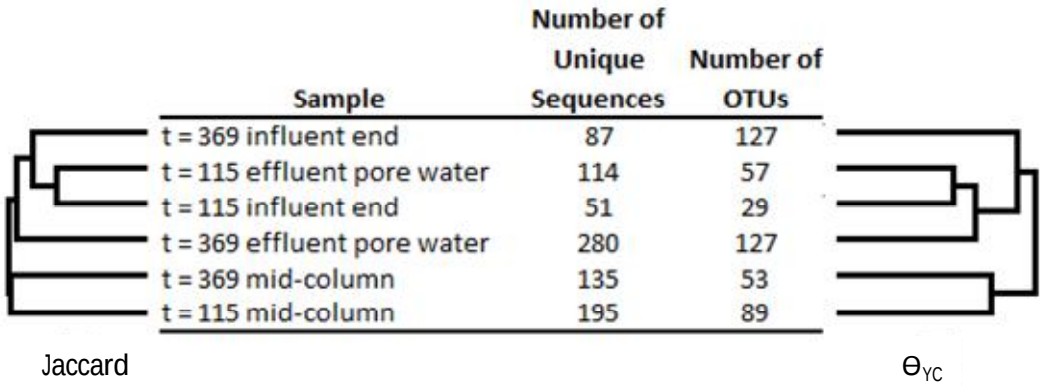

Figure C.2. Dendritic comparison of effluent pore water and solid-phase samples from the two ethene producing columns. The dendrogram to the left represents the similarity of the samples based on the membership-based Jaccard coefficient. The dendrogram on the right represents the similarity of the samples based on the structure based thetaYC coefficient.

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Appendix D

Natural groundwater anion analysis and BDI media composition.

Table D.1. Natural groundwater anion analysis.

Sample ID	Cl (mg/L)	NO_3 (mg/L)	PO_4 (mg/L)	SO_4 (mg/L)
pre-filtration	1.49	0.09	0.10	5.97
post-filtration	1.72	0.12	0.30	6.01
autoclaved	1.45	0.04	0.16	5.82
carboy	1.41	0.03	0.12	5.75
average	1.52	0.07	0.17	5.89
standard deviation	0.14	0.04	0.09	0.12

Table D.2. BDI media composition.

BDI Media

Trace Element Solution	Final Concentration	
HCl	25%	w/w
FeCl2 * 4 H2O	1.500	mg/L
CoCl2 * 6H2O	0.190	mg/L
MnCl2 * 4H2O	0.100	mg/L
ZnCl2	0.070	mg/L
H3BO3	0.006	mg/L
Na2MoO4 * 2 H2O	0.036	mg/L
NiCl2 * 6 H2O	0.024	mg/L
CuCl2 * 2 H2O	0.002	mg/L
Se/W Solution		
Na2SeO3 * 5 H2O	0.006	mg/L
Na2WO4 * 2 H2O	0.008	mg/L
NaOH	0.001	mg/L
Mineral Salts Medium		
NaCl	1000	mg/L
MgCl2 * 6 H2O	500	mg/L
KH2PO4	200	mg/L
NH4Cl	300	mg/L
KCl	300	mg/L
CaCl2 * 2 H2O	15	mg/L

Vitamins	Final Concentration	
biotin	0.020	mg/L
folic acid	0.020	mg/L
pyridoxine HCl	0.100	mg/L
riboflavin	0.050	mg/L
thiamine	0.050	mg/L
nicotinic acid	0.050	mg/L
pantothenic acid	0.050	mg/L
vitamin B12	0.001	mg/L
p-aminobenzoic acid	0.050	mg/L
thioctic acid*	0.050	mg/L
Other Constituents		
Resazurin (0.1% sol.)	0.250	mL/L
Na2SO4 (0.05 mM)	7.102	mg/L
L-cysteine (0.2 mM)	31	mg/L
Na2S * 9 H2O	48	mg/L
NaHCO3 (30 mM)	2520	mg/L

Appendix E

Relative abundance of *Dehalobacter* 16S rRNA gene copies as determined by qPCR.

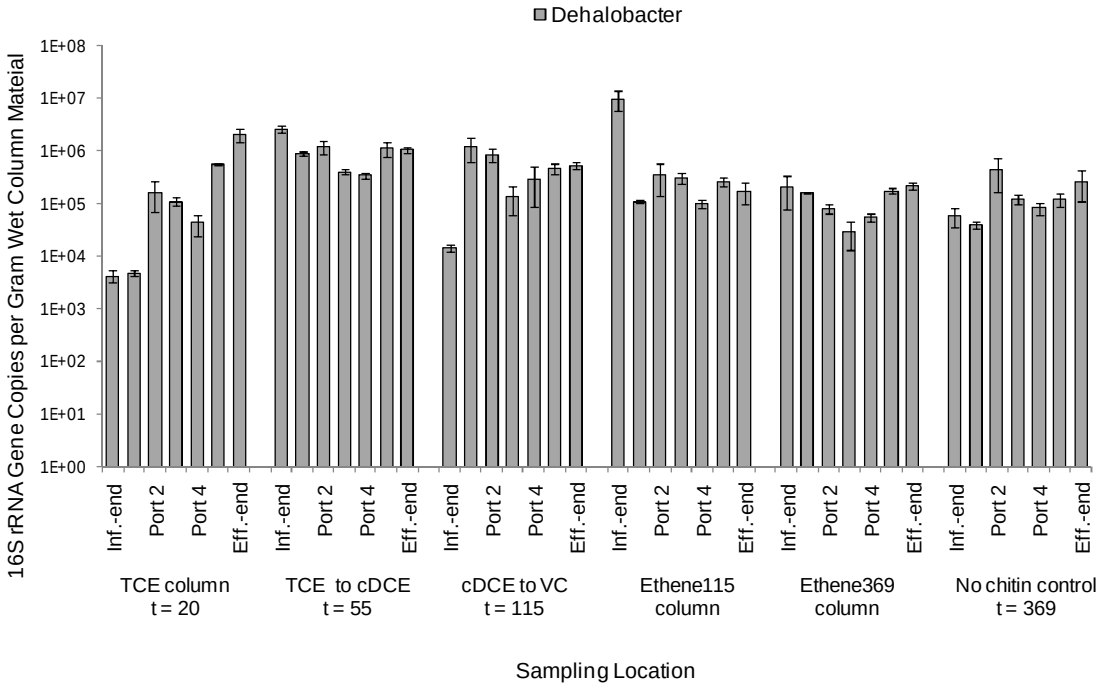

Figure E.1. Number of *Dehalobacter* 16S rRNA gene copies determined with q-PCR in the continuous flow columns over time. Bars represent triplicate averages; error bars represent one standard deviation. Note: *Dehalobacter* was not detected in the un-inoculated controls so those columns are not shown here.

Appendix F

Supplementary chemical data from the continuous flow column experiment.

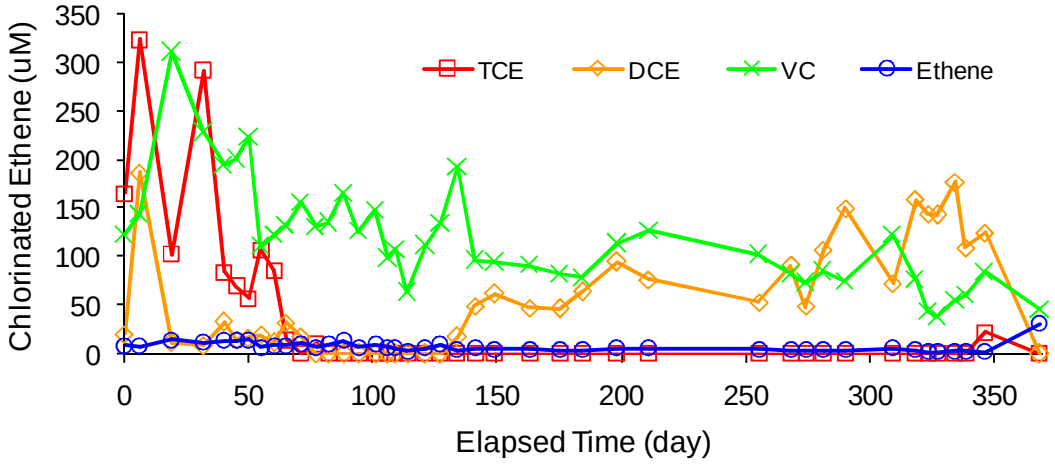

Figure F.1. Typical chlorinated ethene profiles observed in the effluent of a continuous flow column given 100 uM TCE. Data was collected from the VC to ethene column sacrificed on day 369.

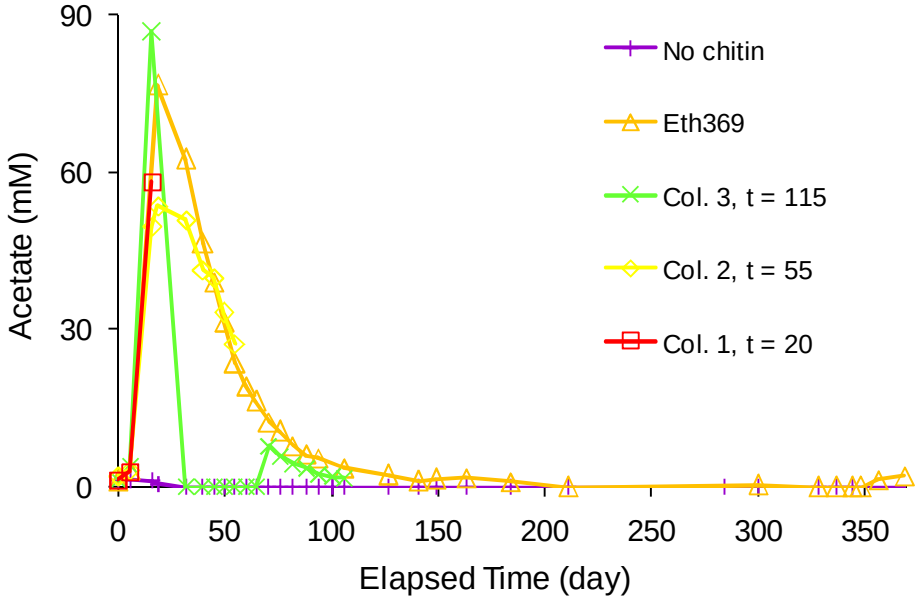

Figure F.2. Acetate acid concentrations observed in the effluents of continuous flow columns. All columns, except the no chitin control, contained 30 grams of crab shell.

CURRICULUM VITA

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EDUCATION

2004 - 2011	Ph.D. in Environmental Engineering, Penn State University
1997 - 1998	M.S. in Environmental Engr. Sciences, University of Florida
1994 - 1997	B.S. in Marine Science, Coastal Carolina University

PROFESSIONAL EXPERIENCE

Spring 2003	Instructor Marine Science, Coastal Carolina University, Conway, SC
2000 - 2003	Environmental Consultant, S&ME, Inc, Atlanta, GA

AWARDS AND SCHOLARSHIPS

2009 - 2011	Teaching Assistantship
2006 – 2009	NSF Graduate Teaching Fellowship in K-12 Education DGE-0338240
Summer 05 & 06	Cecil M. Pepperman Memorial Graduate Fellowship
2004-2005	Research Assistantship

SELECTED PRESENTATIONS AND PUBLICATIONS

McElhoe, J.A., and R.A. Brennan. **2011**. Evaluation of Hydrogen Competition in a Reductively Dechlorination System through FISH Analysis. *Poster presentation*. Environmental Chemistry Student Symposium. The Pennsylvania State University, University Park, PA.

Clemons, J. and J.A. McElhoe. **2008**. Hydrogen Fuel Cell Cars. *Platform Presentation*. 56th Annual Technology Education Association of Pennsylvania (TEAP) Conference. Camp Hill, PA.

McElhoe, J.A., and R.A. Brennan. **2008**. Spatial and Temporal Quantification of Multiple *Dehalococcoides* Strains throughout the Stages of Reductive TCE Dechlorination. *Platform Presentation*. Remediation of Chlorinated and Recalcitrant Compounds Sixth International Conference, Monterey, CA.

McElhoe, J.A., and R.A. Brennan. **2008**. Chemical & Molecular Characterization throughout the Course of Chitin-Enhanced TCE Dechlorination. *Platform presentation* – 2nd place. 10th Annual Environmental Chemistry Student Symposium, The Pennsylvania State University, University Park, PA.

Cossey, K.N., Curtis, N., McElhoe, J.A., and D. Haworth. **2008**. Grad Students & Teachers Work Together to Introduce Fundamental STEM Concepts to K-12. *Poster presentation.* The American Association for the Advancement of Science (AAAS) Annual Meeting, Boston, MA.