REDUCTIVE DECHLORINATION OF TCE BY KB-1™ - INOCULATED MULCH COLUMNS IN THE PRESENCE OF VARIOUS TERMINAL ELECTRON ACCEPTORS

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

By

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May 2016

Dedicated to Mom and Dad,

for their love and support

ABSTRACT

Trichloroethene (TCE), a chlorinated ethene with three chlorine atoms, is not thought to occur naturally in the environment at significant concentrations. However, it has been found in the groundwater and other surface water sites as a result of manufacture, use and disposal of chemicals. Now it has become one of the most common water contaminants. People have paid much attention to the removal of TCE especially after it has been confirmed as a human carcinogen by the U.S. Department of Health and Human Services. The Maximum Contaminant Level (MCL) for TCE (5 micrograms/L) has been strictly regulated in drinking water by the USEPA. In the past several decades, in order to remove TCE and other chlorinated ethenes from water resources, various removal processes have been developed. Among them, biological and reductive processes are more favorable than abiotic and/or oxidative processes when considering the removal outcome and overall cost. Moreover, using biobarriers shows great potential due to its effectiveness, durability and environmental friendliness. For biological TCE removal, KB-1^{TM} culture is a commercialized, reliable culture which can perform complete dechlorination from tetrachloroethene (PCE) to the harmless ethene, and it has both *Geobacter* and *Dehalococcoides (Dhc)* dechlorinators. In this research, a column study was done to observe and measure the dechlorinating performance of KB-1^{TM} in simulated lab-scale biobarriers. Four mulch columns were set up to act as biobarriers and KB-1^{TM} culture was inoculated into the columns to carry out the dechlorination.

Previous research has shown that the presence oxygen or other alternative electron accepters like nitrate and sulfate could limit the performance of the dechlorinators. In previous work it was shown that mulch biobarriers inoculated with KB-1^{TM} could completely dechlorinate 1 mg/L TCE even when influent water was saturated with oxygen. In this research, nitrate and sulfate were pumped into experimental columns to determine the impact on dechlorination ability. Control columns were intended to be identical, but without added nitrate or sulfate in their influents. Gas chromatography (GC) was used to detect methane and dechlorination products at locations along the columns, Polymerase Chain Reactions (PCR) (to detect denitrifiers, dechlorinators and methanogens) and gel electrophoresis were performed on DNA extracted from pore water samples from the columns, and Quantitative-PCR (qPCR) was run to quantify microorganisms of interest (methanogens and KB-1^{TM} *Dhc*) in the columns. According to the GC results and qPCR reports, the KB-1^{TM} *Dhc* populations in the experimental columns were smaller than those in the control columns. Moreover, the methane production rates and dechlorination rates in control columns were also lower than the rates in the control columns. Complete dechlorination to ethene was seen in control columns but with poor material balances. The presence of nitrate and sulfate as low as 0.25 mM impacted dechlorination rates and end products. Though cis-DCE was seen in experimental columns after nitrate and sulfate were quartered, neither VC nor ethene was observed – suggesting that although the *Geobacter* population was activated, the *Dhc* populations were present but inactive.

BIOGRAPHICAL SKETCH

Amber came to the United States to pursue further study in 2014. Before coming to Cornell University, she has obtained a Bachelor of Engineering degree in University of Shanghai for Science and Technology (USST). She decided to take Environmental Engineering as her major even before taking the College Entrance Examination in China because at that time, she thought that an Environmental Engineer could create a super green and sustainable environment. As an environmentalist and pescatarian, she regards it as her life-time goal. At the sophomore year in USST with a major of Environmental Engineering, she knew that this Environmental Engineering major could be more realistic and important, because rather than creating a green world, it is more about turning the world back to a green and sustainable one by solving the existing environmental problems. Therefore, Amber made up her mind to devote herself into this field.

She focused on physical and chemical removal processes during her undergraduate time. In her undergraduate research, she tried to synthesize a new material by writing nanoscale zero-valent Iron onto granular activated and then test the absorption capacity of the new material to hexavalent Chromium in water samples. After a semester's study in Cornell University especially the talk with Professor Richardson, she realized her passion to the bioremediation and curiosity to microbiology. She would like to raise the microbes as pets and rely on them to remediate the contaminated water. Therefore, she made every effort to learn about environmental

microbiology from courses as well as lab skills from other group members, in order to lay foundation for her research and enable her to continue and finish her research.

Her time in Cornell University and the United States is wonderful and life-time memorable although she has made up her mind to go back to her hometown in China after graduation. She wants to apply what she has learnt into practice and show that knowledge and technology are universal. Moreover, she wishes to devote herself into making a more sustainable world soon.

ACKNOWLEDGEMENTS

First, I would like to give my special appreciation to Professor Ruth Richardson and Professor James Gossett for opening the gate of microbiology to me. I also want to thank them for their instructions and suggestions during the study and research process, and also for the encouragements they gave me as well as the great patience they showed in my two academic years studying in Cornell University. Not only the knowledge, but also their devotion to research and adherence to truth. Moreover, there attitude towards doing research, the way they think and react to problems, no matter technical problems or unexpected experiment results.

Second, I would like to give my gratefulness to my beloved parents. They have been supporting me for my education for more than a decade. Now they used the total amount of money that could buy them another decent apartment to continue to support my study in Cornell University while others in their age have been receiving the paid back from their offspring. I feel kind of guilty for now, but I know I will try my best to provide them a better life in the soon future. Moreover, I could not be more grateful for their endless encouragement and comfort when I was struggling and fighting with difficulties and loneliness.

Third, I want to give thanks and appreciation to my colleagues Cristina Fernandez-Baca and Yolanda Brooks for their kindness and patience in helping me with the molecular tools. It is very possible that I could never have a chance to get touch with molecular technology in such level without them. I also want to give my special thanks to Annie Otwell, no matter as a lecturer

in one of the courses or as a colleague and friend. She gave answers to every of my questions with great patience and I guess some of my questions could be quite stupid. Besides, I would like to thank my group member Tiffany Lee, who led me into the mulch column studies and showed me how to do the laboratory work in person. It was enjoyable to be friend with her both inside lab and out of the lab. I also want to thank Larry Ge, who originally set up the columns with Tiffany together and helped me a lot in taking care of the columns. Moreover, I would like to give my thanks to Yitian Sun, for the background he provided me about the mulch column study and the encouragement he gave me when we encountered at the 2015 commencement.

Then I would like to thank all the faculty members of Environmental Processes: Professor Damian Helbling, Professor Leonard Lion and Dr. Monroe Weber-Shirk for their selfless help and encouragement.

Finally, I want to give my thanks to all my friends in Cornell University, the managers and staff in Cornell Dining for providing me a part-time job and promotion to Student Supervisor, as well as all the people who helped me getting over the tough time even just gave me a kind word or warm smile.

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LIST OF ABBREVIATIONS

ASTDR	**Agency for Toxic Substances and Disease Registry**
***cis*-DCE**	***cis*-1,2-dichloroethene**
Dhc	***Dehalococcoides***
DO	**Dissolved Oxygen**
FID	**Flame Ionization Detector**
GC	**Gas Chromatography**
HRC	**Hydrogen Release Compounds**
HRT	**Hydraulic Residence Time**
MCL	**Maximum Contaminant Level**
PCE	**Tetrachloroethene**
PCR	**Polymerase Chain Reaction**
PRB	**Permeable Reactive Barrier**
qPCR	**Quantitative Polymerase Chain Reaction**
RDh	**Reductive Dehalogenases**
TCD	**Thermal Conductivity Detector**
TCE	**Trichloroethene**
USEPA	**United States Environmental Protection Agency**
VC	**Vinyl chloride**
ZVI	**Zero-Valent Iron**

CHAPTER 1 BACKGROUND AND OBJECTIVES

1.1 Introduction

The development of humanity and industry is at the expense of the natural environment. Groundwater contamination, air pollution and other types of pollution have been brought into our environment. Contamination of water resources is becoming even more severe. Many compounds that are not thought to occur naturally in the environment were found in the groundwater as well as some other surface water sites, and they have been severely threatening human life as well as other living creatures on the earth. Chlorinated ethenes, a group of toxic and common groundwater contaminants, have aroused public attention in the past several decades.

Trichloroethene (TCE) is a chlorinated ethene with three chlorine atoms on the alkene molecule. It is nonflammable, colorless liquid with a sweet, burning taste (Agency for toxic Substances & Disease Registry, ATSDR, page last updated on March 3rd, 2011). It is used mainly as a solvent to remove grease from metal parts, also an ingredient in adhesives, paint removers, typewriter correction fluids as well as spot removers. TCE is confirmed to be a human carcinogen (U.S. Department of Health and Human Services, 2005.). It is not thought to occur naturally in the environment. However, in the past decades, it has been found in underground water sources and many surface water sites as a result of the manufacture, use and disposal of the chemicals (Committee on Ground Water Cleanup Alternatives, National Research Council. 1994). Now it is one of the most common groundwater contaminants that is endangering many groundwater supplies across the United States (Bennett et al., 2007).

Tetrachloroethene (PCE), TCE, *cis*-dichloroethene (*cis*-DCE) and vinyl chloride (VC) are all included on the United States Environmental Protection Agency (USEPA) and Agency for Toxic Substances and Disease Registry (ATSDR) 2011 Substance Priority List, with PCE, TCE and VC in the top 40 (ATSDR – Priority List of Hazardous Substances 2011). Besides, VC, the daughter product of natural, reductive transformation of TCE, poses an even more serious threat to humans. According to the USEPA 2008 report, the Maximum Contaminant Level (MCL) for PCE is 0.005 mg/L, for TCE is also 0.005 mg/L, for *cis*-DCE and *trans*-DCE is 0.07 mg/L and 0.1 mg/L, respectively. However, the MCL for VC is only 0.002 mg/L. Therefore, remediation of these chlorinated ethenes, especially the complete dechlorination of them in groundwater, has gained people's attention.

Both *ex situ* and *in situ* groundwater remediation technologies have been applied to TCE treatment, oxidative and reductive, biotic and abiotic. Since TCE is highly oxidized, reductive dechlorination is more favorable than oxidative processes. In recent years, *in situ* bioremediation has become a popular alternative for the remediation of TCE-contaminated sites (Lovley, D. R. et al. 2001; McCarty, P. L. et al., 1997). Among the microorganisms applied to biotically degrade chlorinated ethenes, *Dehalococcoides (Dhc)* is the only documented genus that can completely reduce PCE and TCE to the harmless ethene (Hendrickson, 2002), so cultures containing *Dhc* strains are often added at sites to bioaugment any natural populations in order to speed up bioremediation. Biobarriers are one of many bioremediation options, but they are not always appropriate. Aggressive plume-wide enhanced bioremediation is more common than biobarriers. If the plume is highly concentrated in TCE and it is in a populated area, biobarriers would not be chosen.

1.2 Groundwater Treatment Technologies

1.2.1 Ex situ Remediation

Ex situ bioremediation technologies can most easily be classified by the physical state of the medium to which they are typically applied. Common to the *ex situ* remediation technologies are the processes for removing contaminated materials for treatment. Contaminated media are excavated or extracted (e.g. ground water removal by pumping) and moved to the process location, which may be within or adjacent to the contaminated site (Engineering Issue, United States Environmental Protection Agency, 2006). Until recently, the most conventional method for groundwater remediation has been the pump and treat method (Reddy, 2008). With pump and treat, free-phase contaminants and/or contaminated groundwater are pumped to the surface. Treatment occurs above ground, and the cleaned groundwater is either discharged into sewer systems, surface waters, or re-injected into the subsurface (Cohen, 1997). Pump and treat requires simple equipment and it is effective for source-zone removal where free-phase contamination is present and it was successful during the initial stages of implementation (Reddy, 2008). However, pump and treat performance drastically decreases at later times and significant amounts of residual contamination remained untreated. Due to these limitations, the pump and treat method is now primarily used for free product recovery and control of contaminant plume migration (Reddy, 2008).

In general, *ex situ* groundwater remediation approaches like pump and treat could be somewhat effective, but these methods are costly to operate for decades and require surface

treatment. Moreover, they have resulted in the transfer of contaminants to somewhere else and very often, do not achieve final treatment goals (Semprini, 1995).

1.2.2 In situ Physical and Chemical Remediation

Some common physical and chemical *in situ* remediation technologies are *in situ* air sparging, *in situ* flushing, and permeable reactive barriers.

1.2.2.1 In situ Air Sparging

Air sparging is a remediation technology useful in the treatment of volatile organic contaminants. During the implementation of air sparging, a gas, usually air, is injected into the saturated soil zone below the lowest known level of contamination and as the air comes into contact with the contamination, it will strip the contaminant away or assist in *in situ* degradation. Eventually, the contaminant-laden air encounters the vadose zone, where it is often collected using a soil vapor extraction system and treated on-site (Reddy, 1995; Reddy, 2001). Compared with pump and treat method (*ex situ*), the overall cost of air sparging (*in situ*) can be much lower. However, technical challenges come with the low permeability and stratified soils could be the limitations. Also, if the whole process is not properly designed, it could cause the spreading of the contaminants into clean areas.

1.2.2.2 In situ Flushing

Soil flushing is to pump flushing solution into groundwater via injection wells. The solution then flows down gradient through the region of contamination and then desorbs, solubilizes, and/or flushes the contaminants from the groundwater and aquifer solids. After the

solubilization of the contaminants, the solution is pumped out. The contaminated solution then is treated at the surface using typical wastewater treatment methods, and then recycled by pumping it back to the injection wells (USEPA, 1991; Roote, 1997).

1.2.2.3 Permeable Reactive Barriers

Permeable reactive barriers (PRB) constitute a semi-passive remediation technology that utilize media which cause chemical or biochemical reactions to transform or immobilize contaminants. PRBs could treat the contaminated water with physical and/or chemical methods, although most of the PRBs remove contaminants through biological methods. One of the most widely used media in PRBs for physical and chemical treatment is iron, with a form of zero-valent Iron (ZVI) or iron alloys. ZVI treats contaminated groundwater through a combination of redox processes (some of which may be biologically mediated, such as the reduction of sulfate), precipitation reactions, and sorption (Henderson and Demond, 2007).

1.2.3 In situ Bioremediation

In recent years, successful *in situ* bioremediation has been documented for common and wide-ranging groundwater contaminants, including chlorinated solvents (Hendrickson, 2002). The most common *in situ* biotechnologies include monitored natural attenuation, enhanced bioremediation, and phytoremediation (Reddy, 2008). Results of several pilot and full-scale field investigations suggest that stimulated *in situ* bioremediation is a potentially less expensive, more efficient technology for treatment of chlorinated hydrocarbons (USEPA, 2000). Some examples of bioremediation related technologies are phytoremediation, bioventing, biosparging, bioaugmentation and biostimulation.

Bioremediation may occur on its own (natural attenuation or intrinsic bioremediation). It could also be enhanced by adding electron donors and/or carbon sources that help the growth of the remediating microbes within the medium, which is also known as biostimulation. Moreover, bioaugmentation (addition of specific microbial strains into the contaminated medium to increase the populations of desired microorganisms) could also be an effective way to break down contaminants. Different overall strategies for *in situ* bioremediation of TCE by *Dhc* include those focused on the source zone and plume. Commercialized cultures containing dechlorinators especially various *Dhc* strains include KB-1™ culture (SiREM Labs of Guelph, Ontario, Canada), Bio-Dechlor Inoculum Plus™ (REGENESIS, San Clemente, CA, USA), and SDC-9™ (CB&I Federal Services, LLC). More details on these cultures will be given in section 1.3.2.2.

1.2.3.1 Permeable Reactive Barriers

The use of Permeable Reactive Barriers (PRB) is one of the strategies of bioremediation. Biobarriers are most promising for dilute concentrations in groundwater plumes occurring near the surface. Permeable reactive barriers (PRB) constitute a semi-passive remediation technology that utilize media which cause chemical or biochemical reactions to transform or immobilize contaminants. A simplified figure of a typical PRB is given below in Figure 1.1. In general, permeable reactive media are placed across the flow path of a contaminant plume. As the plume moves through the barrier, the contaminants are transformed to nontoxic products. Additionally, the biobarrier material can sorb contaminants temporarily before the microbes transform them.

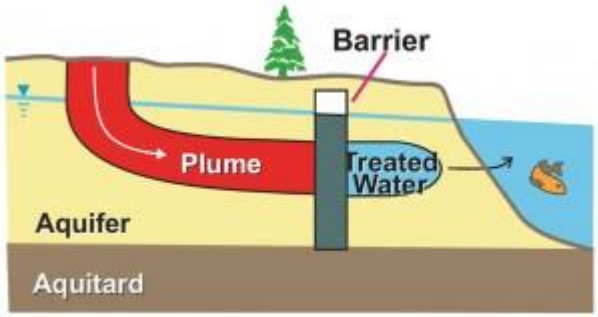

Figure 1.1 Permeable Reactive Barrier. Image from: http://vertexenvironmental.ca/in-situ-remediation/permeable-reactive-barriers/

PRBs are particularly attractive for groundwater remediation because they could be more economic than conventional remediation due to the low operation and maintenance costs (Scherer, 2008). Treating groundwater contaminants via PRBs does not require extracting them up to the surface, because the degradation and/or immobilization of contaminants are *in situ*. Hence no need for expensive above ground facilities for storage, treatment, transport, or disposal other than monitoring wells. Moreover, the operation of PRBs does not require continuous input of energy, because they use a natural gradient of groundwater flow carry contaminants through the reactive zone (Thiruvenkatachari, 2008).

The PRBs remove contaminants by two major processes: (1) Transformation of the contaminant to a less harmful compound; (2) Immobilization of the contaminant within the barrier.

Transformation within a reactive barrier is most commonly an irreversible redox reaction which can convert the contaminant to a less harmful compound. Therefore, it does not necessarily require removal or rejuvenation of the reactive medium before the reactive capacity of the barriers is exhausted (years), unless they are clogged by precipitants and/or microorganisms (Thiruvenkatachari, 2008). The medium may directly or indirectly supply electrons for reduction (e.g., iron metal, iron minerals, organic matter such as mulch or wood chips). These electron donors can then stimulate reduction of electron-accepting contaminants via denitrification, dechlorination, or heavy metal and/or radionuclide reduction) (Scherer, 2008). Evaluation of the media is based on their ability to transform or immobilize the contaminant at sufficiently rapid rates, maintain adequate permeability and reactivity over long time periods, and release only benign compounds as byproducts of the removal process (Scherer, 2000). A wide variety of materials have been employed in PRBs (Nyer, 2003), including organic carbon substrates (e.g. mulch or wood chips, compost) that may create and sustain reducing conditions favorable for anaerobic transformation of chlorinated ethenes and serve as electron donors for reductive dechlorination. Previous research has shown that using stationary permeable barriers created by injecting innocuous vegetable oil into the contaminated aquifer could also achieve *in situ* groundwater remediation. The oil injected provided the electron donor for microorganisms that remediated chlorate- or perchlorate-contaminated water (Hunter, 2002). Inorganic materials could also be employed in PRBs (e.g., quartz sand and zero-valent iron). Furthermore, the inoculation of microorganisms on the PRBs could contribute positive effects to the performance (Van Nooten, 2008). According to previous research by Van Nooten et al., which compared the degradation efficiencies of ZVI containing different iron (III)-reducing *Geobacter*

sulfurreducens strain and/or a bacterial consortium to the degradation efficiencies of noninoculated ZVI in a laboratory-scale column experiment, the reactivity toward iron cycling (defined as green rust production) over time for all inoculated iron columns was increased compared to the noninoculated columns.

Immobilization of the contaminant within the barrier can occur by sorption to the reactive medium or precipitation from the dissolved phase. Both sorption and precipitation processes are generally reversible and therefore may require removal of the reactive materials and accumulated products, depending on the degree to which transformation of sorbed/precipitated contaminants occurs and the geochemistry of the groundwater.

PRBs inoculated with reductively dechlorinating populations (*Dhc* strains) have been confirmed to effectively control the migration of PCE plumes (Lendvay, 2003). In previous research on recirculating-well biobarriers made by Lendvay et al., a side-by-side comparison of bioaugmentation (with *Dhc* -containing PCE-to-ethene dechlorinating inoculum), biostimulation (with continuous lactate and nutrient injection), and a recirculation-only control implementing in a PCE-contaminated aquifer was conducted. The results of this research (i.e., 92% of the total molar concentration of chlorinated ethenes was converted to ethene after 43 days following inoculation in the bioaugmentation site) suggested that biobarriers with reductively dechlorinating populations can be successfully designed once hydrologic information is incorporated.

The material chosen to fill the PRBs in this Masters research was mulch. There are some previous studies that focused on application of mulch PRBs to TCE remediation. Shen and

Wilson conducted a column study to remove TCE from groundwater in 2007(Shen & Wilson, 2007). There were four columns in total with a mean residence time of 17 days, two of them were filled with 50% (volume/volume) shredded tree mulch, 40% sand and 10% cotton gin trash (mulch columns) to act as a PRB, the other two were similar but replaced sand with hematite and/or limestone (hematite and/or limestone), but none of them was bioaugmentated. As the plant mulch decayed in the PRB, it provided electron donor for microbial dechlorination of the contaminants. In addition, sulfate and iron reduction supported by the mulch caused an accumulation of reactive iron sulfur minerals that can abiotically reduce TCE. According to the result, after 793 days of operation, the TCE concentration in the effluent of mulch and sand columns was 2% of the influent; TCE removal rates in mulch columns with hematite and/or limestone were even higher, TCE concentrations in effluent of those two columns were just 0.6% of the influent. In general, conditions were conducive for biological reductive dechlorination in the mulch columns (DO levels producing rate were low, methane was being produced, and the pH was near neutral). However, there was little evidence of significant concentrations of TCE-dechlorination daughter products - Less than 1% of TCE that was removed in the mulch columns could be accounted for as biological reductive dechlorination intermediats (DCE or VC) or end products (ethene). The authors estimated that 80% to 90% of the TCE removal was due to abiotic transformation by FeS minerals formed from high concentration of sulfur in local groundwater (Shen, 2007).

Follow-up research was done by Shen et al. at 2010 in order to study the long-term capacity of plant mulch to support TCE bioremediation. The columns and operational conditions were the same as their study in 2007 but with bioaugmentation: the columns were inoculated

with a culture of dechlorinating bacteria enriched from groundwater from a monitoring well at Altus Air Force Base (AFB), Altus, Okla. After treatment, the TCE concentration declined from 3,200 µg/L to 1.2 µg/L. Dechlorination of TCE to ethene was achieved using plant mulch; however, neither water extractable nor organic-solvent extractable components of the mulch could sustain dechlorination of TCE. This indicates that biodegradation of organics in the plant-derived mulch served as electron donor for dechlorination of TCE. Kinetic analysis of the methane production in the batch tests provides supporting evidence that the plant mulch is able to sustain long-term biological activity in a typical barrier constructed with plant tissues. The mulch in the biobarriers was estimated to support microbial activity for 10 years before need for replacing the mulch or recharging the biobarrier with soluble electron donors (Shen, 2010).

According to a previous Cornell MS student, Yitian Sun, the dechlorinators (both *Dhc* strains and *Geobacter*) inoculated into the column were still found in the column (both suspended in water and attached to mulch) more than 5 months later (Sun, 2014). Therefore, one could say that the mulch columns provided a good habitat for the dechlorinators at least 5 months after inoculation. Moreover, the mulch biobarriers performed dechlorination well, since from Sun's research, the mulch biobarrier inoculated with 1:1000 KB-1^{TM} enrichment culture was able to achieve 73% to 99% complete dechlorination of oxygenated water contaminated with 1 mg/L of TCE (Sun, 2014). However, the impacts of other potential electron acceptors like nitrate and sulfate were not examined in that study.

1.3 TCE Degradation Processes

1.3.1 Oxidative Destruction

Chemicals can be used to oxidize TCE. Permanganate, hydrogen peroxide, and ozone are all effective choices according to the USEPA (Huling, 2006). Alternatively, with bioremediation, TCE can aerobically undergo cometabolism by methanotrophs with carbon dioxide as the end product (McCarty, 1998; Wilson, 1985).

1.3.2 Reductive Dechlorination

Compared with oxidation, reductive dechlorination is more favorable to remove TCE from groundwater because organisms can derive growth-benefit from the transformation process. The oxidative biological processes are cometabolic, require oxygen and a primary substrate (methane and/or toluene) to support the oxidizing bacteria. Also, many of the polluted aquifers are anaerobic, and continuous pumping of oxygen/oxygen-releasing compounds into the aquifers could be costly. Additionally, when the TCE concentration is very high, the solubility of oxygen could be a limiting factor. Reductive dechlorination involves a series of reactions (PCE to TCE to *cis*-DCE to VC to ethene) where the chlorine atoms are replaced by hydrogen atoms one by one and ideally with harmless ethene as the end product. The reductive dechlorination of TCE could be both abiotic and biotic. Biologically, TCE supports growth by organohalide respiration.

1.3.2.1 Abiotic Reductive Dechlorination

Abiotically, chemical reactants which could achieve this goal are mainly carried by ZVI or iron sulfide (FeS) (Reddy, 2008). Among a variety of reactive materials, ZVI has shown the

greatest efficiency for reducing chlorinated compounds in groundwater (US EPA, 1997, 2002). The previous findings by Teerakun et al. (2008) indicated that concentrations of TCE as high as 1000 mg/L did not reduce the efficiency of ZVI to remove TCE. Moreover, abiotic methods could also be combined with other biotic methods to degrade chlorinated ethenes in order to yield a higher efficiency. In previous research by Teerakun et al. (Teerakun, 2011), a three-stage reactive barrier system included both abiotic and biological processes in series to treat a high concentration of TCE (500 mg/L): iron fillings as an iron-based barrier material in the first column; sugarcane bagasse mixed with anaerobic sludge as an anaerobic barrier in the second column; and a biofilm coated on "oxygen carbon inducer releasing" material as an aerobic barrier in the third column. The system reached an 87% overall TCE removal efficiency with a hydraulic residence time of approximately 26 days. However, complete dechlorination was not achieved. The majority of the TCE was only partially dechlorinated to *cis*-DCE and VC.

1.3.2.2 Biotic Reductive Dechlorination

Biotic reductive dechlorination is mainly achieved by various strains of dechlorinating bacteria. The most prominent is *Dhc*. Dechlorinators other than *Dhc*, such as *Geobacter*, *Sulfurospirillum*, *Desulfitobacterium*, and *Dehalobacter* can all partially dechlorinate PCE or TCE to *cis*-DCE (Löffler, 2006). Within the *Dhc* genus, four distinct strains, *Dhc mccartyi* 195 (Maymo´- Gatell, 1999; Maymo´- Gatell, 1997) *Dhc* sp. strain FL2, *Dhc* sp. strain VS, and *Dhc* sp. strain GT (Seshadri, 2005), have been demonstrated to metabolically reduce TCE by anaerobic dehalorespiration. Other strains specialize in DCE and VC reduction only (e.g. *Dhc* strains VS and KB-1-VC) or on other classes of halogenated organics.

Several mixed cultures containing dechlorinators were studied by research groups (Duhamel, 2002; Richardson, 2002; Vainberg, 2009). The results show that the *Dhc* strains are the only genus known to reductively dechlorinate the groundwater pollutants, PCE and TCE, to the nontoxic end product ethene. Several cultures have been isolated from the genus *Dhc* and they can carry out the complete dechlorination (Seshadri, 2005). Because of this proclivity, *Dhc* strains have become important organisms in the implementation of chloroethene bioremediation, and some of the dechlorinating cultures have already been commercialized. Among them, the KB-1™ culture (SiREM Labs of Guelph, Ontario, Canada) is one of the most widely used cultures worldwide. Other commercialized cultures used for bioremediation include Bio-Dechlor Inoculum Plus™ (REGENESIS, San Clemente, CA, USA), and SDC-9™. Bio-Dechlor Inoculum Plus™ is an enriched, microbial consortium containing species of *Dhc* for anaerobic bioaugmentation applications. SDC-9™ (CB&I Federal Services, LLC., [formerly Shaw Environmental, Inc.], The Woodlands, TX, USA) is pathogen-free, non-genetically altered microbial consortium capable of biologically degrading halogenated aliphatic pollutants including PCE, TCE etc. It is composed of anaerobic bacteria including *Dehalococcoides mccartyi* in an aqueous medium. This culture was isolated by enrichment culturing of samples from a chlorinated-solvent-contaminated aquifer in southern California, with lactate as an electron donor and PCE as an electron acceptor (Vainberg, 2009.). The culture has been applied commercially since 2003, and it is sold by licensed distributors under several trade names including RTB-1™, BAC-9™, TSI-DC™, and BioDechlo Inoculum plus™ (California Environmental Protection Agency, State Water Resources Control Board).

The various *Dhc* strains are strict anaerobes; however, they could be used to treat aerobic contaminated water in mulch biobarriers if sufficient, oxygen-scavenging activity exists in the barriers. Based on the research of Yitian Sun, a previous Cornell student who did the research of mulch biobarriers using columns with similar set-ups, within 3 centimeters of the column inlet (port 1) mulch reduced the dissolved oxygen (DO) level of incoming groundwater flow from 7.9 mg/L and generated an environment anaerobic enough for TCE dechlorination and methanogenesis to occur (Sun, 2014). However, an outstanding question is whether mulch based biobarriers could fully dechlorinate TCE if additional competing electron acceptors (such as nitrate and sulfate) were present as well as oxygen and TCE.

1.4 Microorganisms of Interest in This Research

In the dechlorination process, in general, dechlorinators use H_2 as an electron donor (DiStefano, 1992). In an anaerobic system they compete with H_2-utilizing methanogens, SO_4^{2-} reducers and NO_3^- reducers, which can also use H_2 (Yang and McCarty, 1998). Previous research has shown that the presence of alternative electron accepters (nitrate and sulfate, specifically) could show some effects on methane production and dechlorination. The presence of alternative electron acceptors such as NO_3^- and SO_4^{2-}, influences the mineralization and transformation of organic carbon under anoxic conditions (Keller, 2007; Hunt, 2007). Based on the theory mentioned above, a laboratory microcosm study was carried out to elucidate the effects of NO_3^- and SO_4^{2-} on CO_2 and CH_4 production from a freshwater sample (Dodla, 2009). The results showed that both NO_3^- and SO_4^{2-} treatments decreased CH_4 production but NO_3^- almost completely inhibited CH_4 production (>99%) whereas SO_4^{2-} reduced CH_4 production by 78–90%.

Different hypothesis has been made on the mechanism of inhibition and/or competition from the presence of NO_3^- and SO_4^2. Some researchers have speculated that nitrate reducers can outcompete dechlorinators for reducing equivalents under H_2-limited conditions (Lovley, 1994; Chapelle, 1996). According to some other research, SO_4^{2-} could serve as a preferential electron acceptor, therefore inhibit the dehalogenase activity of a specific dehalorespier, *Desulfomonile tiedjei* (Townsend and Suflita, 1997). Alternatively, sulfides may inhibit dechlorination. However, the exact inhibition mechanism is still unclear.

To prove whether bioremediation is occurring or not, one line of evidence is to document the presence and abundance of specific biomarkers unique to the organisms of interest.

Biomarkers are biomolecules (DNA, RNA or protein) that correspond to a specific microbial process or state. They have been used to detect and quantify specific microorganisms in the environment because their presence indicates the existence of a living organism or the occurrence of a specific activity. Among various types of biomarkers, 16S rRNA has been used widely, and distinct 16S rRNA gene sequences are present in different phylogenetic groups of microorganisms. For example, according to some previous studies, 16S rRNA gene sequences are highly conserved among *Dhc* cultures too (Hendrickson, 2002**)** and it has been observed that there is a strong correlation between the presence of the DMC 16S rRNA gene at a site, and the occurrence (or at least the potential) of reductive dechlorination of chlorinated ethenes to ethene. However, presence of the 16S rRNA gene from *Dhc* is not enough to confirm that the *Dhc* are actively conducting complete dechlorination.The reductive dehalogenases (RDh) in the genomes of *Dhc* strains are often used as biomarkers of specific dechlorination steps (see below) (Loffler, 2012) .

In a natural environment, various microorganisms exist in the groundwater and they are surviving on the energy released from fermentation and/or redox reactions. They could co-exist with each other or compete depending on their energy-yielding catabolic reactions. The amount of energy released by different redox reactions, and thus the amount of energy available for ATP synthesis, depends on the redox potential of the terminal electron acceptor. Oxygen (O_2) has the greatest redox potential ($E_0^{'}$ = +0.82 Volts for O_2/H_2O reduction half reaction), and thus aerobic respiration results in the most ATP synthesized. Some *Bacteria* and *Archaea* can use other terminal electron acceptors with lower redox potential when oxygen is not available. Nitrate reduction ($E_0^{'}$ = +0.70 V for NO_3^-/N_2 half reaction) should be more favorable than sulfate reduction ($E_0^{'}$ = -0.22 V ofr SO_4^{-2}/H_2S half reaction) (Madigan et al., 2006). Anaerobic respiration produces less ATP. Though some microorganisms can modify their electron transport chains to use a variety of electron donors and electron acceptors, and, where possible, will switch to the best available electron acceptors available in their environment, most microbes have a particular niche they fill metabolically. Microbes that use more energetically favorable acceptors will out-compete bacteria that use less energetically favorable acceptors.

Methanogens are naturally present in groundwater and surface waters, provided anaerobic niches exist since they are strict anaerobes. Methanogens play an indispensable role in anaerobic wastewater treatments (Tabatabaei, 2010). They are *Archaea* and can produce methane under anaerobic conditions. With respect to TCE dechlorination in biobarriers, we care about methanogens because on one hand, they could act as a marker for the anaerobic condition of the columns. If they could survive in a specific position along the column, it means that that position is anaerobic enough for *Dhc* to survive too. On the other side, which is a negative effect from

methanogens, they would compete with *Dhc* for the electron donor – especially hydrogen gas, which is used by hydrogenotrophic methanogens, and also acetate, which is the carbon source used by *Dhc*.

For methanogen biomarker quantification/detection, a number of primers and probes have been developed to target the 16S rRNA gene sequences of methanogen groups (Castro, 2004; Crocetti, 2006; Marchesi, 2001; Purdy, 2003; Raskin, 1994; Rocheleau, 1999; Sizova, 2003; Sorensen, 1997; Zheng, 2000). Some researchers have developed primers for the gene sequence of the α-subunit of the methyl coenzyme M reductase (*mcrA*) in order to avoid the potential problems with nonspecific amplification using 16S rRNA gene primers (Luton, 2002; Springer, 1995). The *mcrA* is exclusive to the methanogens with the exception of the methane-oxidizing *Archaea* (Hallam, 2003) and shows mostly congruent phylogeny to the 16S rRNA gene, so it could be used as a biomarker to analyze methanogens instead of 16S rRNA.

Besides methanogens, denitrifiers and sulfate reducers can also occur naturally in groundwater, especially in groundwater impacted by excess nitrogen fertilizer in agricultural regions or salt water intrusion in coastal regions. They carry out major roles in nitrogen cycling and sulfur cycling. As with methanogens and *Dhc*, sulfate reducers are strictly anaerobic. Nitrate reducers prefer oxygen as an electron acceptor, but they can reduce nitrate under anoxic condition. The genes of denitrification are well understood and various researchers have developed PCR and qPCR primers for these genes. In analyzing nitrate reducers with molecular tools in this research, nitrite reductase was selected as the biomarker for denitrifiers. This enzyme catalyzes the reduction of nitrite (NO_2^-) to nitric oxide (NO) and is encoded by two different genes: *nirK* and *nirS* (Zumft, 1997). *nirS* is more widespread in denitrifiers and is

highly conserved (Coyne, 1989). Moreover, previous research has evaluated the application of quantitative PCR to the quantification of *nirS* genes in environmental samples (Gruntzig, 2001). For sulfate reduction, dissimilatory sulfite reductase (*dsrA*) is a commonly used biomarker.

As for dechlorinators analysis using molecular tools, reductive dehalogenase genes were the genes of interest and previous molecular investigations of the reductive dehalogenase genes have provided a wealth of sequence information (Seshadri, 2005; Villemur, 2002). The key dechlorinators in KB-1^{TM} culture are *Dhc* and *Geobacter*. For *Dhc*, based on the sequence information mentioned above, degenerate primers to amplify *Dhc* -specific RDh genes were designed by Krajmalnik-Brown et al. (Krajmalnik-Brown, 2004). This allowed for the amplification of many RDh genes from different strains of *Dhc*. However, different *Dhc* have different RDh genes. Given that complete dechlorination is the objective in bioremediation processes, vinyl chloride reductase genes *(vcrA* and *bvcA)* can be used specifically as biomarkers of VC-to-ethene respiring *Dhc* strains. In KB-1^{TM} bioaugmentation culture, the *vcrA* gene was chosen as the biomarker for the main *Dhc* populations. A second vinyl chloride reductase (*bvcA*) is present in a minor *Dhc* population in KB-1^{TM} .For the detection of the dechlorinating *Geobacter* population, the PCE dehalogenase (*pceA)* gene was used. These are the same biomarkers used by a previous Cornell student also analyzing KB-1^{TM} 's dechlorinating populations (Sun, 2014).

1.5 Objectives and Hypothesis

1.5.1 Objectives

The objectives of this research are:

(1) To determine the TCE-dechlorination ability of KB-1^{TM} culture in lab-scale mulch biobarriers to treat water with dissolved oxygen, nitrate and sulfate compared to control columns receiving water with only TCE and oxygen;

(2) To detect various bacteria of interest including KB-1^{TM} dechlorinaters (*Dhc* and *Geobacter*), methanogens, nitrate reducers and sulfate reducers using a molecular tool [Polymerase Chain Reaction (PCR)], and, for methanogens and *Dhc*, to quantify pore-water populations using Quantitative-PCR (qPCR);

(3) To observe the difference in the population ratios of various microorganisms, both between the control and experimental columns, and also at different positions in the columns.

1.5.2 Hypotheses

The hypotheses of this research are:

(1) Complete dechlorination of TCE to ethene in KB-1^{TM}-inoculated mulch biobarriers will be limited in the experimental columns (in the presence of nitrate and sulfate);

(2) Poplulations of KB-1^{TM} dechlorinators (mainly *Dhc* and *Geobacter*) as well as of methanogens will be smaller when nitrate and sulfate are present than in control columns;

(3) The rates of dechlorination at different ports are related to the *vcrA* levels, and the methane productions in different ports are related to the *mcrA* levels.

CHAPTER 2 MATERIALS AND METHODS

2.1 Chemical Reagents

TCE (99.5%, Fisher Scientific) was used to prepare saturated TCE stock solution. Potassium nitrate and potassium sulfate (Fisher Scientific, certified A.C.S.) were added into tap water to make nitrate and sulfate solution filling a liquid reservoir. High purity compressed nitrogen (Airgas), compressed air (Airgas), ultra high purity Hydrogen (Airgas) were used in Gas Chromatography (GC) – with Flame Ionization Detection (FID).

2.2 Mulch Column Set up

A total of four glass columns (5-cm diameter, 60-cm height) were set up on January 15th 2015 to simulate the mulch biobarriers. These mulch-filled columns were prepared as described in Sun (2014). There are supposed to be seven ports in each column. However, the top part of column 4 cracked during the summer of 2015 so column 4 lost port 7 and only had six ports thereafter. A sketch of the columns is presented below in Figure 3.1 in order to visualize and give a more straightforward understanding of the columns. For column1 and column 2, only tap water (with D.O. of around 8mg/L, pH between 6.5 and 7.5) was pumped into them. For columns 3 and 4, aerated tap water containing both Nitrate (NO_3^-) and Sulfate (SO_4^{2-}) with molar concentrations of 1 mM for each (the concentration was quartered later) was pumped into them. Two liquid reservoirs were set up to hold the liquid pumped into the columns. The liquid reservoirs were connected to each column with tubes made of Pharmed and Viton rubbers (Masterflex) as well as a peristaltic pump as a flow-rate controller. The liquid flowed along the

column from the bottom to the top with the flow rate of 0.2 mL/min. Based on the flow rate and packed column porosity, the Hydraulic Residence Time (HRT) was estimated to be 2.69 days.

Pine bark mulch (from Agway in Ithaca, NY) was added to the four columns as the material for the permeable reactive barriers (PRB) and the only source of electron donor. After performing the dry test for the mulch (ASTM D4442: standard test method for direct content measurement of wood –based materials), the moisture content of the mulch was found to be 0.1326 grams of water per gram of ambient mass of mulch. From this moisture content and the addition of water to the packed column, the average porosity was found to be 0.628. In addition to the pine bark mulch, limestone chips (Fisher Scientific Cat. S25201A) were also added into the columns. The limestone chips provide pH buffering to offset acid production (possibly caused by the fermentation process of organics in mulch). The limestone chips were applied as 40% by weight of ambient mulch.

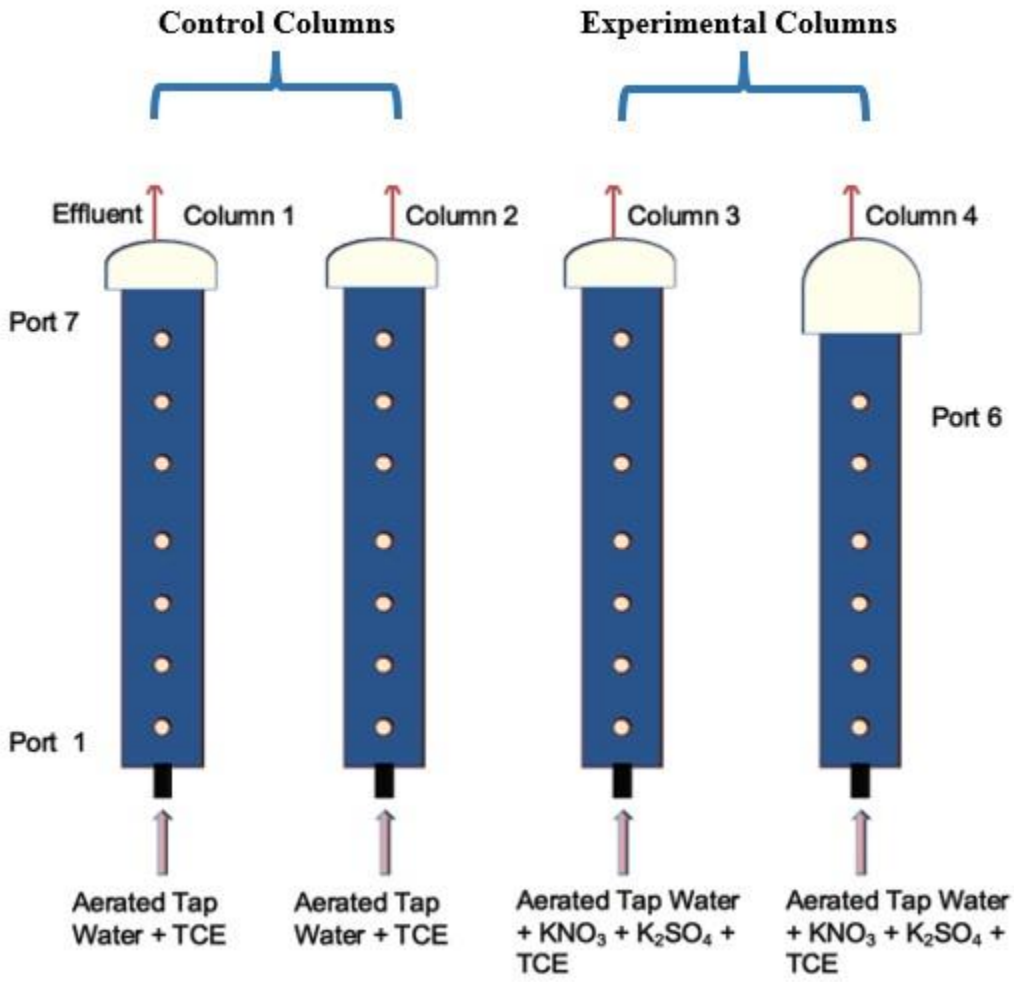

Figure 2.1 Sketch of mulch columns set up including information about reagents in the columns. Flow direction is upward with a 2.69-day retention time. Experimental columns had nitrate and sulfate in influent.

KB-1^{TM} culture was inoculated into the columns at a dilution of 1:100 (9 mL of culture was added via sterile syringe to ports 2 through 5 (2.25 mL each port)). This is equivalent to a total inoculum of approximately 10^9 *Dhc* cells (Waller, 2005).

2.2.1 Liquid Reservoirs

Two liquid reservoirs (two same-sized glass vessels, with total volume of 10L) were set up. One of them was filled with tap water, and the water was then pumped into control columns (column 1 and column2). The other was filled with tap water and potassium nitrate as well as potassium sulfate, and the solution was then pumped into the experimental columns (column 3 and column 4). Initially, 1.39g of potassium sulfate (Fisher Scientific, certified A.C.S. Crystal) and 0.81g of potassium nitrate (Fisher Scientific, certified A.C.S.) were added per eight liters of tap water to make a solution with nitrate and sulfate concentrations of 1 mM each. Seven months into the experiment, the dosages of nitrate and sulfate were quartered. The solution was then pumped into the experimental columns. The solution from two liquid reservoirs flowed into the columns from the bottom to the top via a peristaltic pump. The flow rate set for the liquid reservoirs was 0.2 mL/min (or 288 mL/day) to each column.

2.2.2 TCE Addition via Syringe Pumps

TCE was pumped into the columns via four syringe pumps, one to each column. A saturated aqueous solution of TCE (at 20˚C, 0.11 g/100 mL) was diluted to make a 200 mg/L TCE solution. To make this solution, 3.4 mL of saturated, aqueous TCE as well as 11.6 mL of ultra pure water was added into a 35-mL serum bottle, capped and aluminum-sealed. Then 10 mL volumes of the 200 mg/L TCE solution were taken from the serum bottle and placed into one of the four gas tight glass syringes. The syringes were then loaded onto the syringe pump. The TCE solution was delivered to each column with a dilution factor of 200, so the concentration of

TCE after flowing into the column was designed to be 1 mg/L. The TCE syringes were refilled once per week.

2.3 Gas Chromatography (GC) and Sampling Procedures

Dissolved methane, TCE, *cis*-DCE, VC and ethene in the columns were measured on a Perkin Elmer Autosystem Gas Chromatography with FID (Flame Ionization Detector) as described by Sun (2014).

Two different types of samples were processed. One was for GC analysis, while the other was for DNA extraction and the subsequent molecular work, which will be explained in more detail in section 2.4.1.1.

Seven mL of liquid sample in total was taken from each port using a syringe with a needle. From the 7-mL liquid sample, 5 mL was for GC analysis; therefore the 5-mL sample here was injected into a pre-sealed 9-mL serum bottle. The other 2 mL was for nitrate and sulfate assays (for other group members' use) and was injected into capped plastic tubes then stored in the refrigerator for later use. Since TCE is highly volatile, the 9-mL serum bottles, which the 5-mL liquid was injected into, were capped with rubber stoppers and sealed with aluminum crimps first. After receiving the 5-mL liquid sample, the bottle was shaken for at least five minutes to approach gas-liquid equilibration. The serum bottles were inverted during the shaking process and kept inverted until GC was run on their headspaces in order to avoid any gas leaking. When running GC, 500 µL of headspace gas was taken and injected to the GC-FID to detect TCE, dechlorination products, and methane.

2.4 DNA Extraction

2.4.1 DNA Extraction Methods

2.4.1.1 Sampling

The sampling procedure for biomass for DNA extraction was to take a 15-mL or 30-mL liquid samples from selected sampling ports using a sterile syringe. Initially, port 2 and the effluent were selected to assay the population in lower and upper parts of the columns, respectively. However, there was concern about biomass changes in the effluent tubing. Therefore, the later sampling port was changed from effluent to port 6. The liquid samples were then filtered by vacuum filtration through 47-mm diameter, 0.2-μm pore size Supor 200 membrane filters (Poretics). All the funnels and flasks were covered with aluminum foil and then autoclaved ahead before use in order to avoid contamination during the DNA extraction process. The filter paper with biomass collected was then subjected to DNA extraction immediately or frozen at -20 ºC for later DNA extraction.

2.4.1.2 DNA Extraction

The DNA extraction kit used was the PowerSoil™ DNA Isolation Kit (*MO BIO* Laboratories, Inc., Catalog number: 12888-100), according to the manufacturer's instructions but with 2 minutes in a Bead Beater instead of 10 minutes on a vortexer.

To extract DNA from the filter paper, the first step was to cut the filter paper with collected biomass into small pieces using sterilized tweezers (95% ethanol rinsed and flamed) and scissors, then all the small pieces were put into a PowerSoil™ Kit bead tube.

After extracting DNA, PicoGreen assays were run on the DNA samples to measure the initial total DNA concentration, in order to determine whether a dilution was necessary to do further molecular analysis via PCR and qPCR. For PicoGreen standard curves, lambda DNA was diluted to create standards ranging from 10 µg/mL to 0.005 µg/mL. However, according to the PicoGreen results, the initial total DNA concentrations were quite low. Therefore, no dilution was needed of the DNA extraction samples.

2.5 Calculating Methanogenesis and Dechlorination Rates in Columns

The distance from the bottom of the columns to each port was calculated. Based on the HRT of 2.69 days and the distance between ports, times required by the water to go between ports was calculated and are listed in Table 2.1.

Table 2.1 Distance from the bottom of the columns to each port and the time for liquid to travel between sucessive ports

	Distance from the bottom (cm) to	Distance b/w two ports (cm)	time b/w two ports (d)	time b/w two ports (h)
p1	20	20	0.09	2.23
p2	100	80	0.37	8.90
p3	180	80	0.37	8.90
p4	260	80	0.37	8.90
p5	360	100	0.46	11.13
p6	460	100	0.46	11.13
p7	560	100	0.46	11.13
top	580	20	0.09	2.23

Methane production and dechlorination rates were calculated based on the transit time for water moving between ports and the concentration difference between those ports (using the data in Table A3.1 to Table A3.3 in the Appendix section). For example, to calculate the methane production rate (MPR) in column 1 port 2:

MPR in C1P2 = (methane conc. in C1P3 – methane conc. in C1 P1) / water transit time from port 1 to port 3

Dechlorination rate in a specific port attribuable to *Dhc* strains (DR) here was defined by the net TCE + *cis*-DCE decrease from the influent until that port. It was calculated by using the total *cis*-DCE decrease minus the abiotic loss of *cis*-DCE. According to GC readings, there was little VC and ethene producd in the experimental columns. Thus, *cis*-DCE decrease in experimental columns from the influent to a specific port was considered to be the abiotic loss of TCE and *cis*-DCE up to that port, and the dechlorination rates attributable to *Dhc* strains were calculated only for control columns by subtracting out these abiotic losses from the overall losses of TCE and *cis*-DCE from the experimental columns.

A column-wide MPR and DR (accounting for abiotic *cis*-DCE loss) were caculated. For example:

$$\text{Column-wide MPR} = \frac{methane\ concentration\ in\ C1P7}{water\ transit\ time\ \ to\ P7}$$

TCE concentration in the influent was supposed to be 1 mg/L or 7.6 µM. Therefore, column-wide DR for column 1 is:

Column wide DR for column 1 =

$$\frac{7.6\ \mu M - cDCE\ conc.in\ C1P7 - TCE\ conc.in\ C1P7}{water\ transit\ time\ from\ P1\ to\ P7} - column - wide\ abiotic\ loss\ of\ cDCE$$

Abiotic loss of *cis*-DCE = average of abiotic *cis*-DCE loss in column 3 and abiotic *cis*-DCE loss in column 4

A column-wide abiotic loss of *cis*-DCE in column 3 =

$$\frac{7.6\ \mu M - cDCE\ conc.in\ C3P7 - TCE\ conc.in\ C3P7}{water\ transit\ time\ to\ P7}$$

These calculations assume no reactive loss of methane to oxidation or chlorinated ethene pathways besides reductive dechlorination. Additionally, they neglect sorption.

2.6 Molecular Biological Tools

2.6.1 End-Point Polymerase Chain Reaction (PCR)

PCR amplification of target genes was carried out on an Eppendorf Mastercycler Gradient ThermoCycler. Various primers and PCR programs were applied to detect different microbial groups. The full list of primers used and relevant references are shown in Table 2.2.

Table 2.2 PCR Primers and References

Primers and References					
Target Gene	primer ID	Target Group	5' to 3' Sequence	Amplicon size (bp)	Reference
16S rRNA	8 F	Almost all Bacteria	AGAGTTTGATCCTGGCTCAG	681	Lane, 1991
16S rRNA	690 R		TCTACGCATTTCACC		Lane, 1991
mcrA	*mlas F*	Methanogens	GGTGGTGTMGGDTTCACMCART	490	Steinberg & Regan, 2009
mcrA	*mcrA R*		CGTTCATBGCGTAGTTVGGRTAGT		Steinberg & Regan, 2009
vrcAf	*DCKB1_96900*	*Dhc*	TGCTGGTGGCGTTGGTGCTCT	441	Sun, 2014
vcrAr	*DCKB1_96901*		CTTTACCACTTTTGACGGGCA		Sun, 2014
pceA	*glpce1*	*Geobacter*	TAATGTTGGCGTCATCACTCG	233	Sun, 2014
pceA	*glpce2*		CCCATGTATGAAAGCCTGGGA		Sun, 2014
bvcA	*Bvc925F*	*Dhc*	AAAAGCACTTGGCTATCAAGGAC	92	Kirsti M. Ritalahti, et al, 2006
bvcA	*Bvc1017R*		CCAAAAGCACCACCAGGTC		Kirsti M. Ritalahti, et al, 2006
nirSF	22	Denitrifiers	ACAAGGAGCACAACTGGAAGGT	143	Gruntzig. 2001
nirSR	14		CGCGTCGGCCCAGA		Gruntzig, 2001

PCR with general *16S rRNA* primers served to test for effective DNA extraction since it is a biomarker for nearly all the Bacteria so the possibility of getting PCR products is higher. Lack of PCR product with these primers suggest the presence of inhibitors in the extracted DNA or very low DNA levels. Besides *16S rRNA*, other biomarkers of interest include *mcrA*, *vcrA* and *bvcA*, *pceA*, and *nirS*. They are the biomarkers for methanogens, VC-reducing *Dhc* strains, *Geobacter*, and nitrate reducers, respectively. The sulfate-reducer biomarker, *dsrA*, was not examined because very little sulfate reduction was observed even in the experimental columns. Therefore *dsrA* was not a high priority PCR target.

DNA extracted from *Methanosarcina acetovirans* strain C2a was used as positive control for *mcrA*, and the genomic DNA from *Pseudomonas* strain 21 pure culture was used as the positive control for *nirS* gene primers.

PCR programs used for *16S rRNA*, *mcrA, vcrA, bvcA* and *pceA* were all the same and named "T53C35". The details of this program are shown in Table 2.3:

Table 2.3 PCR Temperature Program for *16S rRNA*, *mcrA*, *vcrA*, *bvcA* and *pceA*, T53C35

PCR Program T53C35		
	step & temp.	remaining time
pre	LID = 105˚C	
1	T = 95˚C	2 min
2	T = 95˚Ç	45 sec
3	T = 53˚C, G= 0	45 sec
4	T = 72˚Ç	1 min
5	Go to step 2	repeat 34 cycles
6	T = 72˚˚C	5 min
7	Hold at 4˚C	
8	end	

PCR targeting *nirS* was initially run under the same PCR program as the others, however, there were multiple bands shown under gel-electrophoresis so the annealing temperature was increased from 53˚C to 55˚C and this improved the results. The program finally used for this research for *nirS* is named “T55C35”, details of the program are shown in Table 2.4:

Table 2.4 PCR Temperature Program for *nirS*, T55C35

PCR Program T53C35		
	step & temp.	remaining time
pre	LID = 105˚C	
1	T = 95˚C	2 min
2	T = 95˚C	45 sec
3	T = 55˚C, G= 0	45 sec
4	T = 72˚C	1 min
5	Go to step 2	repeat 34 cycles
6	T = 72˚C	5 min
7	Hold at 4˚C	
8	end	

2.6.2 Gel Electrophoresis

Agarose (Fisher Scientific, Genetic Analysis Grade) was added as 2% by mass to 0.5X tris-borate EDTA (TBE) buffer to make a gel. For example, 3 g of agarose was added into 150 mL of 0.5X TBE buffer to make a large gel with 100 wells in total. In early research, no stain was added during the gel-making process and the gel had to be stained with Ethidium Bromide after electrophoresis. Since December 2015, GelRed™ Nucleic Acid Get Stain (10,000X, catalog number: 41003) started to be added during the gel making process so the gel could be directly visualized without separate staining. The 10,000X GelRed™ stain was made into 3X to add into the gel, so 4.5 µL of the stain was added per 150 mL agarose mixture. Gels were loaded with 4 µL PCR reaction or 2 µL DNA ladder and run at 100 volts for 30-60 minutes depending on amplicon length.

2.6.3 PCR Clean Up for qPCR Standards

A column clean-up using QIAquick PCR Purification Kit™ was done to PCR products with *vcrA* long amplicon primers and KB-1™ culture DNA to purify PCR products. The cleaned-up products were then sent to Cornell DNA sequencing facility for sequencing to make sure that they were the right products. The sequencing result is shown in Figure B1 in the Appendix section. After making sure that the amplicon is the expected product, they could be made into qPCR standards. They were analyzed by PicoGreen to measure the total DNA concentration. Based on the PicoGreen result, the amplicon was used to create a serial dilution to make a DNA standards for *vcrA* qPCR that cover a range of 6 orders of magnitude in copies/µL. For *mcrA*, qPCR standards were made from DNA extraction of *Methanosarcina acetovirans* strain C2a.

2.6.4 Quantitative-PCR (qPCR) Set Up for mcrA and vcrA

Triplicate amplifications of selected samples were conducted on the iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad). Genes targeted using qPCR were *mcrA* and *vcrA* only, since time was limited. The samples chosen to be analyzed for end-point PCR are from Oct. 19th 2015 (4 samples from port 2 of each column: C1P2,, C2P2, C3P2 and C4P2), Nov. 24th 2015 (8 samples from ports 2 and 6: C1P2, C2P2, C3P2, C4P2, C1P6, C2P6, C3P6 and C4P6), and Feb 15th 2016 (8 samples from ports 2 and 6: C1P2, C2P2, C3P2, C4P2, C1P6, C2P6, C3P6 and C4P6). These dates span changes in the operation and performance of the mulch biobarriers: 6 days before quartering the nitrate and sulfate dosages, 30 days after quartering the dosages of nitrate and sulfate and 115 days after quartering. Samples from Oct. 19th are only

from port 2 in every column since the sampling locations at that time were port 2 and effluent. However, the DNA concentrations in effluent samples' extractions were extremely low, almost undetectable. Therefore the samples from effluent were not used for PCR or qPCR.

For each 25-µL qPCR reaction, 12.5 µL of iQ-SYBR Green Mix (BioRad), 1.17 µL of forward primer and 1.17 µL of reverse primer as well as 7.17 µL of nuclease-free water were added together to make a MasterMix for qPCR. Three µL of sample or standard was used in each qPCR reaction. Triplicate reactions were made for each sample and standard.

2.6.4.1 qPCR Protocols

The thermal cycling program for qPCR was the same as was used in the previous research targeting the same gene (*mcrA* and *vcrA*, respectively), except that 40 cycles were performed. The full program is shown in Table 2.5.

Table 2.5 qPCR Protocol targeting *mcrA* gene, with primers *mlas F* and *mcrA R*, adapted from Steinberg and Regan, 2009

qPCR Protocol with *mcrA* primers *mlas F* and *mcrA R*									
cycle	repeats	step	dwell time	hold	Set point (ºC)	melt curve			
1	1	1	03:30		95.0				
2	40	1	00:30		95.0				
		2	00:45		55.0				
		3	00:30		72.0				
		4	00:30		83.0	SYBR reading taken			
3	1	1	07:00		72.0				
4	90	1	00:10		50.0	Yes	every 0.5 increase in temp.		
5	1	1		Yes	4.0				

Table 2.6 qPCR Protocol targeting *vcrA* gene, with primer of *RDhA14 642F*and *RDhA14 846R,* adapted from Waller, 2005. qPCR protocol was also based on Waller, 2005.

qPCR Protocol targeting *RDhA14 642F* and *RDhA14 846R*									
cycle	repeats	step	dwell time	hold	Set point (ºC)	melt curve			
1	1	1	02:10		94.0				
2	30	1	00:30		94.0				
		2	00:45		48.0				
		3	02:10		72.0				
3	1	1	06:00		72.0				
4	88	1	00:10		50.0	Yes	every 0.5 increase in temp.		
5	1	1		Yes	4.0				

2.6.4.2 qPCR Standards

For the *mcrA* gene, *Methanosarcina acetovirans* strain C2a genome was used to make qPCR standards. The standard curve spanned a range of 6 orders of magnitude, from 10 copies/µL to 10^7 copies/µL by 100-fold dilutions of the high-concentration standard.

For the *vcrA* gene, the long amplicon from previous endpoint PCR was used to make qPCR standards. The long amplicon primers amplify a 44-bp segment that contains the qPCR primer targets plus at least 50 bp on either end. PCR products with *vcrA* long amplicon was first cleaned up by Column Clean Up method using the QIAquick PCR Purification Kit™. Then the

vcrA long amplicons were run PicoGreen with to measure the initial concentration. Triplicates were run in order to measure a more accurate concentration. After that, the known-concentration *vcrA* long amplicons were made into series of dilution to make the qPCR standards. A mean value of 1.12 ng/µL DNA was calculated from the PicoGreen result. Unit conversion from ng/µL to copies/µL was done to the PicoGreen result:

$$\frac{copies}{\mu L} = \frac{copies}{ng\ DNA} * \frac{ng}{uL}$$

$$\frac{copies}{ng} = \frac{copies}{standard\ DNA\ molecule} * \frac{molecules}{base\ pairs} * \frac{1\ base\ pair}{660\ atomic\ mass\ units} * \frac{6.022*10^{23}\ amu}{g} \frac{1\ g}{10^9 ng}$$

The long amplicon length of *vcrA* is 441 (Waller et al. 2005), then:

$$\frac{copies}{ng} = \frac{1\ copy}{standard\ DNA\ molecule} * \frac{1\ molecule}{441\ base\ pairs} * \frac{1\ base\ pair}{660\ atomic\ mass\ units} * \frac{6.022*10^{23}\ amu}{g} \frac{1\ g}{10^9 ng}$$

$= 2.07*10^9$ copies/ng

Therefore, the calculated concentration is 2.32 x 10^9 copies/µL.

The *vcrA* long amplicon was used to make serial dilutions to act as standards for qPCR. The *vcrA* long amplicon from above was diluted into nuclease free water to make a *vcrA* standard with a concentration of 10^8 copies/µL, then the high concentration standard was diluted 10 fold to 10^7 copies/µL, then made a series of 100-fold dilution. The standards range from 10 copies/µL to 10^7 copies/µL.

2.6.4.3 qPCR Results Unit Conversion

In the qPCR report, the quantity of genes of interest is shown in copies/µL extracted DNA. Compared with this unit, copies/mL pore water could be more convenient for comparisons. Thus, a series of unit conversions was made. Since 100 µL DNA sample was extracted from 30 mL of pore water (or 15 mL for the October 19th 2015 samples from port 2), the values from qPCR reports with a unit of copies/µL DNA extraction were conducted using this equation

$$\frac{copies}{\mu L\ DNA\ Extraction} = \frac{copies}{\mu L\ DNA\ Extraction} * \frac{100\ \mu L\ DNA\ Extraction}{30\ mL\ pore\ water}$$

After running qPCR, melt curves and amplification graphs were compared and analyzed to check the qPCR products. The melt curves and amplification graphs for *mcrA* and *vcrA* are shown in Appendix C.

CHAPTER 3 RESULTS AND DISCUSSION

3.1 Column Performance and Analysis

GC analysis was used to compare methane production and extent of dechlorination between control columns and experimental columns as well as changes along the length of the columns. The detailed GC data are presented in Tables A1 to A3 in the Appendix section.

The important dates and overall column operation timeline are shown on Table 3.1.

Table 3.1 Timeline for Column Operation

IMPORTANT DATE	ACTION	NOTE
1/15/2015 day 0	columns set up	
2/15/2015 day 30	water started to flow through the columns	
4/2/2015 day 76	nitrate and sulfate flow started	
4/7/2015 day 110	TCE flow started	
8/3/2015 day 227 day 0 after inoculation	Inoculated column 1,2,3,4 with KB-1 culture	
10/19/2015 day 304, day 77 after inoculation	started to collect biomass from port 2 and effluent for DNA extraction	The extracted DNA from port 2 was used in PCR and qPCR.
10/22/2015 day 307, day 80 after inoculation	took 7 mL liquid samples from every port in each column	GC data from this date were chosen to analyze in this research
10/25/2015 day 310, day 83 after inoculation, day	quartered the nitrate and	

0 after quartering	sulfate dosage	
11/19/2015 day 335, day 108 after inoculation, day 25 after quartering	took 7 mL liquid samples from every port in each column	GC data from this date were chosen to analyze in this research
11/24/2015 day 340, day 113 after inoculation, day 30 after quartering	took 30 mL liquid samples from port 2 and port 6	The extracted DNA was used in PCR and qPCR.
2/15/2016 day 425, day 198 after inoculation, day 115 after quartering	took 30 mL liquid samples from port 2 and port 6	The extracted DNA was used in PCR and qPCR.
3/14/2016 day 451, day 224 after inoculation, day 141 after quartering	took 7 mL liquid samples from odd ports in column 1 and 3; port 2,3,5,7 from column 2 and port 1,3,5,6 from column 4	GC data from this date were chosen to analyze in this research

3.1.1 Dechlorination Ability of the Columns

According to the GC results (data not shown), before inoculation, no dechlorination was observed in any of the four columns. After inoculation, dechlorination could be observed in control columns but barely in experimental columns. After quartering the dosage of nitrate and sulfate, dechlorination could be observed in experimental columns, too, and it was increasing with time. Also according to the nitrate assay (done by other students, data not shown here), approximately 90% of nitrate was reduced after going through the column, both before and after quartering the dosage. As for sulfide production, according to the sulfide assay (done by other students, data not shown here), very little sulfate reduction to sulfide was occurring. However, assays for sulfate (rather than just the product of sulfide) would be valuable to support the conclusion that little sulfate reduction was occurring.

Figure 3.1 shows the dechlorinating performance of columns on Oct. 22nd 2015 (day 307):

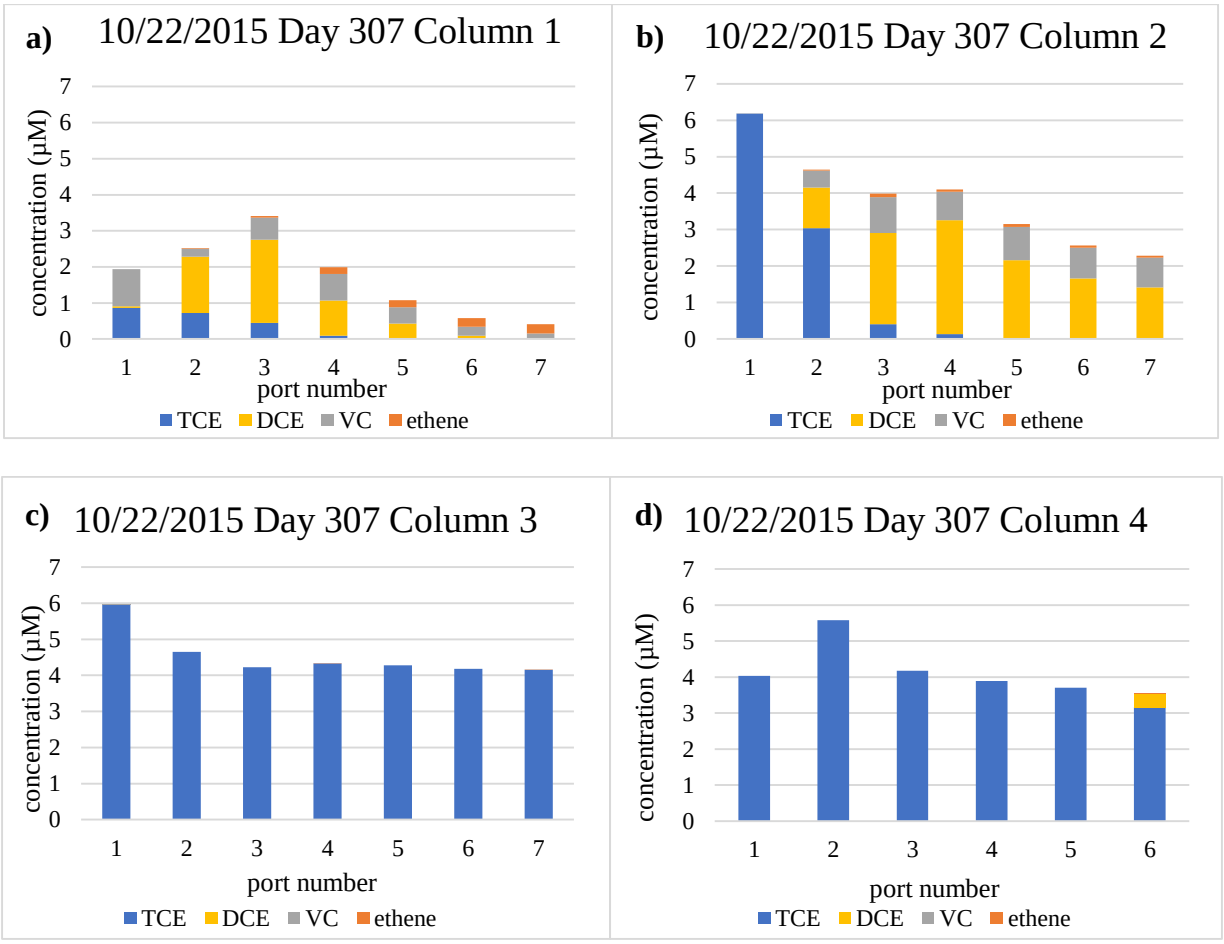

Figure 3.1 Dechlorinating performance of columns on 10/22/2015 Day 307, three days before lowering nitrate and sulfate in experimental columns. Panels a) to d) show the various ethenes in column 1 to 4, respectively. Columns 1 and 2 were control columns (not receiving nitrate or sulfate); Columns 3 and 4 were experimental columns (receiving both nitrate and sulfate).

Figure 3.2 shows the dechlorination performance of columns on Nov. 19th, 2015, Day 335, 25 days after quartering nitrate and sulfate.

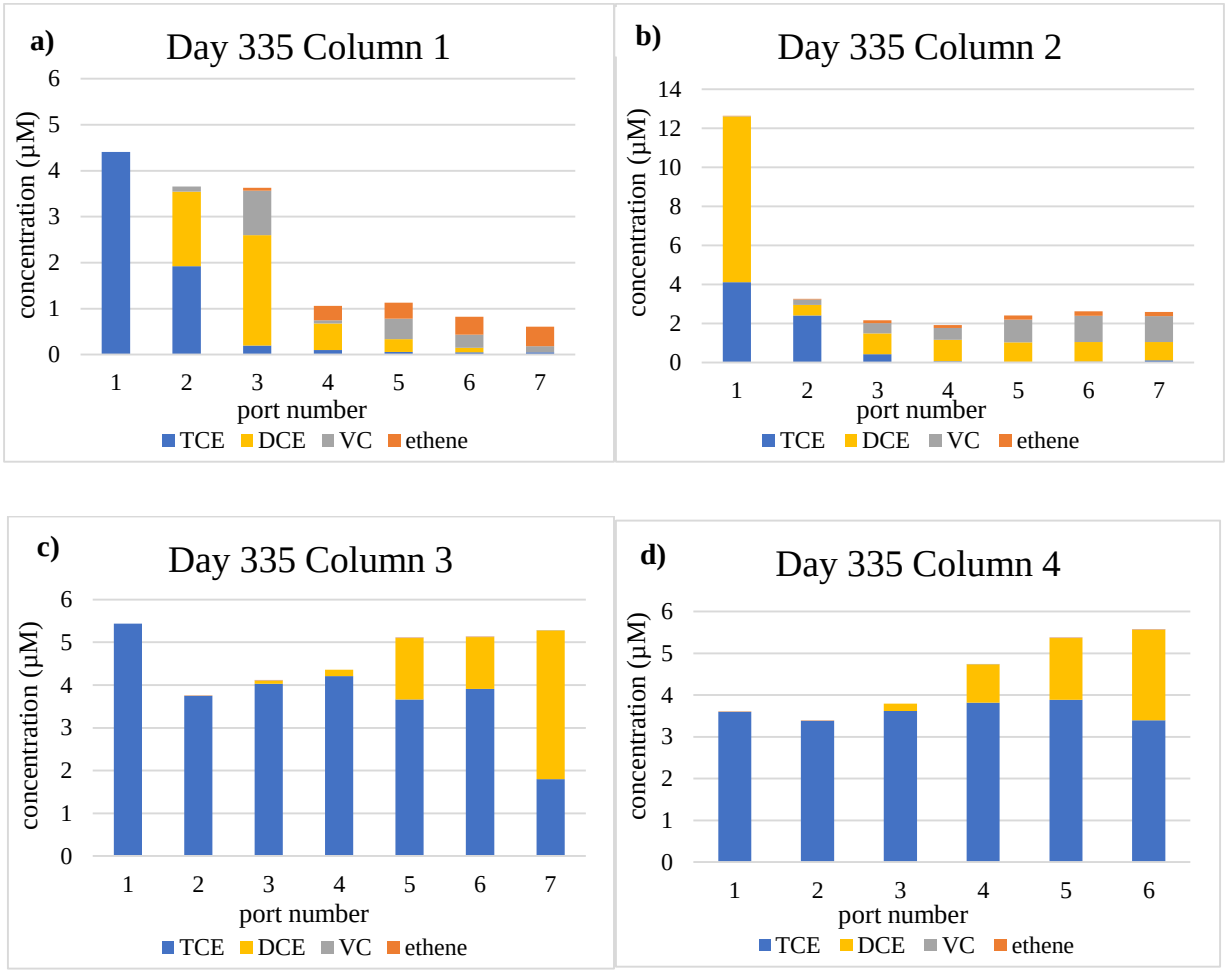

Figure 3.2 Dechlorinating Performance of Columns on 11/19/2015 Day 335. Panels a) to d) show the dechlorination profiles in columns 1 to 4, respectively.

Figure 3.3 shows the dechlorination performance of columns on March. 14th, 2016, Day 451:

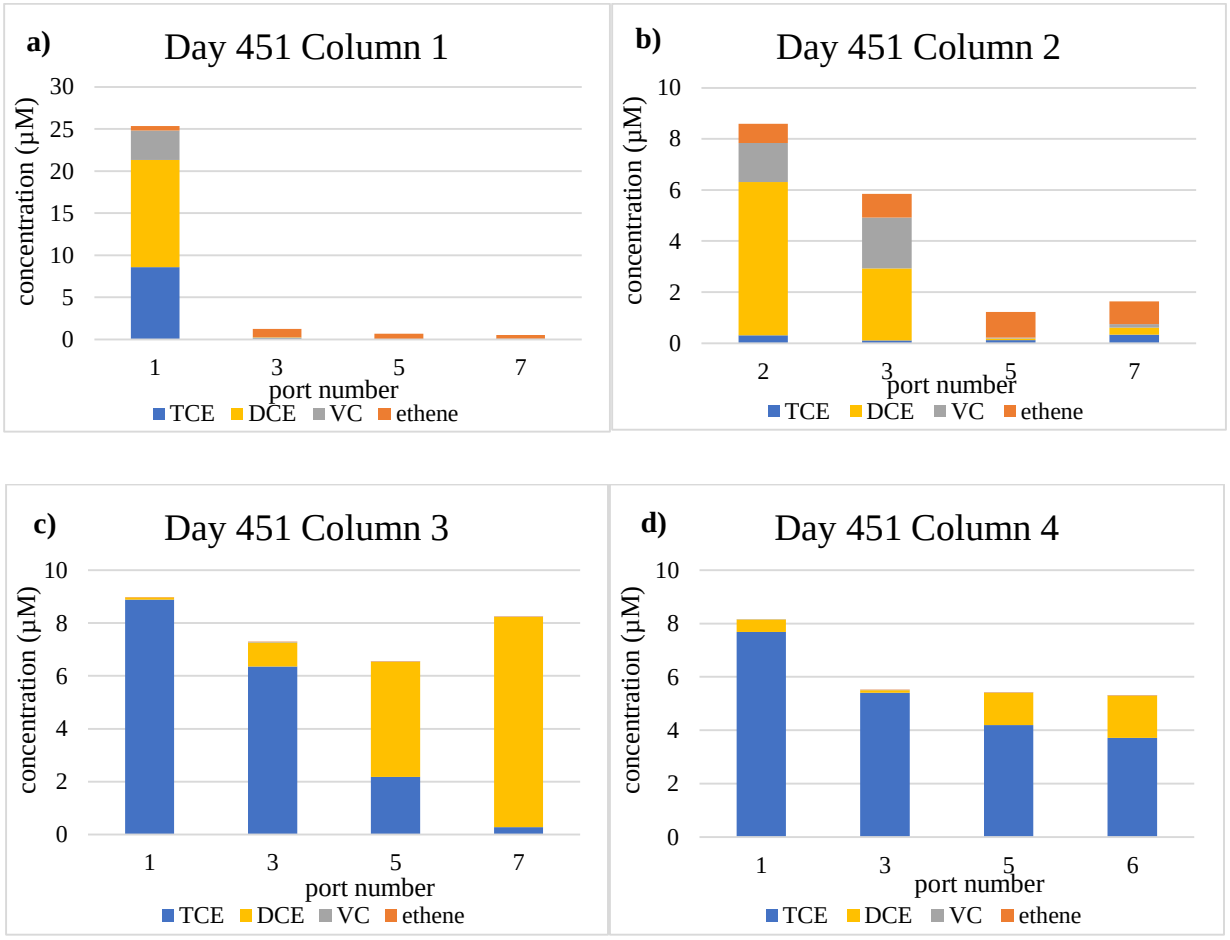

Figure 3.3 Dechlorinating Performance of Columns on 3/14/2016 Day 451. Panels a) to d) show the dechlorination of column 1 to 4, respectively.

The dechlorination results were graphed as stacked columns in order to show the mass balance better. From the current results, the mass balance inside the control mulch columns seems to be problematic. The exact reason is still unclear. One of the hypotheses is the absorption of VC and ethene daughter products onto the mulch filling the columns. Partition coefficients between dissolved and mulch-sorbed VC and ethene levels have not been

determined. Also, some daughter products could escape from the top or other ports when taking samples, though this is unlikely to explain the mass balance discrepancy alone. Another possibility is that there is need for new calibration for a series of dechlorinating products, especially VC and ethene. As for the mass balance problem in experimental columns, it could also be that some of the dechlorination products could be oxidized again by some of the alternative electron acceptors. However, the mass balance is much better in the experimental columns. This suggests the problem is more with calibration curves and/or strong mulch-sorption for VC and/or ethane, since the experimental columns did not apparently produce much of those products.

From the column graphs (Figure 3.1 to Figure 3.3), it could be seen that in control columns, complete dechlorination could be observed and the trend is even clearer in the most recent GC results (Figure 3.3). However, dechlorination in experimental columns was very limited. From Figure 3.1, which shows the dechlorination performances when nitrate and sulfate dosages were 1 mM, it is clear that dechlorination in experimental columns was not occurring. After quartering the nitrate and sulfate dosage on Oct 25th, according to Figure 3.2 and Figure 3.3, dechlorination products were detected – i.e., dechlorination happened. However, complete dechlorination to ethene in experimental columns was not observed. In fact, no ethene and only very low VC levels (up to 0.01 µM) have been observed so far in the experimental columns. This suggests that the *Geobacter* population is the only active KB-1^{TM} dechlorinating population following the reduction in nitrate and sulfate. The *Dhc* populations, if present, are much less active than the *Geobacter*, even after the change.

Figure 3.4 to Figure 3.6 are graphs showing *cis*-DCE productions in port 3, port 4 and port 5 in the experimental columns (columns 3 and 4). These graphs are selected to show the change of dechlorination ability in the experimental columns after quartering the nitrate and sulfate dosage. Note that the Y axis scales are different across figures. Very little VC or ethene were seen at any time in these columns so *cis*-DCE represents the final product of dechlorination in the experimental columns.

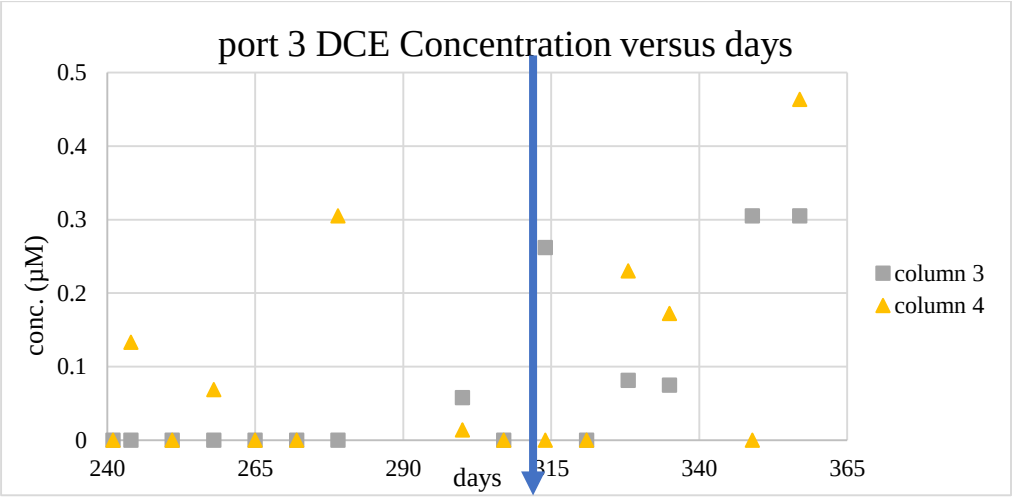

Figure 3.4 *cis*-DCE concentration in experimental columns at port 3 versus days. Arrow denotes date when nitrate and sulfate levels were reduced to 0.25 mM in experimental columns at day 310.

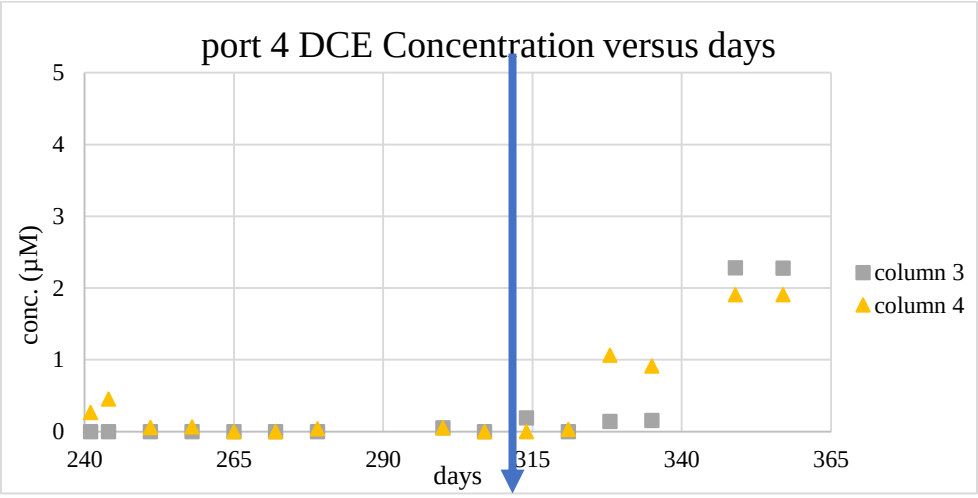

Figure 3.5 *cis*-DCE concentration in experimental columns at port 4 versus days. Arrow denotes date when nitrate and sulfate levels were reduced to 0.25 mM in experimental columns at day 310.

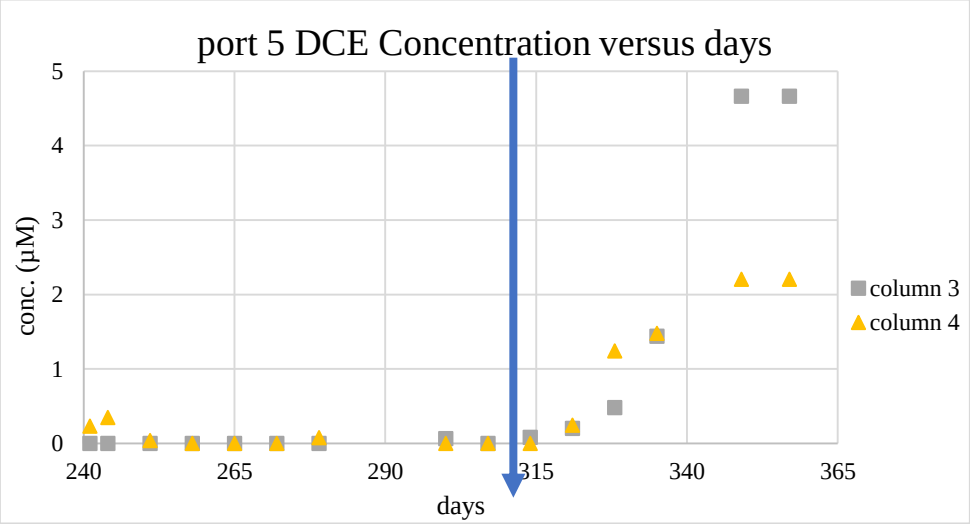

Figure 3.6 *cis*-DCE concentration in experimental columns at port 5 versus days. Arrow denotes date when nitrate and sulfate levels were reduced to 0.25 mM in experimental columns at day 310.

The grey squares and yellow triangles show the *cis*-DCE concentration in the specific position in experimental columns 3 and 4, respectively.

From Figure 3.4 to Figure 3.6, it is apparent that before day 300, there was barely any dechlorination from TCE to *cis*-DCE. However, after day 310, the *cis*-DCE concentration increased and the trend was even clearer by day 350. The quartering of nitrate and sulfate took place at day 310, so an assumption was made that the appearance and/or accumulation of *cis*-DCE is due directly or indirectly to the decrease in nitrate and sulfate concentration in the columns.

3.1.2 Methane Production at Selected Time Points

Methane productions and/or accumulation on three specific dates were selected to show the methanogenesis trends between control and experimental columns. GC samples were selected at

time points closest to the sampling dates for biomass that had DNA extracted. Inoculation was made on Aug. 3rd 2015, day 227. Nitrate and sulfate dosage were quartered on Oct. 25th, 2015, day 310. GC results at around day 310 can be used to assess the methanogenic activity at that time period corresponding to DNA extracted on Oct. 22nd (day 307, three days before quartering the nitrate and sulfate dosage). We also show methane data from Nov. 19th (day 335, 25 days after quartering the dosage) March. 14th (day 451, day 141 after quartering the dosage) .These three time points are also close to the times at which the DNA samples were acquired (Oct. 19th, Nov. 24th and Mar. 14th), so GC results from those three specific dates could also act as a supporting reference for qPCR data analysis.

Figures 3.7 to 3.9 show the comparison of methane concentrations in four columns on the selected dates mentioned above.

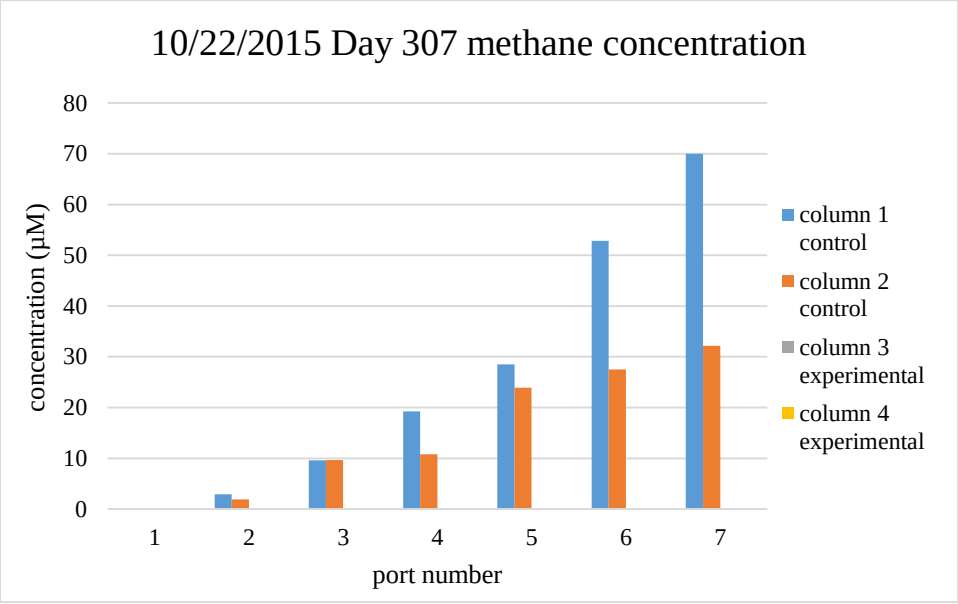

Figure 3.7 10/22/2015 Day 307 methane concentrations in the four columns at each sampling port. This date is just 3 days prior to quartering nitrate and sulfate

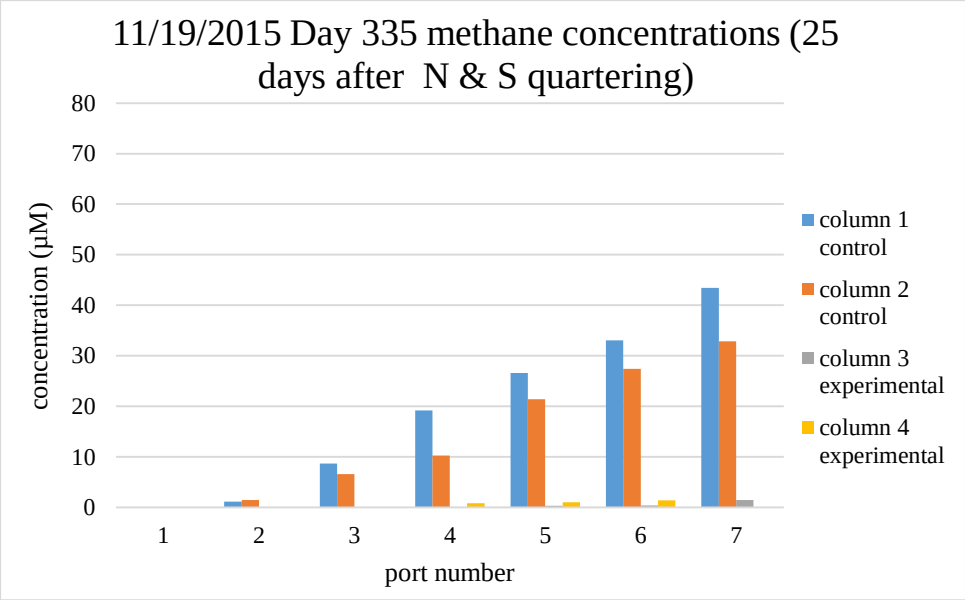

Figure 3.8 11/19/2015 Day 335 methane concentrations (25 days after nitrate and sulfate quartering) in the four columns at each sampling port.

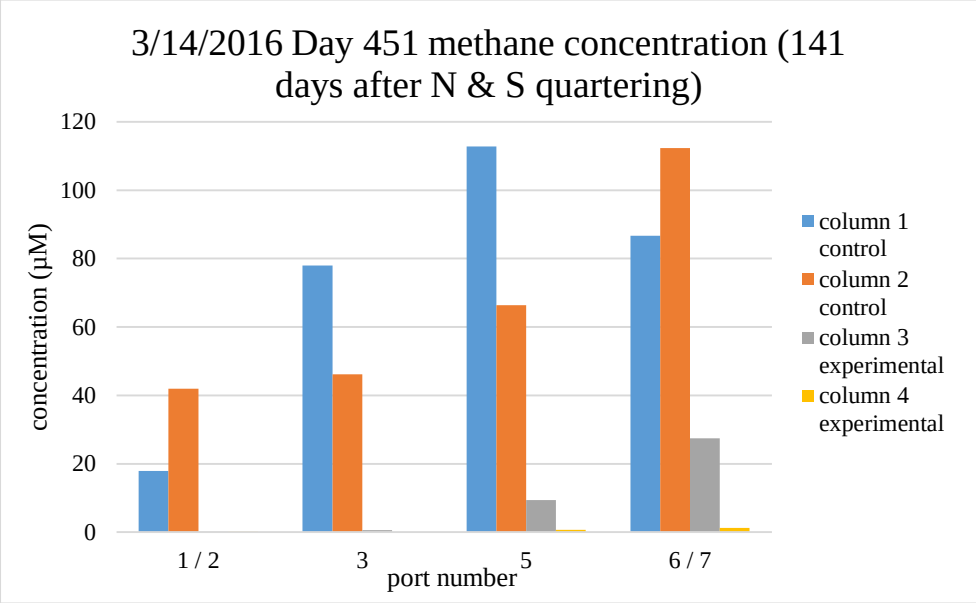

Figure 3.9 3/14/2016 Day 451 methane concentrations at different port locations (141 days after nitrate and sulfate quartering) in the four columns at selected ports (port 1,3,5,7 for column 1 and column 3; port 2,3,5,7 for column 2; port 1,3,5,6 for column 4).

Figures 3.10 to 3.12 show the methane concentrations versus days for port 4, port 5 and port 6/7 for more sampling dates.

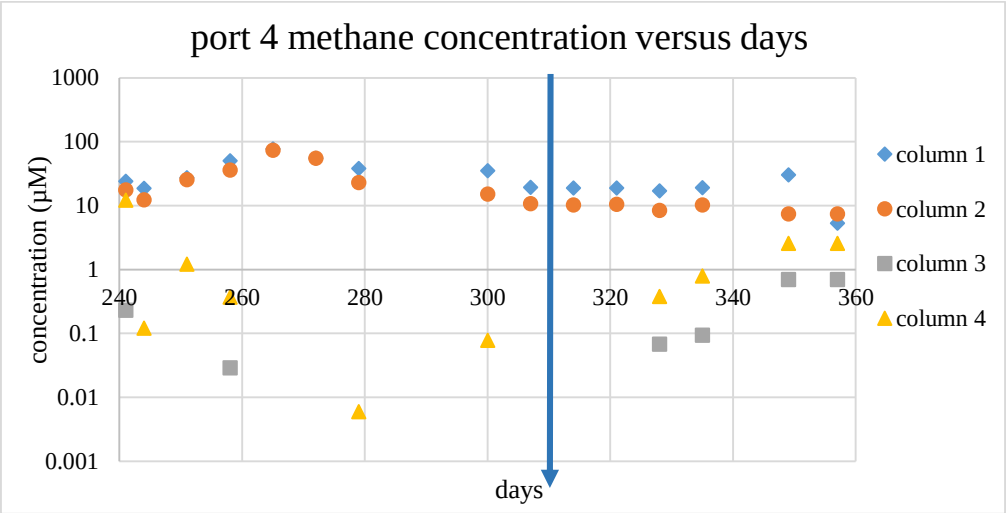

Figure 3.10 Methane concentrations in port 4 in every column versus days. Because the sampling ports were changed to odd-numbered ports after February 2016, data for port 4 only lasts to December 11th 2015, day 357. Blue arrow denotes date when nitrate and sulfate levels were reduced to 0.25 mM in experimental columns (columns 3 & 4)

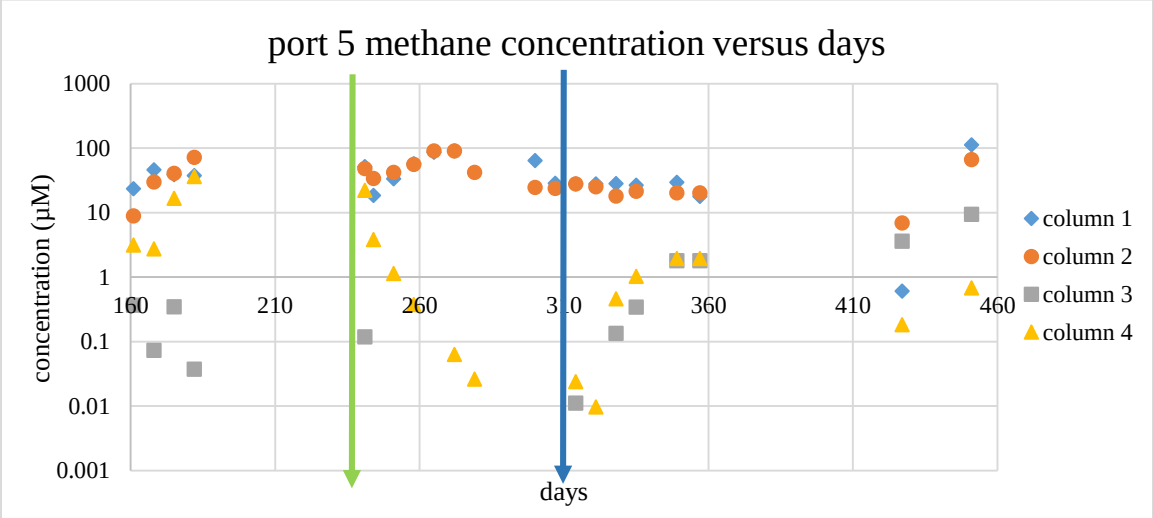

Figure 3.11 Methane concentrations in port 5 at every column versus days. Green arrow denotes date when inoculation was made and the dark arrow denotes date when nitrate and sulfate levels were reduced to 0.25 mM in experimental columns (columns 3 & 4)

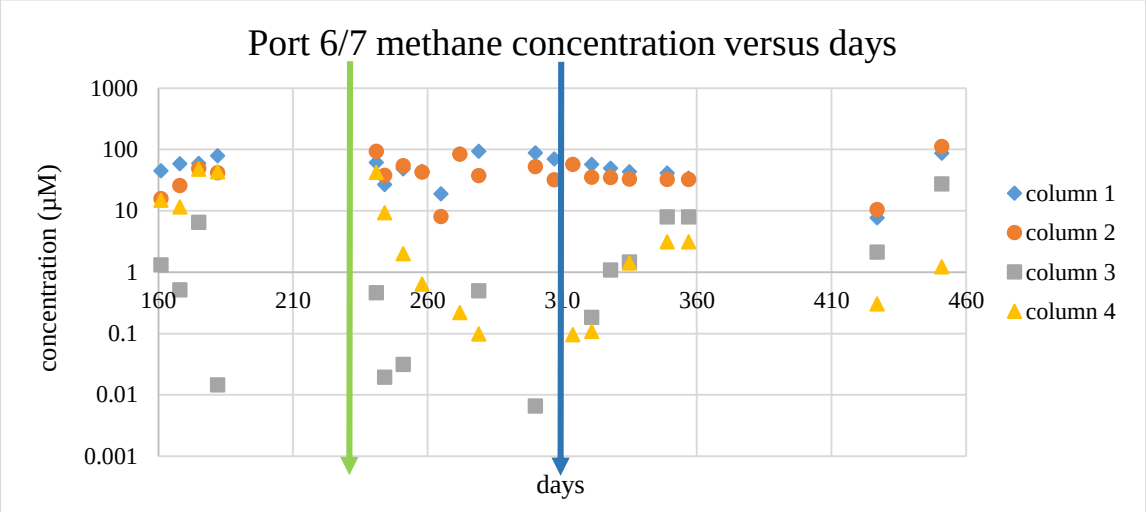

Figure 3.12 Methane concentrations versus days in the final port in each column – port 6 in column 4, and port 7 in columns 1, 2 and 3. All of them are graphed together to show the methane concentrations in the highest positions of the columns. Green arrow denotes date when inoculation was made and the dark arrow denotes date when nitrate and sulfate levels were reduced to 0.25 mM in experimental columns (columns 3 & 4).

From Figures 3.7 to 3.9, it can be seen that methane increased linearly with distance into the control columns suggesting that methane production in control columns was quite consistent throughout the column. However, in the experimental columns, there were ups and downs in methane production. After inoculation on day 227 (where the green arrow appears in the Figures above), methanogens seemed to be affected a lot because the methane production went through a continuous decline. However, a clog in column 4 around this time confounds the methane trends because the true hydraulic residence time of water at that date was longer than the design value of 2.69 days. Since around day 310, which is also the approximate day that nitrate and sulfate dosages was quartered (where the dark blue arrow appears in the Figures above), methanogens in experimental columns seemed to start to recover and the methane production started to increase.

3.2 PCR Products and Gel Electrophoresis Analysis

There are various *Dhc* strains in the KB-1™ culture. To target two of the strains among all the KB-1™ *Dhc* strains, one major strain has the *vcrA* gene, and a different strain, which is a minor population, has *bvcA*. These two biomarkers (*vcrA* and *bvcA*) were selected to detect *Dhc* strains of interest.

A summary of PCR results is provided in Table 3.2.

Table 3.2 Guide to results presented in gel images (Figures 3.13-3.18)

Figure	lane #	Taking Sample Date	Description	Expected Amplicon Length (bps)	Positive or not (Y/N)	Expected PCR products or not (Y/N)
Figure 3.13	1		DNA Ladder			
Figure 3.13	2	11/24/2015	C1P2, *16S rRNA*	681	Y	Y
Figure 3.13	3	11/24/2015	C2P2, *16S rRNA*	681	Y	Y
Figure 3.13	4	11/24/2015	C3P2, *16S rRNA*	681	Y	Y
Figure 3.13	5	11/24/2015	C4P2, *16S rRNA*	681	Y	Y
Figure 3.13	6	11/24/2015	C1P6, *16S rRNA*	681	Y	Y
Figure 3.13	7	11/24/2015	C2P6, *16S rRNA*	681	Y	Y
Figure 3.13	8	11/24/2015	C3P6, *16S rRNA*	681	Y	Y
Figure 3.13	9	11/24/2015	C4P6, *16S rRNA*	681	Y	Y
Figure 3.13	10	11/24/2015	Negative Control		N	Y

Figure 3.14	1		DNA Ladder			
Figure 3.14	2	2/15/2016	C1P2, *mcrA*	490	Y	Y
Figure 3.14	3	2/15/2016	C2P2, *mcrA*	490	Y	Y
Figure 3.14	4	2/15/2016	C3P2, *mcrA*	490	Y	Y
Figure 3.14	5	2/15/2016	C4P2, *mcrA*	490	Y	Y
Figure 3.14	6	2/15/2016	C1P6, *mcrA*	490	Y	Y
Figure 3.14	7	2/15/2016	C2P6, *mcrA*	490	Y	Y
Figure 3.14	8	2/15/2016	C3P6, *mcrA*	490	Y	Y
Figure 3.14	9	2/15/2016	C4P6, *mcrA*	490	Y	Y
Figure 3.14	10	10/19/2015	C1P2, *mcrA*	490	Y	Y
Figure 3.14	11	10/19/2015	C2P2, *mcrA*	490	Y	Y
Figure 3.14	12	10/19/2015	C3P2, *mcrA*	490	Y	Y
Figure 3.14	13	10/19/2015	C4P2, *mcrA*	490	Y	Y
Figure 3.14	14		Negative Control		N	Y
Figure 3.14	15		Positive Control	490	Y	Y
Figure 3.15	1		DNA Ladder			
Figure 3.15	2	11/24/2015	C1P2, *vcrA*	441	Y	Y
Figure 3.15	3	11/24/2015	C2P2, *vcrA*	441	Y	Y
Figure 3.15	4	11/24/2015	C3P2, *vcrA*	441	Y	Y
Figure 3.15	5	11/24/2015	C4P2, *vcrA*	441	Y	Y
Figure 3.15	6	11/24/2015	C1P6, *vcrA*	441	Y	Y
Figure 3.15	7	11/24/2015	C2P6, *vcrA*	441	Y	Y
Figure 3.15	8	11/24/2015	C3P6, *vcrA*	441	Y	Y

Figure 3.15	9	11/24/2015	C4P6, *vcrA*	441	Y	Y
Figure 3.15	10		Negative Control		N	Y
Figure 3.16	1		DNA ladder			
Figure 3.16	2	11/24/2015	C1P2, *bvcA*	92	unclear*	
Figure 3.16	3	11/24/2015	C1P2, *bvcA*	92	unclear	
Figure 3.16	4	11/24/2015	C3P2, *bvcA*	92	unclear	
Figure 3.16	5	11/24/2015	C4P2, *bvcA*	92	unclear	
Figure 3.16	6	11/24/2015	C1P6, *bvcA*	92	unclear	
Figure 3.16	7	11/24/2015	C1P6, *bvcA*	92	unclear	
Figure 3.16	8	11/24/2015	C3P6, *bvcA*	92	unclear	
Figure 3.16	9	11/24/2015	C4P6, *bvcA*	92	unclear	
Figure 3.16	10		Negative Control		-	
Figure 3.17	1		DNA Ladder			
Figure 3.17	2	11/24/2015	C1P2, *nirS*	143	Y	Y
Figure 3.17	3	11/24/2015	C2P2, *nirS*	143	Y	Y
Figure 3.17	4	11/24/2015	C3P2, *nirS*	143	Y	Y
Figure 3.17	5	11/24/2015	C4P2, *nirS*	143	Y	Y
Figure 3.17	6	11/24/2015	C1P6, *nirS*	143	Y	Y
Figure 3.17	7	11/24/2015	C2P6, *nirS*	143	Y	Y
Figure 3.17	8	11/24/2015	C3P6, *nirS*	143	Y	Y
Figure 3.17	9	11/24/2015	C4P6, *nirS*	143	Y	Y
Figure 3.17	10		Negative Control		N	Y
Figure 3.18	1		DNA Ladder			

Figure 3.18	2		Negative Control		N	Y
Figure 3.18	3	11/24/2015	C1P2, *pceA*	233	Y	Y
Figure 3.18	4	11/24/2015	C2P2, *pceA*	233	Y	Y
Figure 3.18	5	11/24/2015	C3P2, *pceA*	233	Y	Y
Figure 3.18	6	11/24/2015	C4P2, *pceA*	233	Y	Y
Figure 3.18	7	11/24/2015	C1P6, *pceA*	233	Y	Y
Figure 3.18	8	11/24/2015	C2P6, *pceA*	233	Y	Y
Figure 3.18	9	11/24/2015	C3P6, *pceA*	233	Y	Y
Figure 3.18	10	11/24/2015	C4P6, *pceA*	233	Y	Y

* According to the gel electrophoresis images, the presence of organisms of interest could be confirmed, although *bvcA* was faint and the lack of a positive control for the PCR limits interpretation of *bvcA* results.

Initially, *16S rRNA* was chosen as the gene of interest since it is a biomarker for almost all the microorganisms so one could have higher possibility to get PCR products. Figure 3.13 shows the gel images of PCR products of the *16S rRNA* as targeting gene, using 8F and 690R as the forward and reverse primers, respectively.

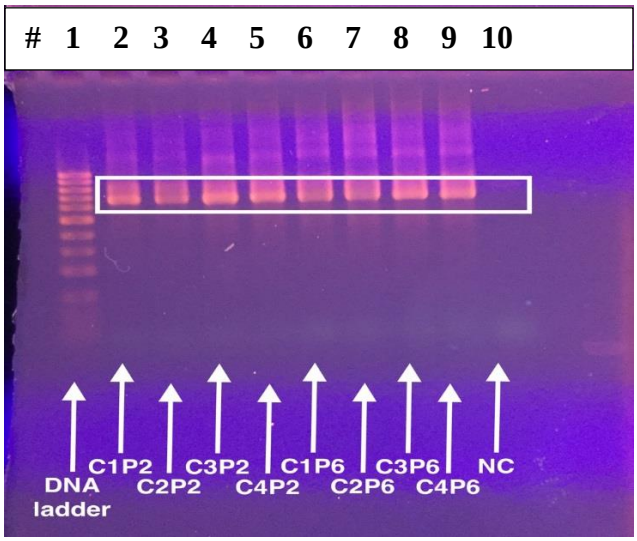

Figure 3.13 Gel image of *16S rRNA* PCR products. White box highlights region of expected amplicon.

In the gel image, bright bands show in the position with amplicon length of around 700 bps. The expected amplicon length of 8F and 690R primers is 681 bps. The negative control lane is blank, so the PCR products are what were expected.

As for methanogens, the *mcrA* gene was chosen to act as a biomarker of methanogens. Figure 3.14 shows the gel images of PCR products with *mcrA* primers *mlas F* and *mcrA R*.

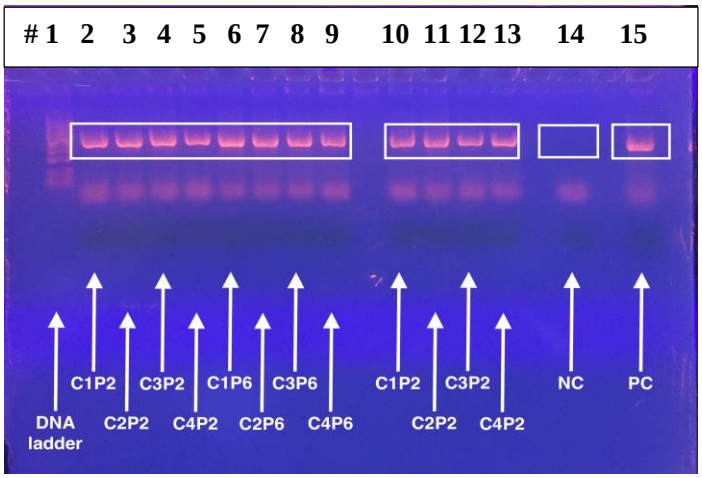

Figure 3.14 Gel image of *mcrA* PCR products for two of the sampling dates. NC= negative control. PC = positive control.

From the gel image, bright bands could be seen appearing at the position consistent with the expected amplicon length for *mlas F* and *mcrA R* primers (490 bps) (Steinberg & Regan, 2009), and the negative control lanes are blank. This suggests the presence of methanogens in every column, port 2 and port 6.

Figure 3.15 shows the gel images of *vcrA* PCR products with, *vcrAF* and *vcrAR* as the forward and reverse primer, respectively.

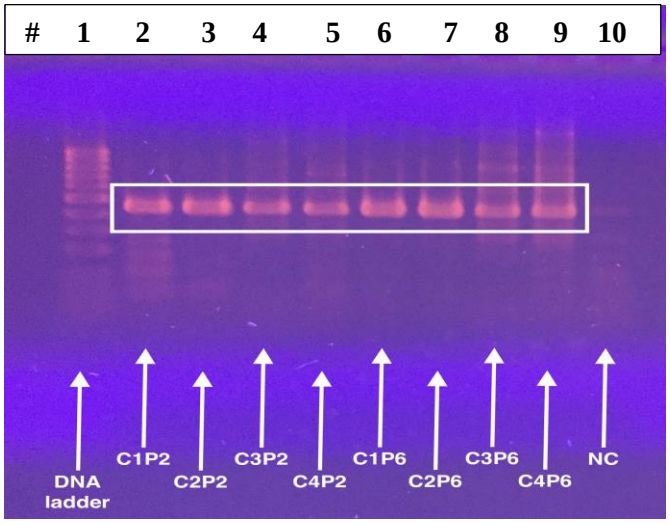

Figure 3.15 Gel image of *vcrA* PCR products with "long amplicon" primers. NC = negative control

From gel image, bright bands could be seen at the position with amplicon length of around 400 to 500 bps, the expected amplicon length for *vcrAF* and *vcrAR* primers is 441 bps and the negative control lane is blank. Therefore, the presence of *Dhc* could be confirmed in every column, port 2 and port 6. The amplicon sequence was later confirmed as vcrA with DNA sequencing results.

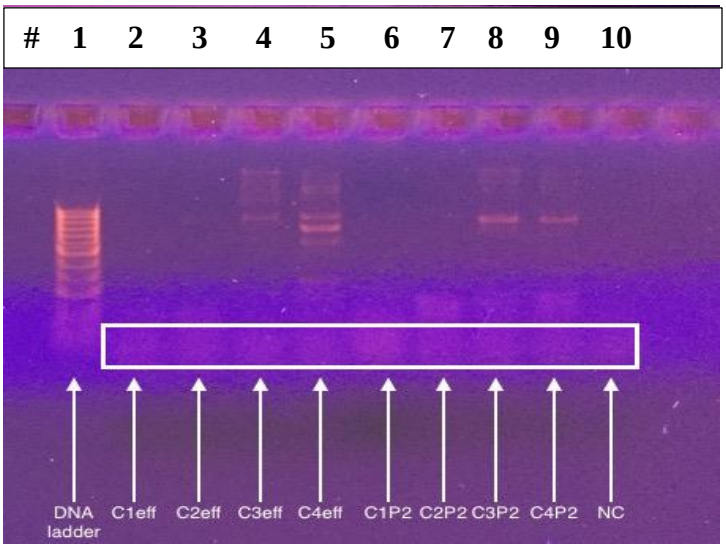

Figure 3.16 Gel image of *bvcA* PCR products

The expected amplicon length for *bvc925F* and *bvc1017R* is 92 bps. However, it is hard to see any clear bands appearing in gel image. One possibility is that the expected PCR product is just 92 bp and it could be too difficult to show clear bands of such short pieces of PCR products in gel electrophoresis. Another possibility is that *bvcA* is a biomarker for a minor stain of *Dhc* in KB-1^{TM} culture, so it is very possible that this minor strain population is too small to detect. Besides, the failure in running PCR and/or gel electrophoresis is also a possibility because according to Figure 3.16, even in negative control lane, there is still a very slight band appearing. A positive control PCR was unfortunately not included on this gel. Additionally, non-specific amplicons are seen for some of the samples from columns 3 and 4 in Figure 3.16.

Figure 3.17 shows the gel images of PCR products with *nirS* as targeting gene, with *nirSF* and *nirSR* as the forward and reverse primer, respectively.

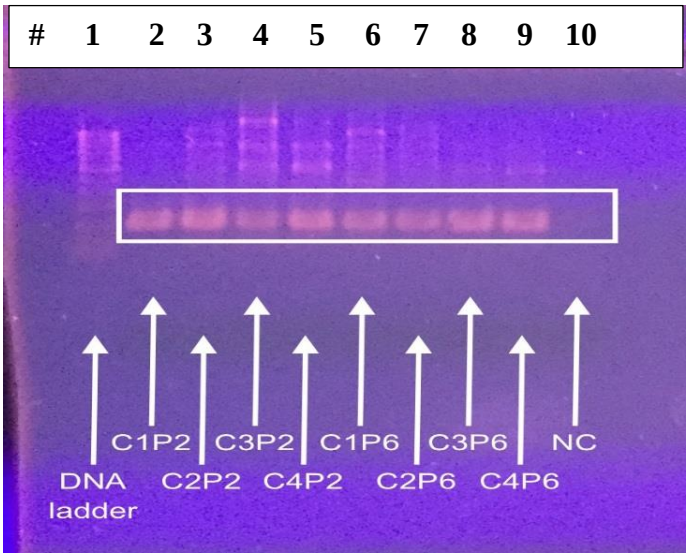

Figure 3.17 Gel image of *nirS* PCR products targeting *nirS*

In this gel image, bright bands appear in all the lanes except the negative control. The position of the bands evinces an amplicon length of 100 to 200 bps, and the expected amplicon length of *nirS* is 143 bps (Gruntzig et al., 2001). Therefore, the PCR products are correct and we could detect nitrate reducers in each column, both port 2 and port 6, even in columns 1 and 2, which did not receive nitrate. Some faint non-specific bands are seen even with the annealing temperature raised to 55 degrees C.

Figure 3.18 shows the gel images of PCR products for *pceA*, with *glpce1* and *glpce2* as the forward and reverse primer, respectively. These primers specifically amplify *Geobacter*'s PCE Reductive dehalogenase (Sun, 2014).

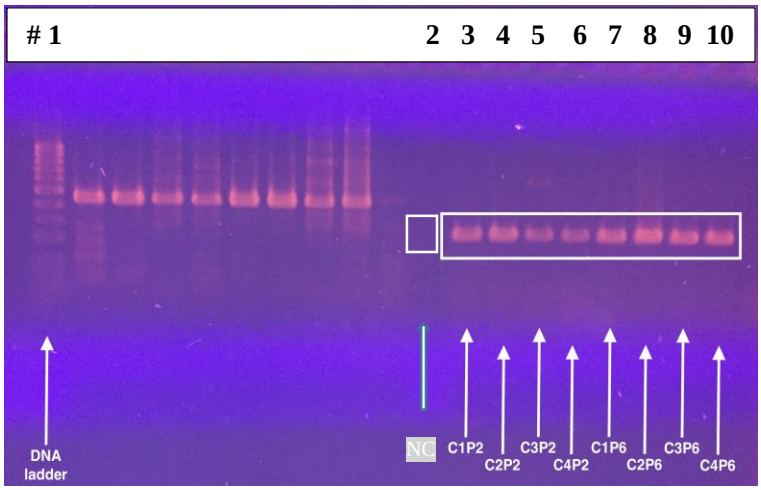

Figure 3.18 Gel image of PCR products targeting *pceA*. The lanes shown in the left part of the image are PCR products targeting *mcrA*. Those PCR products were tested together and shared one DNA ladder.

In the gel image, bright bands show in all the lanes except the negative control (the lane at the left to C1P2). The position of the bands evinces an amplicon length of 200 to 300 bps, the expected amplicon length of *pceA* is 233 bps (Sun, 2014). Therefore, *Geobacter* appears to be present in all columns, both port 2 and port 6.

A summary of PCR and gel electrophoresis results is shown in Table 3.3

Table 3.3 Summary of PCR products and gel images results for DNA samples from day 340

	16S rRNA	*mcrA*	*vcrA*	*bvcA*	*NirS*	*pceA*
C1P2	+	+	+	unclear	+	+
C2P2	+	+	+	unclear	+	+
C3P2	+	+	+	unclear	+	+
C4P2	+	+	+	unclear	+	+
C1P6	+	+	+	unclear	+	+
C2P6	+	+	+	unclear	+	+
C3P6	+	+	+	unclear	+	+
C4P6	+	+	+	unclear	+	+

In general, we detected all the bacteria of interest in both port 2 and port 6, and in every column. However, end-point PCR cannot determine relative or absolute concentrations of biomarker genes in the samples.

3.4 Quantitative PCR (qPCR) Results and Analysis

Quantitative-PCR was performed with primers for *mcrA* and *vcrA* to quantify methanogens and the *vcrA*-containing *Dhc* strain in the mulch column porewater.

3.4.1 qPCR Targeting the mcrA Gene

Quantitative PCR targeting the *mcrA* gene was performed with *mlas F* and *mcrA R* as the forward and reverse primers, respectively. Figures from the qPCR reports are shown in the Appendix C.1 Section with Figure C1 showing the standard curve of the qPCR standards with

samples also represented on the plot, Figure C2 showing the amplification graph for all samples, standards and blanks, and Figure C3showing the melt-curve results.

3.4.2 qPCR Targeting the vcrA Gene

Quantitative PCR targeting the *vcrA* gene was performed with *RDh A14 642F* and *RDhA14 846R* as the forward and reverse primers, respectively. Figures from the qPCR reports targeting *vcrA* genes are included in Appendix C.3 Section with Figure C4 showing the standard curve, Figure C5 showing the amplification graph and Figure C6 showing the melt curve.

Figure 3.19 Concentrations of *mcrA* and *vcrA* in port 2 and port 6 at selected dates are shown in Figure 3.19 in copies/mL of porewater. Panels a) to d) show the copies/mL pore water of *mcrA* genes at port 2, *mcrA* genes at port 6, *vcrA* genes at port 2, and *vcrA* genes at port 6, respectively.

Comparing panel a) and panel b) in Figure 3.19, it is hard to explain the patterns in methanogen populations. There is no distinguishable differences between methanogen levels in control versus experimental columns despite significantly less methanogenesis in the experimental columns — especially at the earliest date (October). Looking at trends over time at the same port, the methanogen populations in column 2 during October and November (day 304 and day 340) were the highest of any value seen on any day for the four columns, but in February (day 427), it went lower, from 10^4 per mL magnitude to 10^3 magnitude in port 2, and from almost 10^5 magnitude to 10^3 magnitude in port 6, respectively. The GC data on February was also a low point for methane production.

Panel c) and panel d) show the *vcrA* population in port 2 and port 6. Comparing these two panels, one could see that the population of the *Dhc* strain with a *vcrA* gene was always higher in control columns than in the experimental columns (10^5 to 10^6 copies/mL pore water in the control columns versus 10^3 to 10^4 copies/mL pore water in the experimental columns, one to two orders of magnitude difference between control and experimental columns). The one exception was the data on February (day 427), when there was more *vcrA* detected in an experimental column (column 3) than a control column (column 1). Though no dechlorination by *Dhc* was observed in the experimental columns for many months after inoculation, a small but significant population survived in the columns.

Figure 3.19 shows the concentrations of *mcrA* and *vcrA* (copies/mL pore water) on the three different days. The charts were graphed in log scale with the same maximum and minimum values in order to show the difference more clearly.

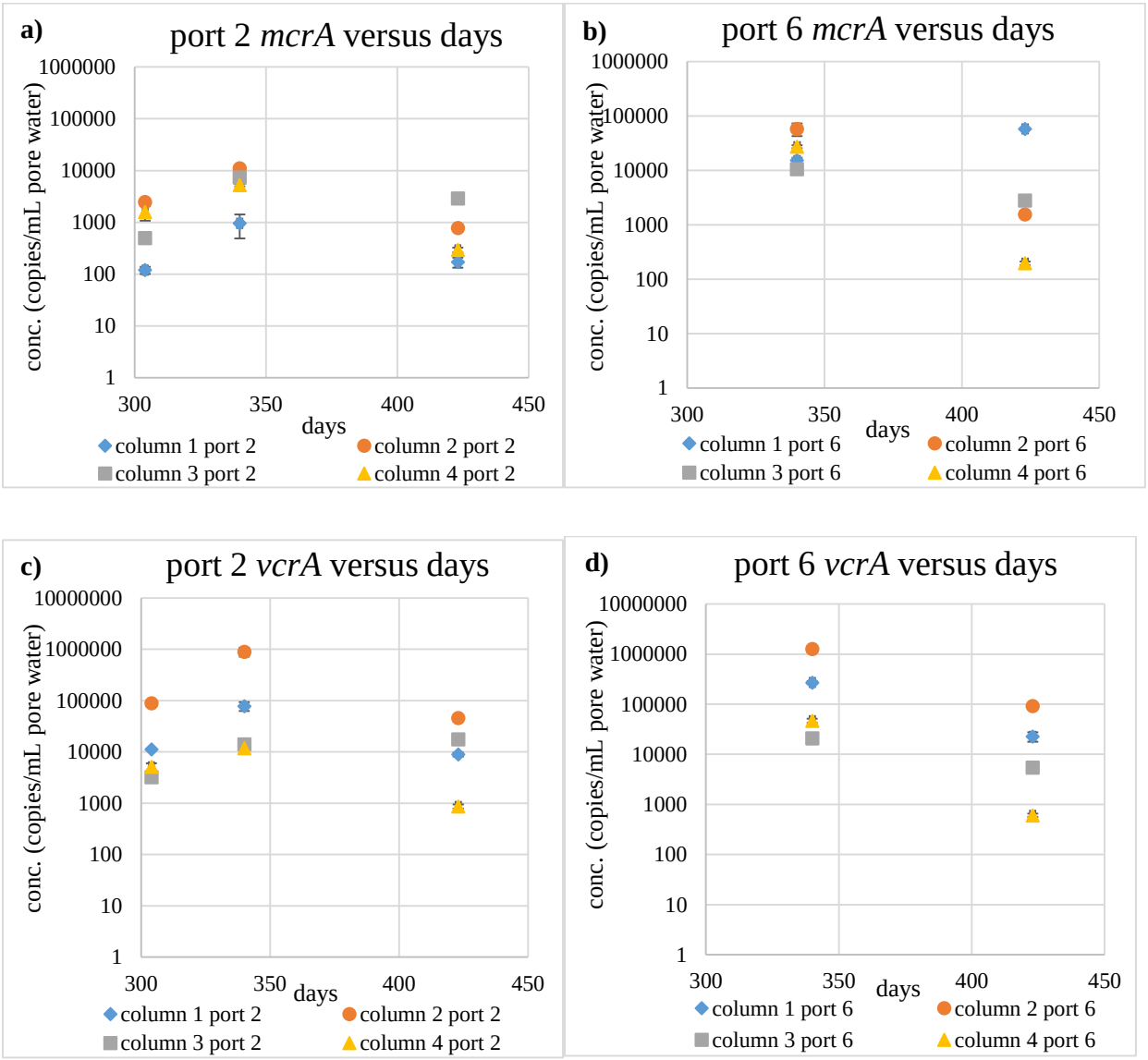

Figure 3.19 Concentrations of *mcrA* and *vcrA* genes in port 2 and port 6 pore water versus experiment day. Panels a) to d) show the copies/mL pore water of *mcrA* genes in port 2 and port 6, as well as *vcrA* genes in port 2 and port 6, respectively. Error bars repesent standard deviation from triplicate qPCR reactions.

Comparing the four charts here, it is easy to see that the porewater concentrations of *vcrA* genes were larger than the concentrations of *mcrA* genes along the columns, in general, with

ratios varying from 81 to 2 (vcrA/mcrA). Because *mcrA* is the biomarker for methanogens while *vcrA* is the biomarker for the main VC-respiring *Dhc* strain in KB-1^{TM}, it could be concluded that the population of methanogens is smaller than the *Dhc*. This is probably because *Dhc* has a larger initial population following inoculation with KB-1^{TM}, has a slower decay rate in the columns, and/or has less tendency to be washed out than the methanogens. It is like that many more *Dhc* and methanogens are in the mulch-attached phase but without sacrifising the columns to extract DNA from the mulch, this MS thesis only assessed the populations in the mobile phase.

The approximate column-wide rates of methane production and dechlorination were calculated in each column from GC data and pore water residence time between ports. More details of the calculation are in chapter 2.2.

Figure 3.20 shows the relationship between the column wide quantity of *mcrA* biomarker genes (as proxy for the population of microorganisms of interest) and the methane production.

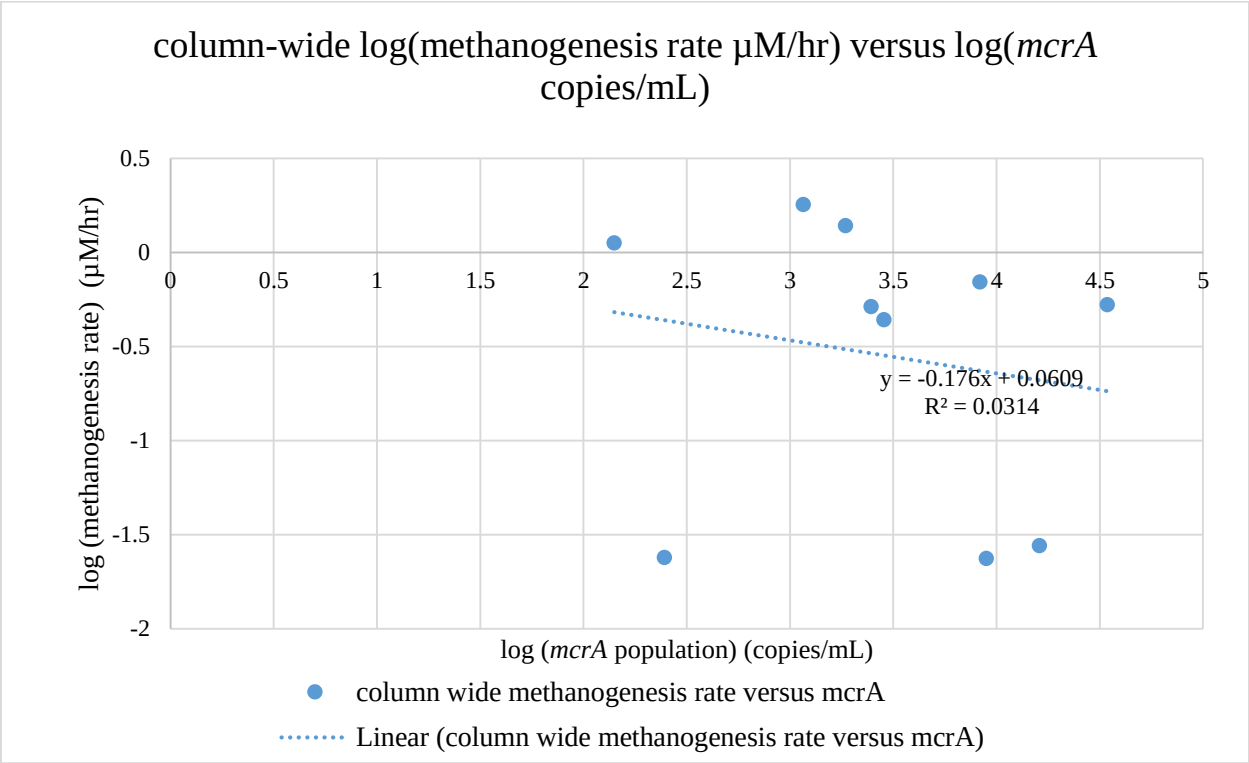

Figure 3.20 Column-wide quantity of *mcrA* genes (log scale) versus methanogensis rate (log scale). Only positive rates were shown in this log-log graph, negative and zero rate were not shown. However, there were *mcrA* even in ports where the local methane production rate was not positive.

A regression was performed column wide methane production and column wide *mcr*A population. According to the report, the P value is 0.62. The fact that value for upper 95% and lower 95% values for slope in the log-log regression (power-law regression) spans 0 along with the high P value and low R^2 value suggests an insignificant relationship. However, this failure could be due to the research limitations and/or the poor data. The full regression statistics summary report is provided in Table D1 in Appendix section D.

Figure 3.21 shows the relationship between the column-wide quantity of *vcrA* biomarker genes (as proxy for the population of microorganisms of interest) and the *cis*-DCE dechlorination rate.

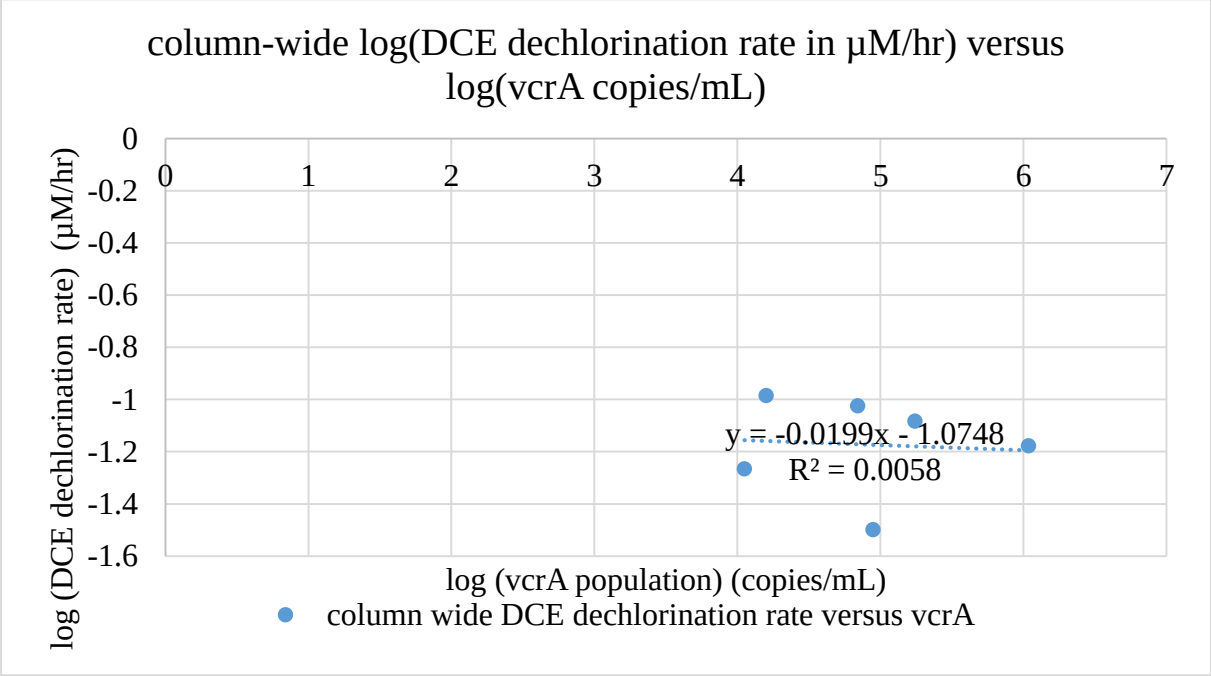

Figure 3.21 Columnwide *cis*-DCE dechlorination wates versus average of *vcrA* genes. Only positive rates were shown in this log-log graph, negative and zero rate were not shown. However, there were *vcrA* populations (see Figure 3.19) even in ports where the local dechlorination rates were not positive including the experimental columns.

A regression test was also run on log of column wide *cis*-DCE dechlorination rate and log of column wide *vcr*A population. The full regression statistics summary report is provided in Table D2 in Appendix section D.

According to the report, the P value is 0.89. The fact that value for upper 95% and lower 95% confidence interval on slope spans 0 along with the high P value and low R^2 value suggests an insignificant linear relationship. However, previous field studies have reported qualitative relationships between the occurrence of *Dhc* population and ethene production (Lendvay et al., 2007). Based on this information and Figure 3.21, the poor relationship might due to research limitations and/or poor data. Even such, this result is consistent with the more dechlorination in control columns shown in GC results and the higher *vcrA* amount in control columns shown in Figure 3.19. As mentioned, there are still *vcrA* genes in the experimental columns despite the fact that negligible dechlorination beyond *cis*-DCE was observed over the whole length of the study. A relationship between *mcrA* gene quantity and methanogenesis is not apparent.

CHAPTER 4 CONCLUSIONS AND FUTURE WORK

From the GC results, complete dechlorination to ethene was observed in control columns, and, on some dates, the dechlorination was complete by the middle ports of the column. However, dechlorination even from TCE to *cis*-DCE was not observed in KB-1^{TM}-inoculated mulch biobarriers with a 1 mM dosage (each) of nitrate and sulfate, let alone the complete dechlorination from TCE to ethene. After quartering the dosage of nitrate and sulfate, dechlorination to *cis*-DCE was quickly stimulated in experimental columns but complete dechlorination was not observed. Though significant populations of *vcrA*-containing *Dhc* were found even after months of high nitrate and sulfate, their activity was very low after the decrease in nitrate and sulfate concentrations. Either the populations were already dead (intact dead cells might still contain the biomarker gene) or the lower levels of nitrate and sulfate are still sufficiently high to prevent active dechlorination by a viable *Dhc* population. It is also worth noting that the pore-water populations are only part of the mulch biomass. Biofilms attached to the mulch will also harbor more cells.

According to the qPCR reports, the difference of *Dhc* population between control and experimental columns is very clear.

Based on the research presented in this Master Thesis, the following conclusion are made:

1. Dechlorination by KB-1^{TM} inoculated mulch biobarriers is limited and dechlorination did not happen when alternative electron acceptors (nitrate and sulfate) are present at 1 mM; only partial dechlorination was seen when nitrate and sulfate were reduced to 0.25 mM.

In control columns, complete dechlorination of TCE was observed within 200 days of inoculation at 1:100 dilution into the column porewater with a 2.7 day residence time.

2. The populations of *vcrA*-containing *Dhc* are smaller in experimental columns than in the control columns but for methanogens, the populations are indistinguishable in the pore water of control and experimental columns.
3. The *Geobacter* population and *vcrA*-containing *Dhc* populations both survived the high nitrate and sulfate phase, but only the *Geobacter* seemed to activate upon lowering the dosage of those alternate electron acceptors.

However, the exact mechanism of inhibition or limitation is still unclear, and future work is needed to figure it out. Future researchers could start by setting up some more columns and adding nitrate and/or sulfate separately into each column to detect the effect of nitrate and sulfate/sulfide alone.

Moreover, other various electron donors (such as butyrate and lactate etc.) could also be added into the columns to provide sufficient electron donors in order to see whether the limitation and/or inhibition is caused by the competition for electron donors. However this has implications for PRB management at the full scale. Also, more work should be done to figure out the reason of the poor mass balance and it should start with getting a new calibration curve for the dechlorination products (especially ethene) as well as a study of sorption of VC and ethene to the mulch. qPCR with other various biomarkers including *nirS, pceA* (from *Geobacter*), and even *dsrA* should also be done in the future and RNA could be analyzed instead of just DNA.

APPENDICES

Appendix A. GC Data of Selected Dates Analyzed in this Thesis

Table A1 Methane, chlorinated ethenes and ethene concentration (with a unit of μmole/L) of 10/22/2015, day 307.

10/22/2015 day 307					
	column 1				
	methane (μmole/L)	ethene (μmole/L)	VC (μmole/L)	*cis*-DCE (μmole/L)	TCE (μmole/L)
p1	0.00	0.00	1.02	0.05	0.86
p2	2.89	0.00	0.22	1.56	0.72
p3	9.56	0.04	0.61	2.30	0.45
p4	19.25	0.18	0.73	0.97	0.10
p5	28.53	0.20	0.45	0.43	0.00
p6	52.83	0.24	0.24	0.10	0.00
p7	70.03	0.25	0.15	0.01	0.00
	column 2				
	methane (μmole/L)	ethene (μmole/L)	VC (μmole/L)	*cis*-DCE (μmole/L)	TCE (μmole/L)
p1	0.00	0.00	0.01	0.00	6.18
p2	1.89	0.00	0.46	1.11	3.04
p3	9.64	0.10	0.98	2.50	0.41
p4	10.77	0.06	0.78	3.13	0.13
p5	23.93	0.08	0.91	2.16	0.00
p6	27.48	0.06	0.84	1.66	0.00
p7	32.17	0.06	0.81	1.42	0.00
	column 3				
	methane (μmole/L)	ethene (μmole/L)	VC (μmole/L)	*cis*-DCE (μmole/L)	TCE (μmole/L)
p1	0.12	0.00	0.00	0.00	5.97
p2	0.06	0.00	0.00	0.00	4.65

p3	0.02	0.00	0.00	0.00	4.22
p4	0.00	0.00	0.00	0.00	4.33
p5	0.00	0.00	0.00	0.00	4.28
p6	0.00	0.00	0.00	0.00	4.18
p7	0.00	0.01	0.00	0.00	4.15
	column4				
	methane (µmole/L)	ethene (µmole/L)	VC (µmole/L)	*cis*-DCE (µmole/L)	TCE (µmole/L)
p1	0.00	0.00	0.00	0.00	4.03
p2	0.00	0.00	0.00	0.00	5.58
p3	0.00	0.00	0.00	0.00	4.18
p4	0.00	0.00	0.00	0.00	3.89
p5	0.00	0.00	0.00	0.00	3.70
p6	0.00	0.01	0.00	0.36	3.16

Table A2 Methane, chlorinated ethenes and ethene concentration (with a unit of μmole/L) of 11/19/2015, day 335.

11/19/2015 Day 335					
	column 1				
	methane (μmole/L)	ethene (μmole/L)	VC (μmole/L)	*cis*-DCE (μmole/L)	TCE (μmole/L)
p1	0.00	0.00	0.00	0.00	4.41
p2	1.13	0.00	0.11	1.62	1.92
p3	8.67	0.06	0.96	2.40	0.20
p4	19.17	0.31	0.07	0.58	0.10
p5	26.60	0.34	0.45	0.28	0.06
p6	33.05	0.39	0.29	0.10	0.05
p7	43.47	0.42	0.14	0.00	0.05
	column 2				
	methane (μmole/L)	ethene (μmole/L)	VC (μmole/L)	*cis*-DCE (μmole/L)	TCE (μmole/L)
p1	0.00	0.00	0.01	8.50	4.11
p2	1.46	0.04	0.28	0.54	2.42
p3	6.60	0.14	0.52	1.07	0.43
p4	10.29	0.15	0.61	1.09	0.08
p5	21.43	0.22	1.16	1.00	0.04
p6	27.40	0.23	1.35	1.00	0.05
p7	32.89	0.22	1.32	0.94	0.11
	column 3				
	methane (μmole/L)	ethene (μmole/L)	VC (μmole/L)	*cis*-DCE (μmole/L)	TCE (μmole/L)
p1	0.00	0.00	0.00	0.00	5.43
p2	0.03	0.00	0.00	0.00	3.75
p3	0.04	0.00	0.00	0.07	4.03
p4	0.09	0.00	0.00	0.15	4.21
p5	0.34	0.00	0.01	1.44	3.66
p6	0.36	0.00	0.01	1.21	3.91
p7	1.47	0.00	0.00	3.48	1.80
	column4				
	methane (μmole/L)	ethene (μmole/L)	VC (μmole/L)	*cis*-DCE (μmole/L)	TCE (μmole/L)
p1	0.00	0.00	0.00	0.00	3.59

p2	0.00	0.00	0.00	0.00	3.38
p3	0.09	0.00	0.00	0.17	3.62
p4	0.80	0.00	0.00	0.91	3.82
p5	1.02	0.00	0.01	1.48	3.88
p6	1.42	0.00	0.01	2.16	3.40

Table A3 Methane, chlorinated ethenes and ethene concentration (with a unit of μmole/L) of 3/14/2015, day 451.

3/14/2016 Day 451					
	column 1				
	methane (μmole/L)	ethene (μmole/L)	VC (μmole/L)	*cis*-DCE (μmole/L)	TCE (μmole/L)
p1	17.93	0.52	3.49	12.75	8.58
p3	78.01	0.98	0.05	0.09	0.13
p5	112.78	0.61	0.01	0.00	0.03
p7	86.70	0.46	0.01	0.00	0.05
	column 2				
	methane (μmole/L)	ethene (μmole/L)	VC (μmole/L)	*cis*-DCE (μmole/L)	TCE (μmole/L)
p2	42.00	0.75	1.53	6.01	0.31
p3	46.16	0.93	1.99	2.81	0.11
p5	66.39	1.00	0.03	0.06	0.13
p7	112.34	0.89	0.13	0.28	0.33
	column 3				
	methane (μmole/L)	ethene (μmole/L)	VC (μmole/L)	*cis*-DCE (μmole/L)	TCE (μmole/L)
p1	0.17	0.00	0.00	0.09	8.88
p3	0.62	0.00	0.02	0.91	6.35
p5	9.42	0.00	0.01	4.36	2.18
p7	27.41	0.00	0.01	7.96	0.28
	column4				
	methane (μmole/L)	ethene (μmole/L)	VC (μmole/L)	*cis*-DCE (μmole/L)	TCE (μmole/L)
p1	0.09	0.00	0.00	0.47	7.68
p3	0.00	0.00	0.00	0.10	5.40
p5	0.68	0.00	0.01	1.21	4.19
p6	1.23	0.00	0.01	1.58	3.71

Appendix B. DNA Sequencing Results

Figure B1 is the screen shot of opening the sequencing result with FinchTV.

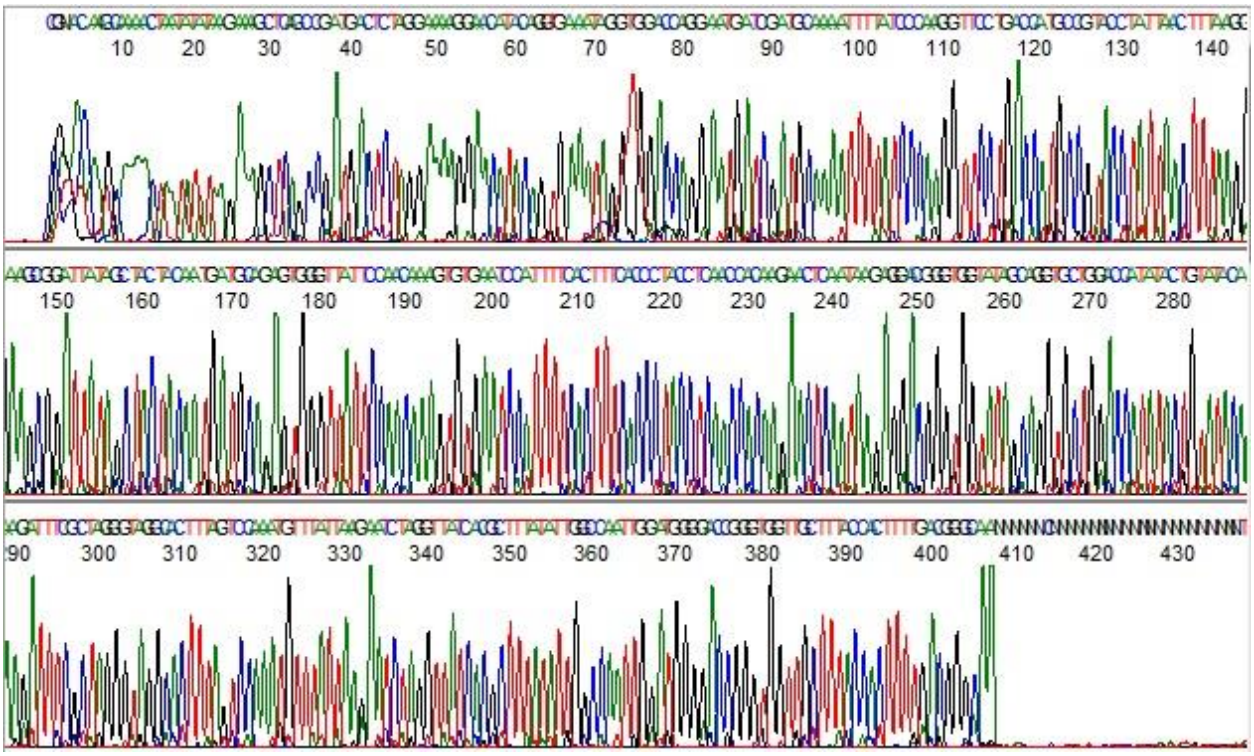

Figure B1 Sequencing result for vcrA long amplicon

From Figure B1, it is clear that there was nothing to be sequenced after 410 bps, which is a very positive sign to show that it was the right amplicon because the expected length of *vcrA* long amplicon is 441bps.

The exact sequence in a text form is shown below:

CGNACAAGCAAAACTAATATATAAGAAAGCTCAGCCGATGACTCTAGGAAAAGGAACAT
ACAGGTGAAATAGGTGGACCAGGAATGATCGATGCAAAATTTTATCCCAAGGTTCCTGACCATGC
CGTACCTATTAACTTTAAGGAAGCGGATTATAGCTACTACAATGATGCAGAGTGGGTTATTCCAA

CAAAGTGTGAATCCATTTTCACTTTCACCCTACCTCAACCACAAGAACTCAATAAGAGGACGGGT
GGTATAGCAGGTGCTGGACCATATACTGTATACAAAGATTTCGCTAGGGTAGGCACTTTAGTCCA
AATGTTTATTAAGAATCTAGGTTATCACGCTTTATATTGGCCAATTGGATGGGGACCGGGTGGTT
GCTTTACCACTTTTGACGGGCA

Note: Underlined region highlights the reverse primer site.

The whole sequence was then BLASTed. According to the BLAST report, there was a 99% identity of this sequence to a *Dhc sp* KB1 clone KB1*RDhAB14* reductive dehalogenase protein gene. Therefore, this is the correct sequence. Then the long amplicons have been confirmed and could be made into a serious of qPCR standards after running PicoGreen to measure the initial total DNA concentration.

Appendix C. qPCR Results

Appendix C.1 qPCR Results on March. 18th Targeting mcrA Gene

Figure C1 shows the Standard Curve of qPCR running targeting *mcrA* gene.

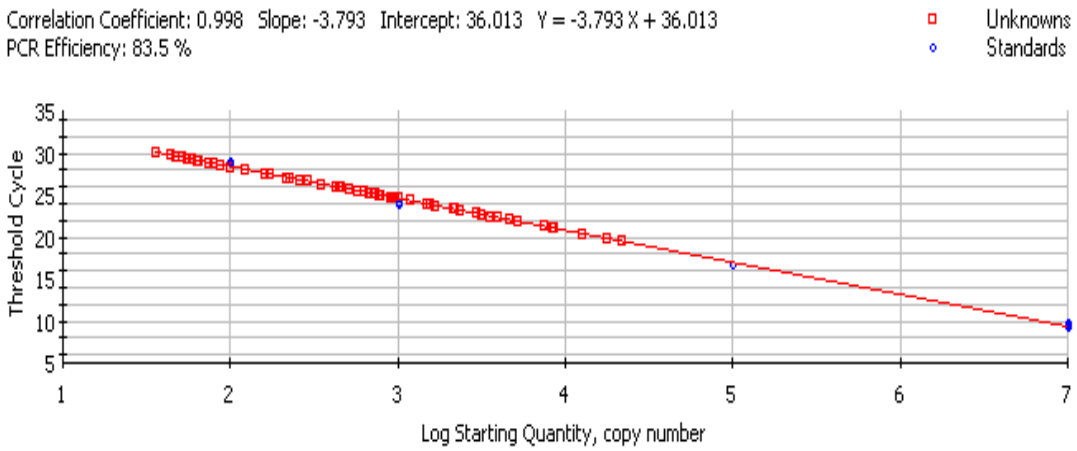

Figure C1 Standard Curve of qPCR amplicons of *mcrA* gene

From this figure, it is easy to tell that the blue circles which show the concentration of standards is quite linear and the red squares which show the concentration of unknown samples are well distributed along the standard curve. However, some of the concentration of the unknown samples are beyond the standards, they are even lower than the most diluted standard. The concentration of these samples could only be estimated based on the standard curve and the accuracy of the concentration of these unknown samples could be doubted.

Figure C2 shows the amplification graph.

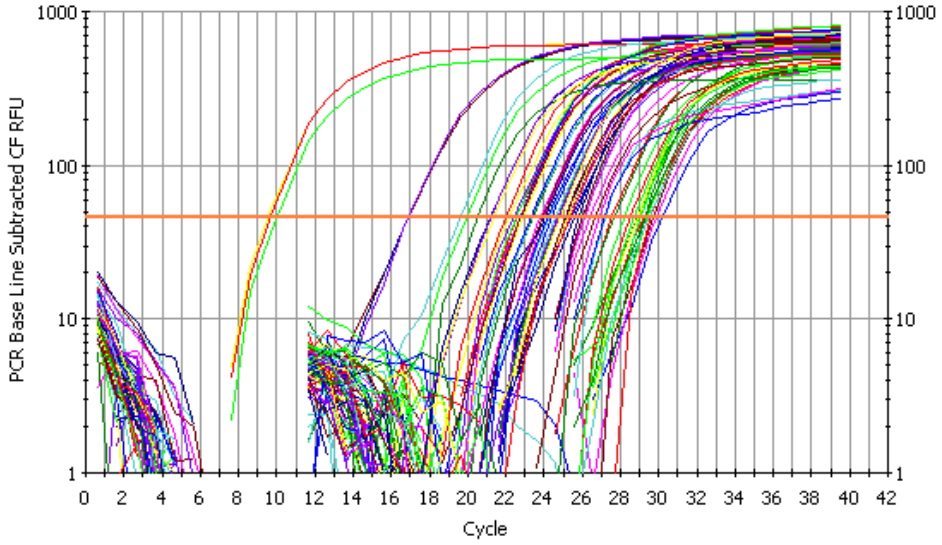

Figure C2 Amplification graph of qPCR for the *mcrA* gene

According to this amplification graph, one could easily see that the triplicates are all very close to each other, especially for the stand-out curves, which are the standards. This is also a good sign of the qPCR running.

Figure C3 shows the melt curve.

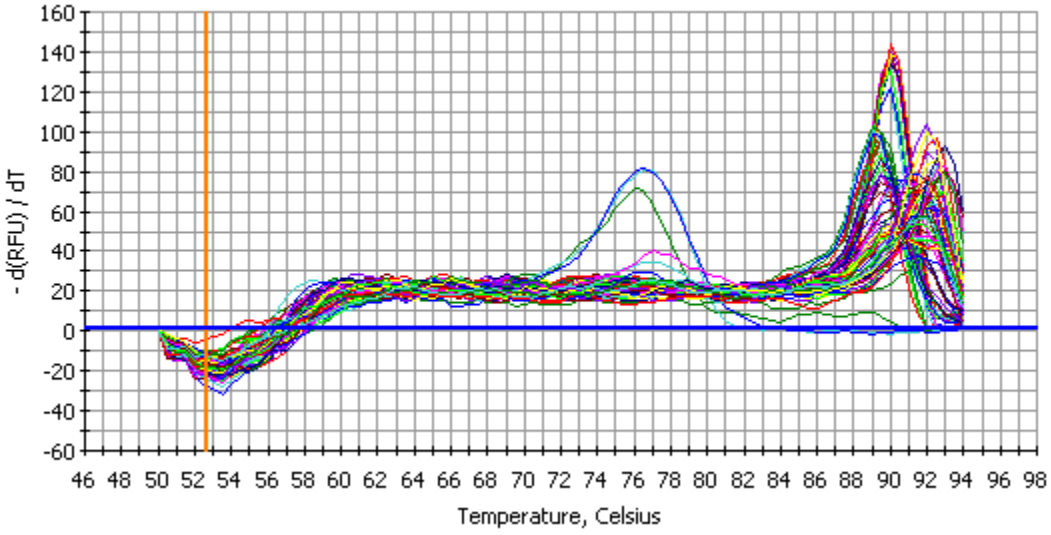

Figure C3 Melt Curve analyses of qPCR amplicons of *mcrA* gene

In this figure, all the qPCR products melt at a temperature of around 90ºC except the wells for blank, which melted at a lower temperature.

Appendix C.2 qPCR Results on March. 19th Targeting vcrA Gene

Figure C4 shows the Standard Curve of qPCR for the *vcrA* gene.

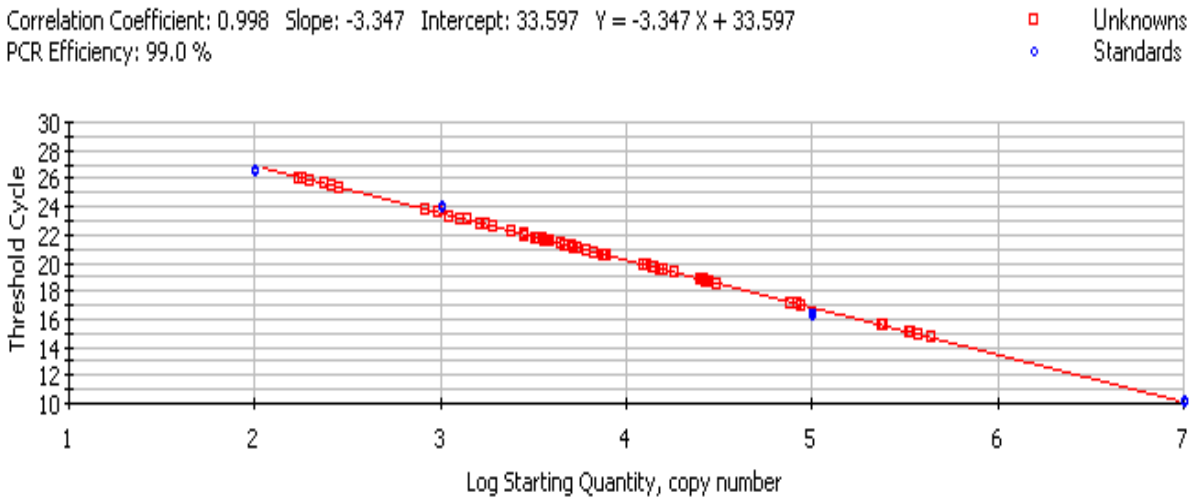

Figure C4 Standard Curve of qPCR amplicons of the *vcrA* gene

According to this figure, it is also clear that the concentration of standards is quite linear and the concentration of unknown samples are well distributed within the standard range.

Figure C5 shows the amplification graph.

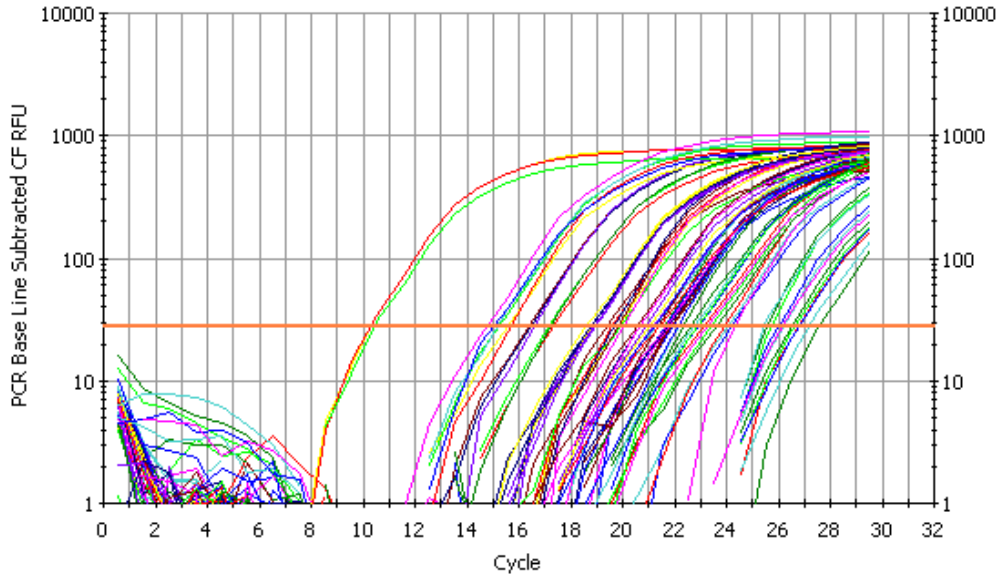

Figure C5 Amplification graph of qPCR for the *vcrA* gene

The trend showing in this figure is quite similar to Figure C2, which is the amplification graph of qPCR targeting *mcrA*, even neater than the *mcrA* one. In this figure, not only the standard with the highest concentration, but also the curves for unknown samples, one could see clear triplicates close to each other.

Figure C6 shows the melting curve.

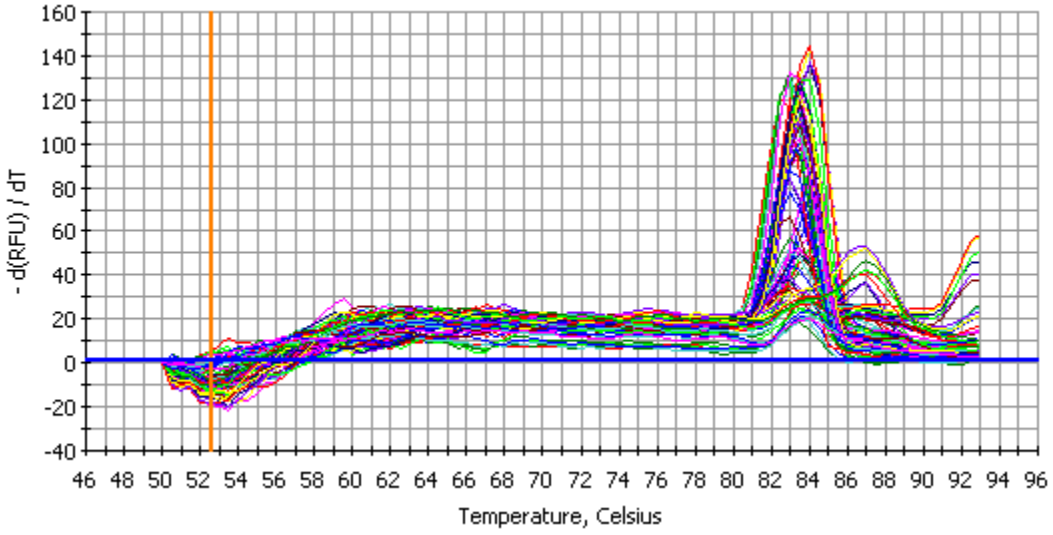

Figure C6 Melt Curve of qPCR amplicons of the *vcrA* gene

In this figure, nearly all the qPCR products melt at a temperature of around 84ºC. Some of the qPCR products melted at a higher temperature. They might be some non-specific qPCR products.

Appendix D.1 Statistics Test Summary Report of Methanogenesis Rate Versus mcrA Levels

Table D1. Regression Statistics Summary Output for methanogenesis rate versus mcrA level

For column wide log methanogenesis rate (µM/hr) versus log mcrA (copies/mL)								
SUMMARY OUTPUT								
Regression Statistics								
Multiple R	0.18							
R Square	0.03							
Adjusted R Square	-0.09							
Standard Error	0.79							
Observations	10							
ANOVA								
	df	*SS*	*MS*	*F*	*Significance F*			
Regression	1	0.16	0.16	0.26	0.62			
Residual	8	4.98	0.62					
Total	9	5.15						
	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.06	1.21	0.05	0.96	-2.73	2.85	-2.73	2.85
X Variable 1	-0.18	0.35	-0.501	0.62	-0.97	0.62	-0.97	0.62

Appendix D.2 Statistics Test Summary Report of cis-DCE Dechlorination Rate Versus vcrA Levels

Table D2. Regression Statistics Summary Output for cis-DCE dechlorination rate versus vcrA level

For column wide log methanogenesis rate (µM/hr) versus log mcrA (copies/mL)								
SUMMARY OUTPUT								
Regression Statistics								
Multiple R	0.08							
R Square	0.01							
Adjusted R Square	-0.24							
Standard Error	0.21							
Observations	6							
ANOVA								
	df	*SS*	*MS*	*F*	*Significance F*			
Regression	1	0.00	0.00	0.02	0.89			
Residual	4	0.18	0.04					
Total	5	0.18						
	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-1.07	0.64	-1.67	0.17	-2.87	0.72	-2.87	0.72
X Variable 1	-0.02	0.13	-0.15	0.89	-0.38	0.34	-0.38	0.34

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