2013

Spatial Relationship between the Trichloroethylene Degrading Bacteria Dehalococcoides Sp., Sulfate Reducers and Archaea during Reductive Dechlorination

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Spatial Relationship between the Trichloroethylene Degrading Bacteria *Dehalococcoides* Sp., Sulfate Reducers and Archaea during Reductive Dechlorination

Pujya Wagle Gautam

**Thesis submitted to the
College of Benjamin M Statler College of Engineering and Mineral resources
at West Virginia University
in partial fulfillment of the requirements
for the Degree of**

**Masters of Science
in
Civil and Environmental Engineering**

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**Morgantown, West Virginia
2013**

Key words: bedrock fracture, bioremediation, DHC, sandy aquifer, silt aquifer, reductive cometabolism, qPCR, TCE.

ABSTRACT

Spatial Relationship between the Trichloroethylene Degrading Bacteria *Dehalococcoides* Sp., Sulfate Reducers and Archaea during Reductive Dechlorination

Pujya Wagle Gautam

Trichloroethylene (TCE) released to the environment is of great concern due to its toxicity and carcinogenicity. The microorganisms involved in bioremediation of TCE such as methanogens (organisms within the domain Archaea), sulfate reducing bacteria (SRB) and *Dehalococcoides* sp. (DHC) are of particular interest in this study. Three different types of bench scale reactors were constructed to model different aquifer types such as sandy, silty and fractured bedrock aquifers (hereafter type 1, 2 or 3 reactors, respectively). This study evaluated the effect of TCE concentration in different types of reactors on the distribution of selected microorganisms with distance from the source of the TCE. It also examined the spatial relationship between *Dehalococcoides* sp., Archaea and SRB with respect to reducing equivalents (e.g., food) in different types of aquifer environment contaminated with TCE. The DNA analysis was performed by using quantitative polymerase chain reaction (qPCR) and fluorescent in situ hybridization (FISH). In this study concentrations of Archaea were higher in all reactors than other microorganisms under study. In the type 1 reactor, with increasing concentrations of TCE, DHC concentrations and SRB concentrations increased. In type 2 and 3 reactors, there were no observed correlations between initial concentrations of TCE and the concentration of the studied microbes. The highest DHC concentration was present near the food source in type 1 and 2 reactors. In type 3 reactors the concentration of microorganisms was higher outside the tube (e.g., fracture) than inside. The spatial relationship between *Dehalococcoides* sp. and various microorganisms that compete for bioremediation substrates supplied is helpful to understand when and where to bioaugment an aquifer undergoing bioremediation of TCE with *Dehalococcoides* sp.

Acknowledgements

My greatest gratitude goes to my supervisor Jennifer L Weidhaas, Ph.D, P.E. who offered her continuous advice, encouragement and undertook tremendous responsibility in supervising the completion of my research and thesis. I thank her for the systematic guidance, patience, motivation, enthusiasm, great effort and immense knowledge she put into training me in the scientific field. I could not have imagined having a better advisor and mentor for my study. It was with her encouragement and help that I could be confident in doing the whole job.

I also would like to thank Dr. Lian-Shin Lin and Dr. Radhey Sharma for their valuable time and serving on my committee.

I acknowledge David Turner, Senior Lab Instrumentation Specialist, Civil and Environmental Engineering, for contributing his time to make the reactors to support this study. Also I would like to thank Dr Jianbo Yao, Division of Animal & Nutritional Sciences and his students for allowing me to use nanodrop quantification spectrophotometer to complete analysis at his laboratory. My thanks go to the chemistry department for allowing me to use the Gas chromatography instrument and Dr Karen Martin, Imaging facility of Mary Babb Randolph Cancer Center at Health science department for training me in image analysis. I am thankful to Dr. Ryan Dupont (USU) and Dr. Paul Hatzinger (Shaw) for providing *Dehalococcoides* cultures studies.

Finally, I take this opportunity to express the profound gratitude to my dear husband Lekhnath Gautam, my beloved parents, siblings and my son Prithul Gautam for their love, encouragement, understanding and continuous support during all this years. I really appreciate the help of my friends and fellow students: Thomas, Autumn, Isabel, Dong Yang (Sunny) and Chenjie. Last but not least, my thanks go to all the other faculty and staff in Civil and Environmental Engineering at WVU especially, Dr. Karen Buzby, and Research Assistant Will Ravenscroft for their help.

Table of contents

ABSTRACT.....	i
Acknowledgements.....	ii
Table of contents	iii
List of Tables	v
List of figures.....	vi
List of Abbreviations.....	ix
CHAPTER 1 INTRODUCTION	1
1.1 Background.....	1
1.2 Exposure and environmental effects of TCE.....	2
1.3 Current treatment methods and issues	2
1.4 Research objectives and hypotheses	5
1.5 Significance of the research.....	5
CHAPTER 2 LITERATURE REVIEW	7
2.1 Trichloroethylene production, use, properties and treatment	7
2.1.1 Physical and chemical properties:	8
2.2 Physical and chemical TCE treatment technologies	9
2.3 Biological TCE treatment.....	10
2.3.1 Aerobic oxidation of TCE.....	11
2.3.2 Anaerobic reductive dechlorination.....	12
2.3.3 Reductive co-metabolism	15
2.4 Electron acceptor/electron donor and redox potential.....	16
2.5 <i>Dehalococcoides</i> sp. (DHC)	23
2.6 Sulfate reducing bacteria (SRB)	26
2.7 Archaea/Methanogens	26
2.8 Molecular screening techniques.....	27
2.8.1 PCR/qPCR	27
2.8.2 Fluorescence <i>in situ</i> hybridization (FISH).....	29
CHAPTER 3 MATERIALS AND METHODS.....	31

3.1	Chemicals	31
3.2	Microcosm preparation.....	31
3.3	Analysis of sulfates, redox potential and COD.....	32
3.4	Construction of type 1 incubation chambers	33
3.5	Construction of type 2 incubation chambers	36
3.6	Construction of type 3 incubation chambers	38
3.7	Growth studies	39
3.8	Microcosm disassembly and cell sampling	39
3.9	DNA extraction method testing and selection	40
3.10	PCR.....	45
3.11	qPCR	47
3.12	Clone library and qPCR standard curve construction	47
3.13	Data analysis.....	49
CHAPTER 4 RESULTS		51
4.1	Analysis of chemical degradation and culture in seed reactor.....	51
4.2	Evaluation of DNA extraction methods by PCR	53
4.3	qPCR results	56
4.3.1	Specific objective 1: To test the hypothesis that increasing TCE concentrations influence the concentration of DHC, SRB and Archaea.	59
4.3.2	Specific objective 2: To test the hypothesis that the concentration of DHC, SRB and Archaea decrease with increasing distance from the source of reducing equivalents (i.e., food)	64
4.3.3	Specific objective 3: To test the hypothesis that after bioaugmentation DHC, SRB and Archaea concentrations increase due to microbial growth.	72
CHAPTER 5 DISCUSSION		75
5.1	Cometabolic mechanism	76
5.2	Spatial variability	76
5.3	Fractured clay or bedrock.....	79
CHAPTER 6 CONCLUSIONS AND RECOMMENDED FUTURE RESEARCH		81
6.1	Conclusions	81
6.2	Recommendations for Future Research.....	82
REFERENCES.....		83
APPENDIX.....		100

List of Tables

Table 1.1. States with the highest reported mass of trichloroethylene.....	1
Table 2.1. Physical and chemical properties of trichloroethylene	8
Table 2.2. Dechlorinating bacteria using TCE as an electron acceptor	14
Table 2.3. Typical redox half-reactions in anaerobic mixed culture with Gibbs free energy	21
Table 2.4. DHC species documented to degrade chlorinated compounds	25
Table 3.1. Specific PCR/qPCR primers and probes	46
Table 4.1. DNA concentration, purity and amplification results of various DNA extraction methods	54
Table 4.2. Detection of DHC, SRB and Archaea in the type 1, 2 and 3 control reactors	57
Table 4.3. Detection of the different orders of methanogens and total Archaea in the seed reactor	57
Table 4.4. Comparison of the average concentration of microorganisms in all areas after 7 days of incubation with different initial nutrient conditions and TCE concentrations	58
 Table A1. Standard Curve for Sulfate Analysis	 100
Table A2. Standard Curve for COD Analysis	101
Table A3. Detailed results for DHC, SRB and Archaea from qPCR analysis in various reactor types and experimental conditions.	102
Table A4. Statistical tests for different reactors and microorganisms	108

List of figures

Figure 2.1. Chemical Structure of TCE.....	7
Figure 2.2. Sequential reductive dechlorination of trichloroethylene under anaerobic condition.	13
Figure 2.3. Flow diagram of fermentation pathway.....	18
Figure 2.4. Redox potential range for favorable dehalogenation, sulfate reduction and methanogenesis.	23
Figure 3.1. Schematic diagram and photograph of the type 1 incubation chambers (figure not to scale).....	34
Figure 3.2. Schematic diagram and photographs of the type 2 incubation chambers.....	37
Figure 3.3. Schematic diagram and photograph of the type 3 incubation chambers	38
Figure 3.4. Schematic diagram of microscope slide (figure not to scale) A) diagram of the three areas sampled for qPCR analysis in type 1 and 2 reactors, and B) the capillary area sampled for qPCR analysis in the type 3 reactor.	40
Figure 3.5. Standard curve for DHC (A), SRB (B) and Archaea (C).	49
Figure 4.1. Sulfate concentrations in the seed reactors over the operational period	51
Figure 4.2. Comparison of culture characteristics of DAPI stained DHC in (A) The KB-1/VC-H ₂ culture from literature at 1000X magnification (Duhamel, 2004) and (B) from this study at 100X magnification observed with epifluorescence microscope with emission wavelength of 450-500nm.	53
Figure 4.3. FISH microscopic images of culture from seed reactor (A) DHC only at magnification of 100X and wavelength 500-570 nm (green) (B) all cells stained with DAPI in same field as A at 450-500 nm wavelength (blue).	53
Figure 4.4. Nanodrop DNA quantification of: (A) Type 1 reactor (B) Type 2 reactor and (C) Type 3 reactor for method 3 ii.	55
Figure 4.5. Detection of (A) bacterial 16S rRNA , (B) <i>Dehalococcoides</i> 16S rRNA , (C) <i>dsrA</i> genes of sulfate reducers and (D) Archaea 16S rRNA.	56
Figure 4.6. DHC, SRB and Archaea gene concentrations with varying TCE concentrations after 7 days in type 1 reactor (average and standard deviation of duplicates in all areas of reactor), type 2 reactor and type 3 reactor.	61

Figure 4.7. Relationship between DHC, SRB and Archaea gene concentration with respect to different TCE concentration in different reactor types after 7 days; A: type 1 reactor (average of all areas), B: type 2 reactor (average of all areas) and C: type 3 reactor (average of all inside and outside capillary tube). Regression with * is statistically significant ($P<0.05$).....	63
Figure 4.8. DHC (A), SRB (B) and Archaea (C) gene concentrations with distance from the agar plug and with different TCE concentrations after 7 days in Type 1 reactor (average and standard deviation of duplicates in three different areas of reactor).....	66
Figure 4.9. Concentration of microorganisms with distance from the agar plug and with differing TCE concentrations after 7 days in the type 2 reactor. DHC (A), SRB (B) and Archaea (C).	68
Figure 4.10. Mean and standard deviations of the log gene concentrations of DHC (A), SRB (B) and Archaea (C) with different concentration of TCE after 7 days in the type 3 reactor.	70
Figure 4.11. Relationship between DHC gene concentration with respect to different TCE concentration in type 1 and type 2 reactors after 7 days; A: area 1, B: area 2. Regression with * is statistically significant ($P<0.05$).	71
Figure 4.12. Relationship between SRB gene concentration in three different areas with respect to different TCE concentration in type 1 reactor after 7 days. Regression with * is statistically significant ($P<0.05$).....	71
Figure 4.13. Average and standard deviations of the log concentrations of DHC, SRB and Archaea gene copies/cm ² in type 1 reactor (A), type 2 reactor (B) and type 3 reactor (C)over time	74
Figure 5.1. Possible location of microorganisms in type 1 reactor in which most microbes are grown near the food amendment competing for electron donors while they still grow farther from food amendment where TCE is present due to diffusion.	78
Figure 5.2. Possible location of microorganisms in type 2 reactor in which TCE and food amendment are together and the microbes compete for them. At farther zone from food there is less and no growth of those microorganisms.....	79
Figure 5.3. Fracture flow microorganisms in type 3 reactor in which TCE and food amendment are together but the space to migrate towards the food is very low. Microbes compete for the TCE located inside the clay as well as water, due to compact structure of clay particle those microbes try to find food.	80

Figure A1. Standard curve for sulfate analysis.....	100
Figure A2. Standard curve for COD analysis.....	101
Figure A3. The log concentrations of DHC, SRB and archaea in type 1 and 2 reactor with all distances.	111
Figure A4. Relative abundance of dechlorinators maintained over the course of 7 days (type 3) reactor. Relative abundance was calculated from qPCR data with genus-specific primer sets..	112
Figure A5. Archaea cells observed on confocal microscope after FISH stained with DAPI and Arch-647 Alexa probe from the seed reactor	112
Figure A6. Sulfate reducing bacteria observed on confocal microscope after FISH stained with DAPI and SRB-555 Alexa probe from the seed reactor	113
Figure A7. Co-distribution of DHC, sulfate reducing bacteria and Archaea in a sludge granule from the seed reactor observed by confocal microscope after FISH staining with DAPI and DHC fluorescein probe, SRB-555 Alexa probe and Arch-647 Alexa probe together (from right to left first is DAPI stained, second DHC, third SRB (upper row), Archaea and last one is all dyes together (lower row)).....	114

List of Abbreviations

ATSDR	Agency for Toxic Substances and Disease Registry
CERCLA	Comprehensive Environmental Response, Compensation, and Liability Act
CLSM	Confocal laser scanning microscope
COD	chemical oxygen demand
DAPI	Diamidino-2-phenylidole dihydrochloride
DCE	dichloroethylene
DHC, Dhc	<i>Dehalococcoides mccartyi</i>
DNAPL	dense non-aqueous phase liquid
<i>dsr</i>	dissimilatory sulfate reduction
Eh	reduction potential
ESTCP	Environmental Security Technology Certification Program
FISH	Fluorescence <i>in-situ</i> hybridization
GAC	granular activated carbon
GC	gas chromatography
K _{oc}	soil-organic carbon partition coefficient
K _{ow}	octanol-water partition coefficient
MCL	maximum contaminant level
NPL	National Priorities List
NRC	National Research Council
PBS	phosphate buffer saline solution
PCE	tetrachloroethene (or perchloroethene)
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
SRB	sulfate reducing bacteria
SERDP	Strategic Environmental Research and Development Program
TCE	trichloroethylene
VC	vinyl chloride
VOC	volatile organic compound

CHAPTER 1 INTRODUCTION

1.1 Background

Trichloroethylene (TCE), CAS number 79-01-6, is a toxic volatile organic compound with excellent solvent properties. It has been extensively used as an industrial chemical. For example TCE has been used as a solvent, metal degreaser, dry cleaning agent, paintstripper, varnish, and in printing ink and industrial paint, among other industrial processes (WHO, 1984; ATSDR, 1997; Doherty, 2000; ATSDR, 2004; EPA consumer factsheet, 2006; NCPH, 2007). The production of TCE was 145 million kg in 1991 (EPA consumer factsheet, 2006). Deliberate and accidental discharge of TCE to water, groundwater and soil at hazardous waste sites has resulted in the spread of TCE in the environment (ATSDR, 2007). TCE is the most frequently detected groundwater contaminant in the United States (Fischer *et al.*, 1987). NRC has reported that TCE only has been found in over 303 groundwater aquifers in the US (NRC, 2012). This compound was designated a priority pollutant by the U.S. Environmental Protection Agency (EPA) in 1976. As of 2007, TCE was detected at 1002 of 1689 National Priority List sites, making it one of the most commonly found contaminants at Superfund sites (ATSDR, 2007). According to Toxic Release Inventory, TCE released to water and land was over 131,995 kg from 1987 to 1993 (EPA consumer factsheet, 2006). The highest concentration was reported in 1982 and the largest releases occurred in Pennsylvania and Illinois (Table 1.1) (EPA consumer factsheet, 2006). The largest direct release to water occurred in West Virginia (Table 1.1) (EPA consumer factsheet, 2006).

Table 1.1. States with the highest reported mass of trichloroethylene releases to water and land, 1987 to 1993 (EPA, Consumer Factsheet)

State*	Water (kg)	Land (kg)
PA	0	15172.7
IL	0	13930
GA	1697.3	7952.3
TX	0	9525.4
MA	0	9035.6
WV	12,822	0

* State totals only include facilities with releases greater than 4536 kg.

Due to its high solubility in water (i.e., 1.366 g/L at 25°C) and environmental persistence, TCE contamination is of great concern. A small amount of TCE is sufficient to contaminate a large amount of groundwater (Aggarwal *et al.*, 2006). Long estimated half lives of TCE (i.e., months to years) (ATSDR, 2004; HSDB, 2012) suggest TCE groundwater contamination will exist for long periods of time. Although extensive research has been completed over the past 3 decades concerning TCE remediation, contamination of groundwater resulting from use of the compound remains a significant problem (Schaumburg, 1990; Shen & Wilson, 2007).

1.2 Exposure and environmental effects of TCE

Trichloroethylene contamination is of great concern because the compound is a known human carcinogen (USEPA, 2012). Exposure to high doses of TCE affects the kidneys and lungs and can result in central nervous system depression, liver changes and cardiac arrhythmias (ATSDR, 2004). The Safe Drinking Water Act Amendments of 1986 established regulation of TCE in drinking water with a maximum contamination level (MCL) of 5 parts per billion (ppb) (USEPA, 2004).

TCE is heavier than water and when released to the subsurface in waste water or groundwater, it sinks down in an aquifer in the form of DNAPL (dense non aqueous phase liquid), when high volumes of TCE are released at once. Remediation becomes difficult once DNAPLs migrate into groundwater aquifers. Fortunately, it has been observed from the previous studies that TCE can be degraded chemically and biologically under aerobic, anaerobic and cometabolic conditions (Castro & Belser, 1990; Maymo-Gatell *et al.*, 1997; McCarty, 1997; Glod *et al.*, 1997; O'Loughlin & Burris, 1999; Inguva & Shreve, 1999; Arnold & Roberts, 2000).

1.3 Current treatment methods and issues

Ex situ and *in situ* remediation efforts with various alternative treatment technologies for TCE have been reported for both remediation of soils and groundwater. Detailed in the next two sections are a high level summary of available *ex situ* and *in situ* remediation technologies.

1.3.1 *Ex situ* TCE remediation technologies

Current water treatment plant operations such as coagulation, sedimentation, precipitation, softening, filtration and chlorination are ineffective at reducing the concentration of TCE to meet MCL standards set by the EPA. Although physical and chemical methods of soil and groundwater remediation, including soil venting, air sparging, air stripping, pump and treat techniques, granular activated carbon (GAC) adsorption have been considered as effective methods to reduce TCE levels in water (Russell *et al.*, 1992; Sijkema *et al.*, 1999); they may not be selected for a variety of reasons (e.g., cost, site complexity, remediation goals). The combination of air stripping and granular activated carbon adsorption of TCE from groundwater has become a popular and effective treatment technology due to lower construction costs of the air stripper and increased life expectancy of the adsorbent (Russell *et al.*, 1992).

1.3.2 *In situ* TCE remediation technologies

Potential *in situ* TCE remediation technologies include, abiotic oxidation and reduction and biological degradation (e.g., *in situ* bioremediation). Increasing interest has been growing in biological processes because the indigenous microbes or introduced microbes in subsurface environments have the potential of converting TCE to harmless products (i.e., ethane, ethene, chlorine) rather than transferring them from one part of the environment to another (e.g., air stripping). Removal of TCE through bioremediation using microorganisms has become an established technology and is considered as a viable remediation option. Biological degradation of TCE has been demonstrated under aerobic and anaerobic conditions (Bouwer & McCarty, 1983; Oldenhuis *et al.*, 1989). When biodegradation of TCE by indigenous microbes is not occurring, *in situ* application of microbes such as *Dehalococcoides mccartyi* sp. (recently renamed from *Dehalococcoides ethenogenes* sp.) (Löffler F.E. *et al.*, 2012), in a remediation approach called bioaugmentation, results in distribution of microorganisms that have the capacity to degrade the chlorinated compounds to non toxic forms in the contaminated area of concern (Russell *et al.*, 1992; Tyagi *et al.*, 2011). Bioaugmentation has been most widely applied for microbial degradation of TCE to ethane and ethene under anaerobic reductive dechlorination

conditions (Vogel *et al.*, 1987) and is the focus of this research. The other major TCE biodegradation approach involves aerobic cometabolism.

The microorganisms associated with or involved in bioremediation of TCE in natural or engineered remediation approaches including methanogens (organisms within the domain Archaea), sulfate reducing bacteria (SRB) and *Dehalococcoides* sp. (DHC) are of particular interest in this study. In a mixed culture, such as that found in groundwater undergoing bioremediation, methanogens, SRB and DHC have to compete with one another for complete reduction of TCE when complex food substrates (e.g. whey powder, sodium lactate, vegetable oil) are provided in a remediation approach called biostimulation.

DNAPL migration in an aquifer depends on the DNAPL's chemical properties and the nature and geological structure within the aquifer. Remediation of chlorinated solvent sites are complex, controversial, expensive and time consuming (Stroo & Ward, 2010) because of DNAPL's likelihood to migrate a large distance and create large dissolved phase groundwater plumes. *In situ* remediation has a wide range of challenges and problems. Previous research indicated the challenges encountered could include geology and hydrology or the location and distribution of chlorinated solvent contamination, as well as unique chemical and biochemical changes (Simkin & Norris 2010). The major problems associated with the biostimulation and bioremediation studied in this research are:

1. Cometabolic mechanism: Physical, chemical and biological processes that occur during remediation.
2. Spatial variability: The spatial variability in the distribution of microorganisms (DHC, SRB and Archaea) and limited amount of spatial information on the relationship between TCE and microorganisms with distance from food source.
3. Fractured clay or bedrock: The dispersion of TCE over large volume and diffusion of TCE in the inaccessible or low permeability zones such as fractured clay or bedrock where TCE can become trapped posing a difficulty for treatment.

The scope of this study was to determine the spatial distribution of selected microorganisms capable of TCE reductive dechlorination with respect to other microbes that compete for food

sources (e.g., SRB and Archaea). Concentrations of Archaea were monitored and used as surrogate for methanogens. Several bench scale models of different aquifer types (e.g., sandy, clay fracture and silt aquifers) were used to evaluate the spatial distribution, survival, growth and competition of microorganisms associated with TCE degradation *in situ* with distance from co-disposed food and TCE.

1.4 Research objectives and hypotheses

The overall objective of this study is to examine the spatial relationship between *Dehalococcoides* sp., Archaea and SRB with respect to TCE and reducing equivalents (e.g., food) in different types of aquifers contaminated with TCE.

The hypotheses that were tested include:

1. Increasing TCE concentrations influence the concentration and distribution of DHC, SRB and Archaea with distance from the TCE
2. The concentrations of DHC, SRB and Archaea decrease in concentration with increasing distance from the source of reducing equivalents (i.e., food),
3. After bioaugmentation DHC, SRB and Archaea concentrations increase due to microbial growth.

Studies were designed to test the hypotheses presented above and experiments were conducted to (1) quantify the abundance of DHC, SRB, and Archaea by quantitative polymerase chain reaction (qPCR) and fluorescent *in situ* hybridization (FISH); (2) observe the spatial relationship between the selected microorganisms with distance from the substrate (food); and (3) determine the concentration of these microbes at low and high concentration of TCE and sulfate. Three types of reactors, each modeling different hydrogeological conditions were constructed and used to address the hypotheses presented above.

1.5 Significance of the research

The results of this study can help in the design of remediation systems and the timing of *in situ* bioaugmentation of contaminated aquifers. Specifically, understanding the spatial relationship between *Dehalococcoides* sp. and various microorganisms that compete for bioremediation substrates supplied to stimulate biodegradation of this toxic compound will aid environmental engineers during the design of remediation systems. It can be helpful to understand when and where to bioaugment an aquifer undergoing bioremediation of TCE with *Dehalococcoides* sp. It can contribute to the improvement of engineered and natural processes applied to *in situ* bioremediation of TCE contaminated sites (sandy, clay and silt types of aquifer) and to improve the implementation of techniques such as biostimulation and bioaugmentation.

The research conducted to address the hypothesis presented in section 1.4 is presented in the following chapters. Specifically, a general introduction to TCE as an environmental contaminant; available physical, chemical and biological treatment methods; redox potential with electron donor and electron acceptor; selected microorganisms in dechlorination process; and molecular screening techniques; is presented in Chapter 2. The materials and methods used to develop experiments addressing the hypothesis are presented in Chapter 3. The results of the experiments addressing the hypotheses and a discussion of the results in the context of the current literature are presented in Chapter 4 and Chapter 5. Included in Chapter 6 is the conclusion made from the study and the recommendations for future work. Finally, a tabulation of additional data from the studies is presented in the Appendix.

CHAPTER 2 LITERATURE REVIEW

2.1 Trichloroethylene production, use, properties and treatment

Trichloroethylene is a chlorinated aliphatic hydrocarbon with the chemical formula C_2HCl_3 (Figure 2.1). It is a non flammable colorless liquid with sweet odor (ATSDR, 1997). The toxic solvent has been widely used in metal degreasing, solvent extraction processes, textile cleaning and as a carrier solvent (WHO, 1984; ATSDR, 1997). Trichloroethylene is also used in chemical production as intermediate, as a refrigerant and in some consumer products like typewriter correction fluid, paint removers, adhesives, spot removers and rug-cleaning fluids (ATSDR, 1997). In the past, it was used as a general anesthetic (ATSDR, 1997). The improper use and disposal in the past has contributed widespread distribution of this toxic and carcinogenic compound in the subsurface as well as soil and groundwater contamination (ATSDR, 1997; Moran *et al.*, 2007). According to the 2007 report by the Agency for Toxic Substances and Disease Registry, TCE contamination was found in at least 1,022 of 1,689 National Priorities List (NPL) sites. The report also estimated from various studies that between 9 and 34 percent of the drinking water supply sources in US may have TCE contamination (ATSDR, 2007).

Figure 2.1. Chemical Structure of TCE

TCE contamination in the environment is of great concern because it is a human carcinogen (Lash *et al.*, 2000; EPA, 2012). Exposure to high doses of TCE affects kidneys and lungs (ATSDR, 2004) and can result in central nervous system depression, liver changes and cardiac arrhythmias (ATSDR, 1997). Safe Drinking Water Act Amendments of 1986 stipulate the maximum contamination level (MCL) of TCE in drinking water is 5 parts per billion ppb (USEPA, 2004).

2.1.1 Physical and chemical properties:

Pure TCE has high aqueous solubility and higher density than water (Table 2.1). Therefore, when released into the subsurface environment in large quantities, it migrates downward in an aquifer due to gravitational force as a DNAPL. Water insoluble compounds tend to partition into another phase for example, volatilizing or adsorbing to organic material. The octanol-water partition coefficient (K_{ow}) is the ratio of a chemical's concentration in the octanol phase to its concentration in the aqueous phase. Log K_{ow} value for TCE is less than 3 (Table 2.1) suggesting this compound does not sorb strongly to the soil (McCarty, 2010). Because of its low solubility and high affinity for soil organic matter, TCE tends to be retained in the soil and slowly released into groundwater (Burris *et al.*, 1995; Mohammad & Kibbey, 2005). By means of dissolution, advection and dispersion, it moves between soil and water and can create large groundwater plumes (Russell *et al.*, 1992). TCE is made up of three chlorine atoms with carbon-carbon double bond, so it is a highly oxidized compound and resists further oxidation (Russell *et al.*, 1992).

Table 2.1. Physical and chemical properties of trichloroethylene

Physical or chemical parameter	Physical or chemical property value	References
Other Names	Acetylene trichloride, ethynyltrichloride, ethylene trichloride, 1-chloro-2,2-dichloroethylene, TCE, 1,1-dichloro-2-chloroethylene, 1,1,2-trichloroethylene, Trichloroethene	ATSDR, 1997
Molecular weight	131.40	Russell <i>et al.</i> , 1992; ATSDR, 1997
Odor	Chloroform like, sweet	ATSDR, 1997
Color	Clear, colorless	ATSDR, 1997
Physical state	Liquid at room temperature	ATSDR, 1997
Density at 20 °C	1.465g/ml	Russell <i>et al.</i> , 1992; ATSDR, 1997

Boiling Point	86.7 °C	Russell <i>et al.</i> , 1992; ATSDR, 1997
Water Solubility	at 25 °C: 1.366 g/L	ATSDR, 1997
Partition coefficient: log K _{ow}	2.42	Russell <i>et al.</i> , 1992; ATSDR, 1997
log K _{oc}	2.03-2.66	
Henry's Law Constant	0.011 atm-m ³ /mol @ 25° C	ATSDR, 1997

2.2 Physical and chemical TCE treatment technologies

For TCE contaminated soil and groundwater remediation, there are two ways for TCE removal in the environment: abiotic and biotic. In abiotic methods TCE is reduced or oxidized through chemical and physical means while the biotic removal methods emphasize the use of microorganisms in contaminated soil and water. Chemical, physical and biological processes under aerobic, anaerobic and cometabolic conditions have been shown to degrade TCE (Castro & Belser, 1990; Maymo-Gatell *et al.*, 1997; McCarty, 1997; Glod *et al.*, 1997; O'Loughlin *et al.*, 1999; Inguva & Shreve, 1999; Arnold & Roberts, 2000).

Physical and chemical methods of treatment and remediation of surface and subsurface water include soil venting, air sparging, air stripping, pump and treat techniques, and granular activated carbon (GAC) adsorption. They have been considered as the effective methods to reduce TCE levels in water ((Russell *et al.*, 1992; Sijkema *et al.*, 1999) but they may not be selected for a variety of reasons. For example, air stripping needs large volumes of air, shifts the contaminant to another medium (atmosphere) and requires large environmental remediation costs (Russell *et al.*, 1992); granular activated carbon (GAC) do not fully remove the contaminant volume or toxicity (Nelson & Bourquin, 1990; Russell *et al.*, 1992) and soil venting technology might cause waste mixture to the soil surface and might be need treatment of off-gases to maintain air quality standards (Russell *et al.*, 1992). The combination of air stripping and granular activated carbon adsorption has become popular and effective treatment technologies due to lower construction costs of the air stripper and increase life expectancy of the adsorbent (Russell *et al.*, 1992).

Another method of TCE remediation in groundwater is chemical reductive dechlorination in which a chlorinated hydrocarbon is reduced by a reactive compound, such as metal sulfides, that can degrade TCE (Butler & Hayes, 1999; Lee & Batchelor, 2003). In this case, an anaerobic environment may be created and the substrate addition may indirectly cause and sustain abiotic reductive dechlorination.

2.3 Biological TCE treatment

Biological remediation of TCE via *in situ* application of microorganisms (i.e., bioaugmentation) or encouragement of the growth of the native TCE degrading microorganisms that have the capacity to degrade the chlorinated compounds to non toxic forms is a feasible, low cost alternative to physical and abiotic methods (Russell *et al.*, 1992; Tyagi *et al.*, 2011).

Bioremediation is an attractive technology to physical and chemical remediation technologies because certain microorganisms (i.e., *Dehalococcoides mccartyi* sp) (Löffler F.E. *et al.*, 2012) can completely degrade TCE to CO₂, water, and chlorine instead of simply a transfer from one medium to another (Russell *et al.*, 1992). Enzymes and cofactors produced during biological degradation enhance the reaction rate. Different types of naturally occurring bacteria enzymatically reduce chlorine atoms to chloride and nontoxic metabolites (Field & Sierra-Alvarez, 2004). The most relevant microbial degradation processes occurring during bioremediation of TCE include (1) aerobic oxidation, (2) direct anaerobic reductive dechlorination and (3) reductive cometabolism. There are two reactions in the anaerobic reductive bioremediation process: direct anaerobic reductive dechlorination and cometabolic anaerobic reductive dechlorination.

Cometabolism is a process in which the chlorinated compound is reduced by a non-specific enzyme or co factor produced during microbial metabolism of another compound (primary substrate) in an anaerobic environment but does not yield any energy or growth benefit for the microbe mediating the reaction (USEPA, 2000). A special type of co-metabolism involves the oxidation of chlorinated solvents by various oxygenase enzymes, which is known as co-oxidation (Alvarez-Cohen & Speitel, 2001). Under anaerobic conditions, a common form of co-

metabolism is the reaction of reduced enzyme cofactors with chlorinated solvents, resulting in their reductive dehalogenation (Krone *et al.*, 1989; Gantzer & Wackett, 1991). These organisms and their biological degradation mechanisms are discussed in the sections below. Anaerobic reductive dechlorination and/or reductive cometabolism are the methods that were utilized in these studies.

2.3.1 Aerobic oxidation of TCE

Through aerobic oxidation, highly chlorinated VOCs like carbon tetrachloride, penta- and hexachloroethane, and PCE are not bio-oxidizable (Nelson *et al.*, 1988; Wackett & Gibson, 1988; Tsien *et al.*, 1989; Vannelli *et al.*, 1990; Field & Sierra-Alvarez, 2004). With increasing number of chlorine atoms or carbon-chlorine bonds, the microbial degradation rate of chlorinated VOC decreases. Highly chlorinated species such as tetrachloroethylene (PCE) degrade anaerobically more rapidly, whereas less chlorinated species such as dichloroethylene (DCE) are more rapidly degraded by aerobic processes and may accumulated in anaerobic environments (Lee *et al.*, 1998).

Aerobic co-oxidation is a widespread process with great importance for bioremediation. Microorganisms (e.g., *Pseudomonas* sp, *Methylococcus* sp) use chlorinated solvents as electron donors and carbon sources to support growth, or they cometabolically transform them while degrading other substrates, a process known as co-oxidation (Field & Sierra-Alvarez, 2004). TCE or higher chlorinated ethenes can be degraded by aerobic co-oxidation mechanisms (Wackett *et al.*, 1994; Alvarez-Cohen & Speitel, 2001). Aerobic degradation of TCE, DCEs and vinyl chloride (VC) involves oxygenase enzymes (Nelson *et al.*, 1986; Field & Sierra-Alvarez, 2004). These enzymes are present in a large number of microbial species such as toluene, methane, or ammonia oxidizing bacteria (Nelson *et al.*, 1988; Wackett & Gibson, 1988; Tsien *et al.*, 1989; Vannelli *et al.*, 1990; Russell *et al.*, 1992; Alvarez-Cohen & Speitel, 2001); (Nelson *et al.*, 1986; Coleman *et al.*, 2002). Suitable primary substrates for energy and/or carbon sources for these microorganisms include methane, ethane, ethene, propane, propene, ammonia, toluene and phenol (Wilson & Wilson, 1985; Nelson *et al.*, 1986; Folsom *et al.*, 1990; Vannelli *et al.*, 1990; Semprini, 1997; Van Agteren *et al.*, 1998; Alvarez-Cohen & Speitel, 2001; Arp *et al.*, 2001). In

this case, there is no formation of vinyl chloride and complete mineralization takes place (Russell *et al.*, 1992). The most studied organism capable of TCE co-oxidation is the methane co-oxidising bacteria *Methylosinus trichosporium* that produce the enzyme methane monooxygenase (MMO) (Russell *et al.*, 1992). Methanotrophs, under aerobic conditions utilize methane (CH₄) as their primary substrate and cometabolically biodegrade chlorinated solvents. Methanotrophs are generally most active at the interface between aerobic and anaerobic zones (Lorah & Voytek, 2004). The methane produced under anaerobic conditions is utilized by methanotrophs to oxidize TCE in aerobic zones and form epoxides that are chemically unstable and can be transformed rapidly by abiotic hydrolysis to nonvolatile products, including aldehydes and acids (Lorah & Voytek, 2004).

2.3.2 Anaerobic reductive dechlorination

Anaerobic reductive dechlorination is one of the most common and most efficient bioremediation approaches for TCE. Ground water can be limited in oxygen, so biological treatment of TCE by reductive dehalogenation can be a very effective biological process for degradation of highly chlorinated compounds in environments that are not susceptible to oxidative transformation.

Microorganisms have demonstrated degradation of TCE by sequential replacement of chlorine atoms of TCE via reductive dehalogenation under anaerobic conditions (Freedman & Gossett, 1989; DiStefano *et al.*, 1991; Mohn & Tiedje, 1992; Maymo-Gatell *et al.*, 1997; McCarty, 1997; Rosner *et al.*, 1997; Kengen *et al.*, 1999). Reductive dehalogenation is the mechanism yielding ethene and chloride in anaerobic environments where organic electron donors or hydrogen are present. In the mechanism, one electron and one hydrogen atom are transferred onto the carbon atom and HCl is released (Holliger *et al.*, 2004). Large chlorinated solvents such as PCE can be dehalogenated to less chlorinated forms by microorganisms under anaerobic conditions by sequential replacement of chlorine atoms by hydrogen atoms as shown in Figure 2.2 (Freedman & Gossett, 1989; DiStefano *et al.*, 1991; Vogel *et al.*, 1994; McCarty, 1997; Maymo-Gatell *et al.*, 1997).

Figure 2.2. Sequential reductive dechlorination of trichloroethylene under anaerobic condition

Microorganisms from several phylogenetic groups (Table 2.2) are able to reduce PCE to TCE, and TCE to cis-1,2-dichloroethene (cis-DCE) (Neumann *et al.*, 1995; Schumacher *et al.*, 1997; Sung *et al.*, 2003) which reduces to VC and finally VC to non toxic ethane (Freedman & Gossett, 1989; Maymo-Gatell *et al.*, 1997; McCarty, 1997; Kengen *et al.*, 1999; Morrill *et al.*, 2005) (Figure 2.2). Dechlorination of TCE to cis-DCE is a relatively fast process but is significantly slower as number of chlorine decreases (step from VC to ethene) (Maymó-Gatell *et al.*, 1999; Middeldorp *et al.*, 1999). The intermediate product VC is more toxic than TCE (Pill *et al.*, 1991). VC is a known human carcinogen (Russell *et al.*, 1992).

Different phylogenetically diverse microorganisms use TCE as a terminal electron acceptor and gain energy from reductive dechlorination (Freedman & Gossett, 1989; Holliger *et al.*, 1993; Sharma & McCarty, 1996; McCarty, 1997) (Table 2.2), including dehalorespiring (Schumacher *et al.*, 1997; Maymó-Gatell *et al.*, 1995; Scholz-Muramatsu *et al.*, 1995; Sun *et al.*, 2002; Grostern & Edwards, 2006; Grostern *et al.*, 2009), sulfate-reducing bacteria (Wrenn & Rittmann, 1996) and methanogens (Vogel *et al.*, 1987). However, only the members of the genus *Dehalococcoides mccartyi* sp. (recently renamed from *Dehalococcoides ethenogenes* (Löffler F.E. *et al.*, 2012) is able to degrade toxic chlorinated compound to the environmentally acceptable final products of chloride and ethane or ethene (Table 2.2) through sequential reductive dechlorination in presence of appropriate electron donors (e.g., lactate), and nutrients under anaerobic conditions (Sung *et al.*, ; Maymo-Gatell *et al.*, 1997; Richardson *et al.*, 2002; Cupples *et al.*, 2003; He *et al.*, 2003a, Freedman & Gossett, 1989; Holliger *et al.*, 1993).

Table 2.2. Dechlorinating bacteria using TCE as an electron acceptor

Microorganisms	Species	Dechlorination products	References
Dehalorespiring bacteria	<i>Dehalospirillum multivorans</i>	TCE-→ <i>cis</i> -DCE	Scholz-Muramatsu <i>et al.</i> , 1995
	<i>Dehalobacter restrictus</i>	<i>cis</i> -DCE	Holliger <i>et al.</i> , 1998
	<i>Clostridium bifermentans</i>	<i>cis</i> -DCE	Chang <i>et al.</i> , 2000
	<i>Dehalococcoides mccartyi</i>	TCE→ <i>cis</i> -DCE→VC→Ethene	Löffler F.E. <i>et al.</i> , 2012
Methanogens	<i>Methanosarcina</i> sp.	TCE-→ <i>cis</i> -DCE→VC	Fathepure 1987, 1988,
Sulfate reducing bacteria	<i>Desulfomonile tiedjei</i>	TCE-→ <i>cis</i> -DCE→VC	Fathepure 1987, Freedman 1989, Cole 1995
	<i>Desulfuromonas</i> sp.	TCE-→ <i>cis</i> -DCE	Löffler 2003;He 2003, Sung <i>et al</i> 2003
Iron reducing bacteria	<i>Geobacter</i> sp.	TCE-→ <i>cis</i> -DCE	Duhamel 2002, 2007
TCE = Trichloroethylene			
<i>cis</i> -DCE = <i>cis</i> -Dichloroethylene			
VC =Vinyl chloride			

In dehalorespiration, TCE serves as terminal electron acceptor while hydrogen supplied by fermentation of organic substrates serves as the direct electron donor (DiStefano *et al.*, 1992).

2.3.3 Reductive co-metabolism

Microorganisms that do not gain energy from degradation of contaminants; facilitate a process called “cometabolism” (Alvarez Cohen 1991). They cometabolically transform chlorinated compounds while they are degrading non-halogenated primary substrates. Slow reductive dechlorination of chloroethenes and other haloorganic compounds is a cometabolic process facilitated by hydrocarbon-degrading bacteria (Alvarez Cohen 1991; Ensley 1991; Semprini 1997). The last transformation step from VC to ethene occurs slowly in a cometabolic process (Holliger *et al.*, 2006). Cometabolic reductive dechlorination can occur with the help of cofactors, such as vitamin B12 and coenzyme that catalyzes the replacement of chlorines by hydrogen atoms (Fathepure & Boyd, 1988). Current literature suggests that anaerobic reductive dechlorination is carried out by only a few classifications of microorganisms, including certain species of methanogens, sulfate-reducing bacteria (Bagley DM, Gossett JM. 1989; Chapelle *et al.*, 1993; Bradley & Chapelle, 1996) and dehalorespiring bacteria (Maymo-Gatell *et al.*, 1997). These types of bacteria do not precede energy-yielding reaction but are co-metabolic (Lee *et al.*, 1998). The reactive co-enzymes are typically found more commonly in methanogens (Fathepure & Boyd, 1988; Maymo-Gatell *et al.*, 1997; Yager *et al.*, 1997).

Since TCE is not a growth substrate, it can be degraded via co-metabolism in which an oxygenase, the corresponding enzyme for initiating growth substrate oxidation, fortuitously transforms TCE (Folsom *et al.*, 1990; Arcangeli & Arvin, 1997). Methanogens and sulfate reducing bacteria typically have slower TCE degradation rates than DHC (Bagley DM, Gossett JM. 1989).

Reductive cometabolism under anaerobic conditions has been observed for groundwater contaminants such as chloroform, carbon tetrachloride and all chlorinated ethanes, mainly by methanogenic and acetogenic mixed cultures, and by sulfate and iron reducers (Egli *et al.*, 1987; Egli *et al.*, 1988; Chen *et al.*, 1996). Slow conversion of TCE by methanogens and acetogenes

with H₂ as the electron donor has been observed (Fathepure *et al.*, 1987; Egli *et al.*, 1988; Fathepure & Boyd, 1988). Fathepure & Boyd, 1988 suggested in his study that the dechlorination of chloroethylenes *in situ* could be enhanced simply by stimulating methanogenesis. The reductive dechlorination of TCE to cis- DCE by sulfate reducing bacteria has been reported by Bagley and Gossett. Dechlorinating capability and rate of a sulfate-reducing enrichment is slower than in mixed methanogenic enrichments (Bagley DM, Gossett JM. 1989). Chapelle *et al.*, 1996 in the site study found that reductive dehalogenation was mostly favored in methanogenic and sulfate-reducing zones. Fathepure and Boyd (1987) suggested that PCE dechlorination by *Methanosarcina* sp. is a cometabolic process.

In an anaerobic environment, *Dehalococcoides* coexist with sulfate reducing bacteria, acetogens and methanogens, who all compete for available hydrogen. However these other microorganisms might also assist DHC directly by providing necessary micronutrients to dechlorinators during the dechlorination process (Duhamel et al 2007, Yager 1997). The hydrogen and acetate produced from the transformation of electron-donating substrates during fermentation have been shown to be used by *Dehalococcoides* (Yang & McCarty, 1998; Duhamel & Edwards, 2007). At lower concentrations of the hydrogen, for anaerobic reductive dechlorination to occur, dechlorinators must successfully compete against other microorganisms that also utilize hydrogen (Yang & McCarty, 1998). The extent of dechlorination has been shown to increase as the amount of primary carbon substrate increases (Fathepure & Boyd 1988). Without an electron donor, reductive dechlorination cannot be sustained (Fathepure & Boyd, 1988; Freedman & Gossett, 1989). VC, the toxic intermediate, is cometabolically reduced by *Dehalococcoides ethenogenes* (Maymó-Gatell *et al.*, 2001).

2.4 Electron acceptor/electron donor and redox potential

Hydrogen produced by fermentation is often the primary electron donor for dehalogenation (DiStefano *et al.*, 1992; Smatlak *et al.*, 1996; Fennell *et al.*, 1997). Dehalogenating bacteria compete for hydrogen with other hydrogen-consuming microorganisms in natural aquatic sediment systems such as Fe(III)-reducing, Mn(II)-reducing, nitrate-reducing, sulfate-reducing, and methanogenic bacteria during anaerobic condition (Chapelle *et al.*, 1993; Smatlak *et al.*,

1996; Yang & McCarty, 1998). Methanogens coexist with sulfate reducing bacteria in most environmental systems depending on rate of hydrogen production and availability of sulfate (Lovley *et al.*, 1982). Although fermentation products such as acetate have been identified as direct electron donors, the microorganisms require hydrogen as the electron donor for complete dechlorination (Maymo-Gatell *et al.*, 1997). So, hydrogen may be the most important electron donor for anaerobic dechlorination. The hydrogen and acetate produced from the transformation of electron-donating substrates during fermentation are used by *Dehalococcoides* for dechlorination (Yang & McCarty, 1998; Duhamel & Edwards, 2007).

Lactate in the form of sodium lactate is commonly used as a substrate for anaerobic dechlorination in field bioremediation application and was utilized in this study. Sodium lactate disassociates to form lactate and a sodium ion as follows:

The lactate molecule can then be fermented by more than one process shown in Figure 2.3.

Figure 2.3. Flow diagram of fermentation pathway

The following reactions show the steps for the production of hydrogen through microbial degradation of lactate and its associated fermentation products (Macbeth *et al.*, 2004). The first step of the lactate degradation pathway is shown in Equation 2.

Bicarbonate ion and a hydrogen ion may form carbon dioxide and water:

Combining equations (1) and (2), the fermentation of lactate to acetate and hydrogen can be written as the following balanced reaction:

The acetate produced in this reaction may be used directly as an electron donor for reduction reactions or may be further fermented to produce hydrogen.

In the propionate pathway (second step), lactate degrades to propionate ($\text{CH}_3\text{CH}_2\text{COO}^-$), acetate, and carbonate (Equation 5). Further, propionate degrades to acetate (Equation 6) and the acetate is further oxidized to carbonate and hydrogen under extremely reducing conditions (Equation 7) (Macbeth *et al.*, 2004).

Hydrogen produced by fermentative organisms from Equations 2, 4, 6, and 7 is rapidly consumed by other bacteria, including SRB, methanogens, and dechlorinating microorganisms. The overall reactions for dehalogenation, sulfate reduction and methanogens are in Table 2.3. For example, in an anaerobic reductive dechlorination, molecular hydrogen (H_2) disassociates in the following half reaction:

Then, TCE is reduced by the substitution of a chloride ion with a hydrogen ion and the transfer of two electrons:

The dechlorination of TCE using hydrogen as the electron donor can be written as follows:

In these reactions, two electrons are transferred with molecular hydrogen (H_2) as the electron donor (which is oxidized) and the chlorinated molecule as the electron acceptor (which is reduced).

In anaerobic environments that have a low redox potential; anaerobes including fermentative bacteria, acetogenic bacteria, SRB, dehalogenating bacteria and methanogens that grow on lactate, propionate and butyrate, compete for the available common substrates (H_2). Typical redox half-reactions in an anaerobic mixed culture are listed in Table 2.3 with theoretical stoichiometric reactions describing the complete biodecomposition of compounds under methanogenic conditions.

Table 2.3. Typical redox half-reactions in anaerobic mixed culture with Gibbs free energy
(Thauer *et al.*, 1977; Dolfig & Janssen, 1994 and Zeikus, 1977)

Equation	ΔG KJ/Reaction
Acetogenic reactions	
$\text{CH}_3\text{CHOHCOO}^- + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{HCO}_3^- + 2\text{H}_2 + \text{H}^+$	-4.2
$\text{CH}_3\text{CHOHCOO}^- \rightarrow \text{CH}_3\text{CH}_2\text{COO}^- + \text{CH}_3\text{COO}^- + \text{HCO}_3^- + \text{H}^+$	-992.2
$\text{CH}_3\text{CH}_2\text{COO}^- + 3\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{HCO}_3^- + 3\text{H}_2 + \text{H}^+$	+76.1
$\text{CH}_3\text{CH}_2\text{CH}_2\text{COO}^- + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COO}^- + 2\text{H}_2 + \text{H}^+$	+48.1
$\text{CH}_3\text{COO}^- + 4\text{H}_2\text{O} \rightarrow 2\text{HCO}_3^- + 4\text{H}_2 + \text{H}^+$	+104.6
Methanogenic reactions	
$\text{CH}_3\text{COO}^- + \text{H}_2\text{O} \rightarrow \text{HCO}_3^- + \text{CH}_4$	-31
$\text{HCO}_3^- + 4\text{H}_2 + \text{H}^+ \rightarrow \text{CH}_4 + 3\text{H}_2\text{O}$	-136
$4\text{HCO}_2^- + 4\text{H}^+ \rightarrow \text{CH}_4 + 3\text{CO}_2 + 2\text{H}_2\text{O}$	-145
$4\text{CH}_3\text{OH} + 2\text{H}_2\text{O} \rightarrow 3\text{CH}_4 + \text{CO}_2 + 4\text{H}_2\text{O}$	-106.5
$\text{CH}_3\text{OH} + \text{H}_2 \rightarrow \text{CH}_4 + \text{H}_2\text{O}$	-112.5
$\text{CH}_3\text{COO}^- + \text{H}^+ \rightarrow \text{CH}_4 + \text{CO}_2$	-36
Sulfate reducing reactions	
$\text{CH}_3\text{COO}^- + \text{SO}_4^{2-} \rightarrow 2\text{HCO}_3^- + \text{HS}^-$	651.6
$\text{HS}^- + \text{H}^+ \rightarrow \text{H}_2\text{S}$	-5.19
$\text{SO}_4^{2-} + 4\text{H}_2 \rightarrow \text{H}_2\text{S} + 2\text{H}_2\text{O} + 2\text{OH}^-$	237.2
$\text{SO}_4^{2-} + 8\text{e}^- + 10\text{H}^+ \rightarrow \text{H}_2\text{S} + 4\text{H}_2\text{O}$	225
$\text{SO}_4^{2-} + 4\text{H}_2 + \text{H}^+ \rightarrow \text{HS}^- + 4\text{H}_2\text{O}$	-152
$\text{SO}_4^{2-} + 8\text{e}^- + 9\text{H}^+ \rightarrow \text{HS}^- + 4\text{H}_2\text{O}$	164
$\text{CH}_3\text{COO}^- + \text{SO}_4^{2-} \rightarrow 2\text{HCO}_3^- + \text{HS}^-$	-47.6
$\text{CH}_3\text{CH}_2\text{COO}^- + 0.75\text{SO}_4^{2-} \rightarrow \text{CH}_3\text{COO}^- + \text{HCO}_3^- + 0.75\text{HS}^- + 0.25\text{H}^+$	-37.7
$\text{CH}_3\text{CH}_2\text{CH}_2\text{COO}^- + 0.5\text{SO}_4^{2-} \rightarrow 2\text{CH}_3\text{COO}^- + 0.5\text{HS}^- + 0.5\text{H}^+$	-27.8
$\text{CH}_3\text{CHOHCOO}^- + 0.5\text{SO}_4^{2-} \rightarrow \text{CH}_3\text{COO}^- + \text{HCO}_3^- + 0.5\text{HS}^-$	-80.2
Dehalogenating reactions	
$\text{HC}_2\text{Cl}_3 + \text{H}_2 \rightarrow \text{H}_2\text{C}_2\text{Cl}_2 + \text{Cl}^- + \text{H}^+$	-168.6
$\text{HC}_2\text{Cl}_3 + 4\text{e}^- + 4\text{H}^+ \rightarrow \text{C}_2\text{H}_4 + 3\text{Cl}^- + \text{H}^+$	558
$\text{HC}_2\text{Cl}_3 + 2\text{H}_2 \rightarrow \text{H}_4\text{C}_2 + 3\text{Cl}^- + \text{H}^+$	-390.76

Smatlak *et al.*, 1996 suggested that the dechlorinators would successfully compete for hydrogen only at very low hydrogen concentrations. Minimizing methanogenic competition for the available hydrogen, dechlorination can be maximized by using organic substrates which ferment slowly, resulting in the steady release of low-levels of hydrogen (electron donor) over time (Smatlak *et al.*, 1996). Ballapragada *et al.*, 1997 concluded that dechlorinating bacteria compete with methanogens for hydrogen at low hydrogen concentrations of 9 to 50 nmol/L.

PCE and TCE have a higher redox potential so they are electron acceptors and yield more energy on their complete dechlorination to ethene (Vogel *et al.*, 1987). As shown in Figure 2.4, for the most rapid and extensive anaerobic reductive dechlorination to occur, redox conditions should be in the sulfate reducing to methanogenic range with the optimal hydrogen concentrations range from 2 nmol/L (mid-range of sulfate reduction) to 11 nmol/L (mid-range of methanogenesis) (Yang & McCarty, 1998). The range of estimated redox potential for dehalogenation ranges from approximately of 580 mV to -250 mV at a pH of 7 and a temperature of 25 °C.

Figure 2.4. Redox potential range for favorable dehalogenation, sulfate reduction and methanogenesis.

2.5 *Dehalococcoides* sp. (DHC)

Dehalococcoides sp. are small spherical or coccoid shaped microorganisms with a diameter of approximately 300-500 nanometers (He *et al.*, 2005), 1 μ m wide and 0.1-0.2 μ m thick (Löffler F.E. *et al.*, 2012). They use chlorinated carbons as the terminal electron acceptor, acetate as a carbon source and hydrogen as the electron donor which may be produced by fermentation of organic substrates such as sodium lactate. *Dehalococcoides* spp. has also been shown to dechlorinate chlorobenzenes, chloronaphthalenes, polychlorinated biphenyls, and dibenzodioxins (Adrian *et al.*, 2000, Fennell *et al.*, 2004, He *et al.*, 2003b). DHC gets H_2 from other neighboring microorganisms like acetogens and fermenting bacteria. Oxygen is toxic to them (Sharma & McCarty, 1996; McCarty, 1997). *Dehalococcoides* strains grow slowly with doubling times of 0.8–3 days (Maymo-Gatell *et al.*, 1997; Cupples *et al.*, 2003; He *et al.*, 2003a; Duhamel *et al.*,

2004) and the yield per μmole of chloride released is 6.3×10^7 to 3.1×10^8 . Enzymes associated are reductive dehalogenases (RDases) and hydrogenase (H_2ase).

Dehalococcoides bacteria belong to *Chloroflexi*, or green non-sulfur bacteria (Löffler *et al.*, 2004; Löffler F.E. *et al.*, 2012). The DHC strains shown to degrade TCE to date are shown in Table 2.4. Strain BAV1 is the only organism that reduces PCE to ethene through direct metabolism (Maymo-Gatell *et al.*, 1997; Maymó-Gatell *et al.*, 2001; He *et al.*, 2003a; He *et al.*, 2005) and Strain 195 and FL2 reduce PCE to VC and then VC is reduced by cometabolism (Maymo-Gatell *et al.*, 1997).

Table 2.4. DHC species documented to degrade chlorinated compounds

<i>Dehalococcoides</i> sp. strains	RDase functional gene	Contaminants degraded by DHC species	Major end products	References
Strain 195	<i>pceA</i> , <i>tceA</i>	PCE, TCE, <i>cis</i> -DCE	VC, ethene	Maymo-Gatell <i>et al.</i> , 1997
Strain CBDB1	<i>pceA</i>	Tetrachlorobenzene, Trichlorobenzene, Hexachlorobenzene,	Trichlorobenzene or dichlorobenzene	Adrian <i>et al.</i> , 2000; Jayachandran <i>et al.</i> , 2003
Strain BAV1	<i>bvcA</i>	DCE, VC	ethane, ethene, chloride	He <i>et al.</i> , 2003a
Strain FL2	<i>tceA</i>	TCE, DCE	VC, ethene	He <i>et al.</i> , 2005
Strain GT	<i>vcrA</i>	TCE, DCE, VC	ethene	Sung <i>et al.</i> ,
Strain VS	<i>vcrA</i>	TCE, <i>cis</i> -DCE, VC	ethene	Cupples <i>et al.</i> , 2003
Strain MB	-	PCE,TCE	<i>trans</i> -DCE	Cheng & He, 2009
KB-1/VC	-	PCE, TCE, <i>cis</i> -DCE, VC	ethene	Duhamel <i>et al.</i> , 2004

2.6 Sulfate reducing bacteria (SRB)

Dissimilatory microbial SO_4^{2-} reduction is an important metabolic activity in many reduced environments and is mediated by a metabolically diverse group of microorganisms: the sulfate-reducing bacteria (Odom *et al.*, 1993). Sulfate reducing bacteria are named for a class of microbes which conducts dissimilatory sulfate reduction and use sulfate as the terminal electron acceptor for growth (Postgate, 1979). The sulfate ion acts as oxidizing agent for the dissimilation of the organic matter (Postgate, 1979) while some species cannot use sulfate but reduce sulfur to sulfide (Hamilton, 1985). They are taxonomically diverse and physiology versatile (Barton & Hamilton, 2007). Organic acids such as acetate, propionate, and butyrate derived primarily from fermentation serve as carbon sources for SRB. However, SRB were found to grow on environmental contaminants such as petroleum hydrocarbon constituents and halogenated compounds (Ensley & Suflita, 1995). To date, 19 genera of SRB have been described (Rooney-Varga *et al.*, 1998). Dissimilatory sulfite reductase (DSR) is a key enzyme in dissimilatory sulfate reduction in SRB. DSR catalyses the reduction of sulfite to sulfide, a reaction found only in dissimilatory sulfate-reducing prokaryotes (Kondo *et al.*, 2004). Currently, functional DSR genes: *dsrAB* *dsrA* and *dsrB* with similar physiological characteristics have been used as targets in qPCR (Wagner *et al.*, 1998; Chang *et al.*, 2001; Joulain *et al.*, 2001; Kondo *et al.*, 2004).

2.7 Archaea/Methanogens

Methanogens are prokaryotic microorganisms that fall under Domain Archaea, produce methane as an endproduct of their metabolism and are divided into five major orders, *Methanobacteriales*, *Methanosarcinales*, *Methanococcales*, *Methanomicrobiales*, and *Methanopyrales* (Liu & Whitman, 2008). The cell morphologies may be sarcina, rods, spheres or coccids and spirals and are gram positive (Zeikus, 1977). Methanogens vary greatly in their morphological and physiological characteristics and are found in wide variety of anaerobic environments (Liu & Whitman, 2008). In contrast to sulfate reducing bacteria, methanogens use a limited number of substrates for growth. Hydrogen, carbon dioxide and acetate are the most important substrates which are provided by complex anaerobic bacterial communities through fermentation

(Hackstein, 2010). All methanogenic bacteria can use hydrogen as an energy source and utilize acetate, formate and CO₂ and/or other methyl group compounds such as methanol, methylthiols and methylamines as the substrates (Thauer 1998, Zeikus, 1977). Methanogens are abundant in habitats where electron acceptors such as O₂⁻, NO₃⁻, Fe³⁺, and SO₄²⁻ are limiting (Liu & Whitman, 2008).

Methanogenic Archaea belonging to the order *Methanobacteriales*, *Methanomicrobiales*, *Methanococcales*, and *Methanopyrales* use H₂ as substrate for growth and CH₄ production. Some of them are capable of using formate as well as H₂ to reduce CO₂ to CH₄ (Balch *et al.*, 1979). *Methanosarcinales* have the widest substrate range and are capable of metabolizing two or more substrates (Balch *et al.*, 1979, Zeikus, 1977). They use H₂ or acetate or methyl group compounds such as methanol, methylated amines, and methylated sulfides as substrate (Balch *et al.*, 1979, Thauer 1998, Zeikus, 1977). *Methanosarcina* species are difficult to isolate and maintain in pure culture (Zeikus, 1977). Methane production includes a complex biochemical pathway characterized by a number of unique coenzymes. Much of the biologically generated methane is derived from acetate in sediment and sludge digestors (Zeikus, 1977).

2.8 Molecular screening techniques

2.8.1 PCR/qPCR

Polymerase chain reaction (PCR) is a technique which generates millions of copies of genes exponentially from minute amount of deoxyribonucleic acid (DNA) in a short period of time (less than 3 hours). Even one copy of DNA sequence in a sample can be amplified and detected (Arya *et al.*, 2005). The PCR process involves a series of chemical reactions performed at various temperatures in the so-called PCR cycle. Each cycle consists of three steps. The first step is called denaturation, where doublestranded DNA in a sample is denatured or separated into single strands by heating the sample in a thermocycler at 94 to 96 °C. The second step is hybridization or annealing in which sample is cooled slightly by lowering the temperature to 50-60 °C. During this second step two types of synthetic primers are added to the denatured DNA to hybridize or bind with the complementary DNA sequence if present in a sample. Primers are short single stranded DNA oligonucleotides. These primers are complementary to either the 5' or

3' ends of target DNA. Once the primers are hybridized with the DNA; a specialized enzyme called taq polymerase is used to add free nucleotides (i.e., A, G, C and T representing adenine, guanine, cytosine and thymine respectively). Last step is extension; the temperature is increased up to 72°C. At this point any single stranded DNA sequences are filled in by the polymerase enzyme with the remaining free nucleotides in the chemical mixture. After one round of synthesis, the amount of sample DNA is doubled. The temperature is again increased to denature the recently formed DNA duplex. Denaturation (heating) followed by hybridization and synthesis (cooling) is repeated to create or “amplify” millions of copies of the original DNA sequence. In each cycle the number of copies is doubled so the target sequence increases exponentially up to two million copies (depending on the number of cycles performed).

Quantitative polymerase chain reaction (qPCR) technology allows one to determine the amount of DNA amplified in each cycle. Quantitative real-time PCR is a highly sensitive technique that can be used for detection and quantification of specific genes of microorganisms in a DNA extraction. Numbers of copies of DNA is considered a surrogate for the number of microorganisms in a sample. There are two mechanisms for qPCR detection, both of which are based on the measurement of the increase in fluorescence as the number of copies of a microorganisms gene increase during the PCR cycle. The first method, called TaqMan™ qPCR involves a dye-containing probe that emits fluorescent light as the reaction proceeds. The second method, called SYBR Green qPCR, involves the incorporation of a dye into double stranded DNA. The SYBR Green dye fluoresces brightly when bound to double stranded DNA and weakly when associated with single stranded DNA. In both types of qPCR, the amount of emitted fluorescence is proportional to the amount of PCR products. Quantitative PCR is subject to detection limits, and is not an absolute method to determine the presence of a targeted species.

The 16S rRNA gene, encoding the 16S small ribosomal subunit, has served as a highly suitable target for studying bacterial or Archaeal species. 16S rRNA serves as phylogenetic marker to detect diversity of Bacteria and Archaea. However, in the case where multiple different orders of microbes can do one function (e.g., sulfate reduction), use of 16S rRNA gene-targeted fingerprinting method is not possible, therefore specific genetic markers (i.e., functional genes) are targeted. The average 16S rRNA gene concentrations of the methanogenic Archaea from

wastewater anaerobic sludge in the literature ranged from 10^6 to 10^{10} copies/ml (Yu 2005). A range of 10^2 to 10^5 copies/ml 16S rRNA DHC gene were detected in a wastewater treatment plant anaerobic sludge in the study done by Loeffler, et al. (2012) and 10^3 to 10^5 DHC cells/ml in groundwater (Lu *et al.*, 2006 a). Similarly a range of 10^5 to 10^8 copies of *dsrA*/ml of nucleic acid extract from estuarine sediments was recorded by Kondo (2004). *Dehalococcoides* contains only one copy of the 16S rDNA gene on its chromosome (Freeborn *et al.*, 2005, <http://rrndb.mmg.msu.edu>) and Archaea ranged from 1 to 4 16S rDNA gene (<http://rrndb.mmg.msu.edu>). Therefore qPCR values reported as gene copies/ml are typically well correlated with numbers of cells/ml.

2.8.2 Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) is a technique to identify specific genes within an organism's chromosome (*in situ*) based on the ability of an introduced single-stranded DNA or RNA to hybridize the complementary target DNA sequence contained within the organism (Amann *et al.*, 1996; Amann *et al.*, 2006; Amann & Fuchs, 2008). The method comprises of four basic steps. The first step is fixation in which the target cells have to be fixed with aldehydes and alcohols (Amann *et al.*, 2006; Amann & Fuchs, 2008) before hybridization. The purpose of the fixation is to prevent lysis of the cells and protect the morphology of cell. The next step is hybridization of the introduced fluorescently labeled probes to the target sequence contained in the fixed cells. During hybridization both the target and the probe sequences are denatured. The labeled probe binds to the target and forms specific hybrids in presence of buffer. Blue fluorescent counter staining can be performed with aromatic diamidines like diamidino-2-phenylidole dihydrochloride (DAPI) (Zimmer & Wähnert, 1986), which are non-specific dyes that bind to DNA sequences. In third step, washing of the sample is performed to rinse off excess probe molecules. The last step is identification of fluorescent organisms and potentially quantification by fluorescence microscope or confocal laser scanning microscope (CLSM).

FISH is fundamentally different than PCR and qPCR in that qPCR amplifies DNA millions of times making new copies and allows quantification of the genes while FISH is a visualization

technique in which a cell is permeabilized, allowing a dye to enter the cell, bind to DNA thus making the cell fluorescent and allowing for quantification of cells.

CHAPTER 3 MATERIALS AND METHODS

3.1 Chemicals

All materials used for growth and maintenance of microcosms were obtained from Fisher Scientific Company, LLC, (Fairlawn, New Jersey, USA city, state) unless otherwise specified and were reagent grade or better unless otherwise specified. Sodium lactate (60% w/w syrup), TCE (99.9%, Acros organics, New Jersey, USA), agarose (molecular genetic analysis grade), potassium sulfate and magnesium sulfate and yeast extract are used as substrates and/or as analytical standards. The reagents used for analysis of sulfate are: HCl (trace metal grade), NaCl, ethanol (200 proof, Decon Labs Inc., USA), glycerol (enzyme grade) and barium chloride. The DNA extraction kits (DNeasy ® Bood & Tissue Kit) and PCR purification kits (QIA quick® PCR Purification Kit) were obtained from Qiagen Sciences (Maryland, USA). Maxima® Probe/ROX qPCR master mix (2X) (Fermentas Life sciences Inc.), iTaq™ Universal Probes Supermix (Bio-Rad laboratories Inc, Hercules, CA, USA), Power SYBR®Green PCR master mix (2X) (Applied Biosystem, Foster city, CA, USA), iTaq™ Universal SYBR Green Supermix (Bio-Rad laboratories Inc, Hercules, CA, USA) and PCR mix (PCR MasterMix (2X), Thermo scientific) were used for amplification of DNA. For cloning and plasmid extraction, TOPO TA® cloning kit (Invitrogen, Carlsbad, CA, USA) and Pure Link™ Quick Plasmid Miniprep Kit (Invitrogen, Germany), respectively were used.

3.2 Microcosm preparation

Two dechlorinating cultures were used to inoculate the reactors including (1) the Bachman Road culture obtained from Dr. Ryan Dupont at Utah State University, which contains *Dehalococcoides mccartyi* sp. (Löffler F.E. *et al.*, 2012) and (2) the SDC-9™ culture from Dr. Paul Hatzinger of The Shaw Group, Inc., which contains *Dehalococcoides mccartyi* (Löffler F.E. *et al.*, 2012). These cultures were transferred into 500 ml sterile glass bottle with 400 ml solution and amended with fresh minimal salt medium containing: 4.3 g/L of NH₄Cl, 50 g/L of KH₂PO₄, 20 g/L of MgCl₂·6H₂O, 1 g/L of CaCl₂·2H₂O, 100mg/L of FeCl₂·4H₂O, 5 g/L NaHCO₃; 0.0002% (200 mg/L) of resazurin; and 0.05% of yeast extract and 10 mls of a trace element solution

(Hendrickson et al., 2002; Löffler et al., 1997). The trace element solution contained 100 mg/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 170 mg/L CoCl_2 , 118 mg/L ZnSO_4 , 19 mg/L H_3BO_4 , 20 mg/L $\text{Na}_2\text{MO}_4 \cdot 2\text{H}_2\text{O}$. Resazurin (Acros) was used as a visual indicator of low-redox-potential conditions. Sodium lactate (10mM) was added as electron donor from a neat stock (60% syrup) and 10 to 20 mg/L of TCE from stock solution (1g/L) was added to microcosms initially and periodically throughout the incubation period (approximately every 7 days). Additionally, 10 mg/L sulfate was added to the reactor whenever the sulfate concentration was low. The reactors were filled with little headspace and purged with nitrogen gas. The reactors were incubated at room temperature on shaker at 120 RPM and wrapped in tin foil to limit photo degradation of TCE.

Cell growth was determined by observing the turbidity in the reactor, by microscopic examination with an epifluorescent microscope and qPCR. Microscopic examination was performed by pipeting 5 μl of culture onto a microscope slide. The cells were fixed to the slide by passing the bottom of the microscope slide through a flame. Then 5 μl of DAPI solution (4',6-diamidine-2'-phenylindole dihydrochloride, 50 $\mu\text{g/ml}$, Roche) was added and the slide was allowed to air dry in the dark. The slide was viewed with epifluorescence microscope with filter sets 2 and 9 (Zeiss Axiostar Plus) at a magnification of 100X. The images were captured with a Motic Images plus version 2.0 software. Samples were analyzed at time zero and then a week (5 to 15 days) for sulfate reduction after addition of sulfate to the reactor. The pH in the seed reactor was checked every week by pH paper.

3.3 Analysis of sulfates, redox potential and COD

Sulfate was analyzed according to Kolmert *et al.*, 2000. Standard sulfate solutions were prepared from the range of known concentrations 0 to 5 mM by dissolving K_2SO_4 in DI water. The calibration curve was fitted with a third degree polynomial curve over the range 0 to 5 mM sulfate (Figure A1 in Appendix A). The sample was analyzed by mixing 1 ml of conditioning reagent (contains 150 g NaCl, 100 ml glycerol (126 g), 60 ml concentrated HCl and 200 ml 95% ethanol and is made up to 1 L with deionized water) to 1 ml of sample (sample was diluted to fit into the range of the standard curve (0 to 5 mM)). Then 60 mg barium chloride powder was added to it and mixed by shaking for 30 sec. This mixture was transferred into cuvette and measured

absorbance at 420 nm against a blank using a spectrophotometer (Biomate 3, Fisher). From the mean absorbance values and standard curve made over the range of 0 to 5 mM, sulfate was calculated from the polynomial equation. The 0–5 mM concentration corresponds to typical sulfate concentrations in cultures of sulfate-reducing bacteria; hence no or little dilution of samples was needed.

Redox potential was measured by using oxidation reduction potential meter (Eutech Instruments pH 190 series).

COD was measured by Test Method B –Micro COD by sealed digestion and spectrometry as described in the protocol by ASTM 2012. First, 1.2 ml of potassium dichromate digestion solution was placed in a culture tube. Then 2.8 ml of silver sulfate catalyst solution was added and mixed. It was allowed to cool. Samples were always syringe filtered through 0.45 μ m filter (Fisherbrand) to remove cells. Then 2 ml of filtered sample was added and the tube was shaken vigorously. The mixture was placed in a heating device at 150 °C for 2 hour. It was cooled at room temperature. Then spectrophotometric readings were done by measuring absorbance at 420 nm. The COD value was determined from standard curves constructed from various concentrations (0, 5, 10, 20, 30, 40, 50, 75 and 100 mg/L) of potassium hydrogen phthalate (Figure A2 in Appendix A). 0.425 g potassium hydrogen phthalate was added to 500 ml water to make 1g/L as stock solution. The stock solution was used to make standards of various concentrations.

3.4 Construction of type 1 incubation chambers

Incubation chamber description: 10.5 X 6.5 X 1 cm plexiglass reactors were used for incubation chamber type 1 as shown in Figure 3.1 below. As shown in Figure 3.1 each reactor contained a chamber capable of holding 10 ml of solution (“1” in the figure), a microscope slide for sealing the chamber (“2” in the figure), and a small hole at back of the chamber for addition of the culture (“3” in the figure). Chambers were sterilized between inoculations by bleaching. 10% bleach was sprayed into the chambers for 20-30 sec.

Figure 3.1. Schematic diagram and photograph of the type 1 incubation chambers (figure not to scale).

Culture used for inoculation: 2 ml of dechlorinating culture was taken from the reactor and centrifuged for 10 min at 5000 x g. Supernatant was discarded and the pellet was washed by adding 1 ml of 1X PBS buffer (Phosphate Buffered Saline, 10X Solution), then vortexing, followed by centrifugation again for 10 min at 5000 x g. The supernatant was discarded. The washed culture was then resuspended in 200 µl of 1X PBS solution. A total of 100 µl of culture, containing on average 8.69×10^{10} 16S rRNA gene copies/ml was added to type 1 reactors as inoculum.

Media: A total of 50 mg agar (electrophoresis grade) along with 2 mg of magnesium sulfate was mixed in 1 ml of yeast extract solution to make a 5% v/v gel. It was heated in a water bath at 100 °C until it melted. To the melted agar-yeast extract solution, 75 µl of sodium lactate was added and immediately different concentrations of TCE (0.1, 10, 50 and 100 mg/L) were added and mixed by stirring with pipette tip. A total of 9 ml of 1X phosphate buffer solution (PBS) was added at the open well of the incubation chamber 1 (Figure 3.1). A total of 70 µl of the mixture (TCE, agarose, sulfate and sodium lactate mix) was pipetted onto the slide, allowed to harden (approximately 2 seconds) and then the slide was immediately inverted upside down (approximately within 5 seconds after drop of agar was added) in the reactor containing the PBS solution which was sparged with nitrogen gas (Air Gas Mid America Inc, Bowling Green, Kentucky). The reactor was sealed by plumber's putty (Oatey plumber's putty) at the edges of the microscope slide and the plexiglass well. The putty was applied at the outside the chamber so there was no contact between the putty and the PBS in the incubation well. The side opposite the microscope slide with the solid face contained a small hole used to inject the culture and the headspace was filled with approximately 1 ml of 1X PBS with 100 µl of culture from resuspension and the hole was sealed with tape (Figure 3.1). It was then incubated at 25 °C for 1 week, in the dark. Resazurin was added to the 1X PBS solution to aid in the observation of the redox condition. Resazurin is an oxazine dye. It is blue in its oxidized state and upon reduction it changes color to pink (partially reduced) and then clear (completely reduced) as oxygen becomes limiting in the chamber (Twigg, 1945; Guerin *et al.*, 2001). In first stage of reduction due to loss of the oxygen atom, it changes to pink and in second step it completely changes to colorless (Guerin *et al.*, 2001). The reactors on the first day of inoculation was blue; turned to pink after 6 hours and after 24-30 hours of inoculation it turned to colorless until incubation period.

Controls: Negative controls included (1) a growth inhibited control made by adding 2 mg of sodium azide to the agar mixture, (2) a no microorganism added control, and (3) a no TCE added control. Various other treatments included (1) a high TCE, high sulfate, and high culture treatment, (2) a high TCE, low sulfate, and low culture treatment, (3) a high TCE, low sulfate, high culture treatment and (4) a high TCE, high sulfate, low culture treatment.

3.5 Construction of type 2 incubation chambers

Two glass cover slips were taped to opposite sides of a microscope slide leaving approximately 2 cm space between the cover slips (Figure 3.2). The substrate was prepared as described above and 70 μ l of the media as described in Section 3.5 was added in the middle of two cover slips. The agar/TCE mixture was immediately covered by another cover slip from the top (Figure 3.2). A total of 10 μ l of inoculum (from stock reactor) after washing with 1X PBS solution (washing steps are described in section 3.4) was injected from the edge of cover slip and covered the entire microscope slide with parafilm to make anaerobic condition. The microscope slide was then placed on two caps within a petri dish (Figure 3.2), which contained 10 ml of water to maintain humid conditions in the incubation chamber during incubation. Further humid conditions were maintained by sealing around the edge of petri dish wrapping with parafilm. The type 2 incubation chambers were incubated at 25 °C in an incubator in the dark for 1 week. Resazurin was added for the observation of the redox condition during injection of the culture. Due to opacity of the chamber, the color change was not visible from outside. When the chamber was opened on last day of incubation, it was colorless. The controls were same as described in Section 3.5.

Food in
between two
cover slips

Food covered
from top by a
cover slip

Sealed with
parafilm and placed
in a humid chamber

Figure 3.2. Schematic diagram and photographs of the type 2 incubation chambers

3.6 Construction of type 3 incubation chambers

The substrate was prepared as described in Section 3.5. Approximately 10 μ l of the agar/TCE mixture was pipetted by capillary tube (Figure 3.3), placed on the microscope slide and immediately covered by cover slip from the top (Figure 3.3). A total of 10 μ l of inoculum (from stock reactor) after washing with PBS solution (washing steps as described in section 3.4) was injected from the edge of cover slip and covered by parafilm to make anaerobic conditions and then placed on two caps within a Petri dish (Figure 3.3), which contained 10 ml of water to maintain humid conditions in the incubation chamber during incubation. Further humid conditions were maintained by sealing the entire microscope slide with parafilm. The capillary containing chambers were incubated at 25 °C in an incubator in the dark for 1 week. Resazurin was added for the observation of the redox condition during injection of the culture. Due to opacity of the chamber, the color was not visible from outside. When the chamber was opened on last day of incubation, it was colorless. The controls were same as described in Section 3.5.

Figure 3.3. Schematic diagram and photograph of the type 3 incubation chambers

3.7 Growth studies

All the reactors were set up by same way as discussed in above 3.5, 3.6 and 3.7 section but incubated over different days: 0 day, 1 day, 2 days, 3 days, 5 days and 7 days. After set up of the type 3 reactor, the day 0 sample was taken after 0.5 hrs. For the type 1 and 2 reactors the day 0 sample was taken after 2 hrs.

3.8 Microcosm disassembly and cell sampling

After 1 week, or as necessary, microcosms were disassembled and the microcosm biofilms were sampled for the three groups of microorganisms under study. In type 1 microcosms, first the liquid inside the chamber was removed by syringe from hole. Then all the putty was removed and the microscope was slowly dissembled from the chamber to minimize disturbance of the cells on the microscope slide. In type 2 and 3 reactors, the parafilm was unwrapped and the cover slip was slowly pulled out with the help of sterile tweezer. Microorganism biofilms were scrapped off the glass microscope slides with a scraper blade that had been sterilized by autoclave at 121 °C for 15 minutes (Figure 3.4A and B) and were transferred to 1.5 ml Eppendorf tubes, containing 100 µL of 10 mM Tris (pH 7.7) and vortexed. Cells scrapped from the type 3 reactors for DNA extraction, was conducted by first holding the tube by bleach sterilized tweezers and dipping it into 70% bleach for 5 seconds, to remove cells from the exterior of the capillary tube. Then it was inserted inside the 1.5 ml Eppendorf tube containing 100 µl of 10 mM Tris (pH 7.7) and vortexed. The agar was visible inside the tube after vortexing also. The external area near the tube on the microscope slides was then scrapped off like in other 2 of types of reactors. During DNA extraction as described in method 3 of section 3.9, the agar inside the tube was melted and dissolved in the liquid and was not observed inside the tube.

Figure 3.4. Schematic diagram of microscope slide (figure not to scale) A) diagram of the three areas sampled for qPCR analysis in type 1 and 2 reactors, and B) the capillary area sampled for qPCR analysis in the type 3 reactor.

3.9 DNA extraction method testing and selection

Various DNA extraction methods were then tested to determine which technique yielded the highest quantity and best quality DNA from the microscope slides or scrapped cells. The protocols tested are presented below. Where necessary, the yield and quality of the DNA generated by each method was assessed by monitoring absorption at 260 nm, using a Nanodrop® ND-1000 version 3.7.1 spectrometer (Fisher). All reagents listed below were molecular biology grade unless otherwise noted. After extraction all DNA was stored at -20 °C until use.

Method 1: Boil

The scraped material was resuspended in 100 µl of sterile double-distilled DNase, RNase free water in a 1.5 ml sterile tube and incubated for 20 minutes in a water bath at 99 °C, then centrifuged for 5 minutes at 4000 x g. The supernatant was transferred to a separate 2 ml sterile tube and was directly used for PCR or qPCR (Sepp R et al., 1994). Also, to the pellet remaining in the original 2 ml sterile tube that was boiled, 100 µl of sterile double-distilled DNase, RNase free water was added and the tube was left on vortex at low speed to dissolve any remaining

DNA in the cell pellet for 2 hours. The yield and quality of the DNA was assessed as described above.

Method 2: Phenol:chloroform:isoamyl alcohol treatment

DNA extraction by this method was accomplished using the phenol/chloroform/isoamyl alcohol protocol as described by Gall *et al.*, 1993 and Poljak *et al.*, 1995 and with some modifications (elution buffer and the volume). The scraped material was resuspended in 200 µl of digestion buffer (50 mM Tris-HCl, 1 mM EDTA and 1% Tween-20) with 800 µg/ml of proteinase K in a sterile 1.5 ml tube and then incubated at 56°C for 1 hour in a water bath. Then 200 µl of phenol:chloroform:isoamyl alcohol, 25:24:1 (v:v:v) and 10 mM Tris-HCL (pH 8.0) was added to each tube and shaken vigorously for 1 minute followed by centrifugation at 6000 x g for 10 minutes. After centrifugation, the supernatant (which contains DNA) was transferred into another 2 ml tube; and 0.1 (100 µl) volume of sodium acetate (3M, pH 5) with 900 ul of cold 70% ethanol was added. The mixture was incubated at -20° C for 40 minutes. After centrifugation at 14,000 x g for 7 minutes at 4 °C, the supernatant was discarded and the precipitate (DNA) was air dried (Poljak *et al.*, 1995) and re-dissolved in 40 µl of 10 mM Tris. The yield and quality of the DNA was assessed as described above.

Method 3: Freeze-Thaw and Boil-Cool

The scraped material was resuspended in 100 µl of 10 mM Tris-HCl (pH 8.3) in a sterile 1.5 ml tube, briefly vortexed and frozen at -70°C for 1 hour. Afterwards, the sample was thawed at room temperature, incubated at 100°C in a water bath for 20 minutes, chilled on ice for 10 minutes, briefly vortexed and spun down at 4,000 x g for 2 minutes (Poljak *et al.*, 1995). The yield and quality of the DNA was assessed as described above.

- (i) The scraped material was resuspended in 100 µl of 10 mM Tris-HCl (pH 8.3) in a sterile 1.5 ml tube, briefly vortexed and frozen at -70°C for 1 hour. Afterwards, the sample was thawed at room temperature, incubated at 100°C in a water bath for 20 minutes, chilled on ice

for 10 minutes, briefly vortexed and spun down at 4,000 x g for 2 minutes (Poljak and Barlic 1998). The yield and quality of the DNA was assessed as described above.

(ii) This method was followed according to de Roda Husman *et al.*, 1995. The cells were suspended in 100 µl of 10 mM Tris-HCl (pH 8.1) in a sterile 1.5 ml tube. The cell suspension was vortexed and frozen at – 20 °C overnight. It was thawed at room temperature, boiled for 20 minutes approximately at 99°C in a water bath, cooled on ice for 10 minutes and spun down at 4000 x g for 2 minutes. The yield and quality of the DNA was assessed as described above.

Method 4: Freeze- thaw method

The scrapped cells were suspended in 100 µl of 10 mM Tris-HCl (pH 8.1) in a sterile 2 ml tube and frozen at -20 °C and then thawed at room temperature for 1 hour each. The freeze and thaw cycle was repeated a total of 3 times and spun down at 4000 x g for 2 minutes. The yield and quality of the DNA was assessed as described above.

Method 5: Lysis Method

Two separate types of DNA extraction by lysis with lysozyme or SDS were tested as described below. These methods involved extraction of the DNA from the cells on the slide, prior to scrapping the cell debris and DNA from the microscope slide into a 1.5 ml tube.

(i) A total of 60 µl of lysozyme was added to the surface of the slide over an area approximately 1 cm², and the slides were frozen for 1 hour at -20 °C. The slides were then thawed for 1 hour at room temperature. The freeze and thaw cycle was repeated a total of 3 times. The cells were scraped from the microscope slide and resuspended in 100 µl of DNase RNase free water. It was centrifuged for 5 minutes at 4000 x g. The yield and quality of the DNA was assessed as described.

(ii) A total of 60 µl of SDS added on the slide and the slides were frozen for 1 hour at -20 °C. The slides were then thawed for 1 hour at room temperature. The freeze and thaw cycle was repeated a total of 3 times. The cells were scraped from the microscope slide and resuspended in

100 μ l of DNase RNase free water centrifuged for 5 minutes at 4000 x g. The yield and quality of the DNA was assessed as described.

Method 6: Proteinase K/Tween-20 lysis method

In this method, the scrapped cells were suspended in 100 μ l of lysis buffer (0.1 mg/ml proteinase K, 0.1% (v/v) Tween 20, 50 mM potassium chloride, 10mM Tris-HCl pH 8.3 and 1.5 mM magnesium chloride) in a sterile 1.5 ml tube and incubated overnight at 37 °C in an incubator. The samples were heated at 96 °C for 10 minutes to inactivate the proteinase K enzyme and then centrifuged for 3 minutes at 11,000 x g (de Roda Husman *et al.*, 1995). The yield and quality of the DNA was assessed as described above.

Method 7: DNA extraction using Holmes-Bonner Buffer

This method was followed as described by Holmes 1973 with slight modifications. The scrapped cells were suspended into 100 μ l of Holmes-Bonner Buffer (1 M Tris (pH 7.5 – 8), 5 M NaCl, 0.5 M EDTA, 260.8 g/L Urea, 10% SDS and water to reach volume, stirred over mild heat) and 300 μ l DI water. Then 1 volume of phenol and 1 volume of chloroform (can alternately use phenol: chloroform [1:1]) was added and the samples were mixed by inverting the tubes manually for 1-2 minutes and centrifuged at 14,000 x for 5 minutes. The upper layer was transferred to a new tube taking care not to include any part of the interphase. The extraction was repeated one additional time and the upper layer was transferred to another sterile 2 ml tube. One volume of chloroform was added and the mixing and centrifugation described above was repeated. Then 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 to 3 volumes of cold, 100% ethanol was added and stored at -70 °C for 1 hr. The sample was then centrifuged at maximum for 15 minutes and the ethanol was discarded. Cold 70% ethanol (approximately 1 ml) was added and the solution was centrifuged at 14,000 x g for 15 minutes and the ethanol was carefully removed (Holmes & Bonner, 1973). The open tube containing the DNA was stored upright on a rack covered with Kim wipe tissue (Kimtech Science brand) and allow it to dry. Then 100 μ l of 10 mM Tris was added to the DNA in the tube and it was left to dissolve the DNA at room temperature for 10 minutes. The yield and quality of the DNA was assessed as described above.

Method 8: Phenol: chloroform extraction and (i) isopropanol clean up method, (ii) ethanol cleanup, or (iii) PCR cleanup kit

The scraped cell were suspended in 500 μ l TE buffer (Tris 10 mM, pH 7.4; EDTA 1 mM) and extracted two times with approximately 500 μ l of 2:1 (v:v) chloroform: methanol mix. The solution was then vortexed and centrifuged for 3 minutes at 14,000 $\times$ g. The upper (aqueous) phase containing the DNA (Supernatant 1) was transferred into a separate 2.0 ml Eppendorf tube and centrifuged at 14,000 $\times$ g, for 5 minutes. This extraction was then repeated. Then 50 μ l TE buffer and 50 μ l of 1 M Tris solution (pH 9) were added. Then 5 μ l of 20 mg/ml lysozyme (100 μ g/ ml final) and 100 μ l of *RNaseA* (50 μ g/ml final) was added to the cell suspension and mixed gently by inverting or reversing the tube several times. It was then incubated overnight at 37 °C in an incubator. Then 25 μ l of proteinase K (100 μ g/ml final) was added, the solution was mixed gently and incubated at 37 °C for an hour. Then 60 μ l SDS (10%) was added and incubated for additional 2 to 3 hours at 55°C. The DNA was extracted by adding 600 μ l phenol and 800 μ l chloroform and mixed by gently reversing the tube a few minutes. The solution was then centrifuged at 14,000 $\times$ g for 3 minutes. Supernatant was transferred to another 2 ml tube and then the extraction was repeated. After this process one of the following process used for DNA cleanup on the supernatant.

- i. A total of 0.1 volumes of sodium acetate (3 M pH 5.2) and one volume of isopropanol was added and mixed by gently inverting the tube several times. It was incubated for 1 hour at 4°C and centrifuged at 14,000 $\times$ g for 10 minutes. The supernatant was carefully removed by pipette and the pellet was dried to remove all liquid. The pellet was then resuspended by adding 50 μ l of 10 mM Tris (pH 7.4). The yield and quality of the DNA was assessed as described above.
- ii. A total of 0.1 volumes of sodium acetate (3 M pH 5.2) and 1 volume of 70% ethanol was added and incubated for 30 minutes at -20°C and centrifuged 14000 $\times$ g for 30 minutes at 4°C. The supernatant was carefully removed and 100 μ l of 70 % ethanol was added. The solution was then centrifuged 14000 $\times$ g for 5 minutes at 4 °C in a refrigerated microcentrifuge. All liquid was then removed. The pellet was resuspended in 50 μ l of 10 mM Tris (pH 7.4). The yield and quality of the DNA was assessed as described above.
- iii. In this method, the DNA was cleaned by using QIAquick® PCR Purification Kit (Qiagen) following the manufacturer instructions. Briefly, in this method, the samples were mixed with

Qiagen buffer, filtered with DNeasy spin columns and washed. Then eluted by 25 μ l of buffer EB (10 mM Tris, pH 8.5) instead of 50 μ l from manufacture's protocol. The yield and quality of the DNA was assessed as described above.

Method 9: Sonication

The scrapped cells were resuspended in 1.5 ml tubes containing 100 μ l of 10 mM Tris solution. The tube was then sonicated in a water bath sonicator (Fisher) for 1 hour and was centrifuged at 10,000 x g for 1 minute. The supernatant was transferred and cleaned up by the ethanol precipitation cleanup method as described in Method 8 (ii). The DNA was dissolved in 100 μ l of Tris (pH 7.4). The yield and quality of the DNA was assessed as described above. For PCR and qPCR purposes 5 μ l aliquots were used from each DNA extraction method described above.

3.10 PCR

PCR amplification was performed in a total volume of 25 μ l. The PCR reaction mixture contained 1X PCR master mix (Thermo Scientific), 0.2 μ M of each universal bacteria 8F and 1492R primers (Table 3.1), template DNA (5 μ l), 0.5 mg/ml of Ultrapure™ BSA (Ambion®) and enough PCR grade water (DNase, RNase free) to make up a 25 μ l solution. PCR was conducted on an Eppendorf thermocycler (Eppendorf version 1.628) and the thermocycler program was 95 °C for 15 minutes; 40 cycles of: 95 °C for 60 seconds, 53.5 °C for 60 seconds, 72°C for 90 seconds, extension at 72 °C for 7 minutes. PCR-amplified DNA was detected by using 1% (wt/vol) agarose (electrophoresis grade) gel electrophoresis run in 1X TAE buffer at 100 volts for 30 minutes. The gel was stained by adding 2 μ l of 10 mg/ml solution of ethidium bromide prior to loading. A total volume of 4 μ l of PCR product with 1 μ l of loading dye (Agarose gel loading dye 6X, Nalgene) was added to the gel and DNA was detected by UV light using a Gel logic 100 imaging system (molecular imaging software v 4.0.3).

The primers that were used in the PCR and qPCR for amplification of the 16S rRNA and *dsr A* gene target in the reactors are presented in Table 3.1.

Table 3.1. Specific PCR/qPCR primers and probes

Primer/ probe Name	Sequence	Target Gene	Amplicon length (bp)	References
8F	5'-AGA GTT TGA TCC TGG CTC AG-3'	Bacteria 16S rRNA	1502	Hendrickson <i>et al.</i> (2002)
1492R	5'-GGT TAC CTT GTT ACG ACT T-3'	Bacteria 16S rRNA		Hendrickson <i>et al.</i> (2002)
DHC-F	5'-GGT AAT ACG TAG GGA AGC AAG CG-3'	<i>Dehalococcoides</i> sp. 16S rRNA	98	Holmes <i>et al.</i> (2006), He <i>et al.</i> (2007)
DHC-R	5'-CCG GTT AAG CCG GGA AAT T-3'	<i>Dehalococcoides</i> sp. 16S rRNA		Freeborn <i>et al.</i> (2005), He <i>et al.</i> (2006), Holmes <i>et al.</i> (2006)
16S DHC Probe	FAM-ACA TCC AAC TTG AAA GAC CAC CTA CGC TCA CT-BHQ-1	<i>Dehalococcoides</i> sp. 16S rRNA		Freeborn <i>et al.</i> (2005), He <i>et al.</i> (2006), Holmes <i>et al.</i> (2006)
Arch 344 F	5'-ACG GGG YGC AGC AGG CGC GA-3'	Archaea 16S rRNA	462	(Raskin <i>et al.</i> , 1994)
Arch 806 R	5' -GGA CTA CCC GGG TAT CTA AT -3'	Archaea 16S rRNA		(Takai & Horikoshi, 2000)
DSR1F+	5'-ACS CAC TGG AAG CAC GGG GG- 3'	<i>dsr A</i> functional gene	221	(Kondo <i>et al.</i> , 2006)
DSR-R	5'GTG GMR CCG TGC AKR TTG G- 3'	<i>dsr A</i> functional gene		(Kondo <i>et al.</i> , 2006)
MBAC-F	5'-CGW AGG GAA GCT GTT AAG T-3'	<i>Methanobacteriales</i> sp. 16S rRNA	343	(Yu <i>et al.</i> , 2005)
MBAC-R	5'-TAC CGT CGT CCA CTC CTT-3'	<i>Methanobacteriales</i> sp. 16S rRNA		(Yu <i>et al.</i> , 2005)
MCOC-F	5'-TAA GGG CTG GGC AAG T-3'	<i>Methanococcales</i> sp. 16S rRNA	337	(Yu <i>et al.</i> , 2005)
MCOC-R	5'-CAC CTA GTY CGC ARA GTT TA-3'	<i>Methanococcales</i> sp. 16S rRNA		(Yu <i>et al.</i> , 2005)
MMIC-F	5'-ATC GRT ACG GGT TGT GGG-3'	<i>Methanomicrobiales</i> sp. 16S rRNA	506	(Yu <i>et al.</i> , 2005)
MMIC-R	5'-CAC CTA ACG CRC ATH GTT TA C-3'	<i>Methanomicrobiales</i> sp. 16S rRNA		(Yu <i>et al.</i> , 2005)
MSAR2-F	5'-GTA AAC GAT RYT CGC TAG GT-'3	<i>Methanosarcinales</i> sp. 16S rRNA	354	(Yu <i>et al.</i> , 2005)
MSAR2-R	5'-GGT CCC CAC AGW GTA CC-3'	<i>Methanosarcinales</i> sp. 16S rRNA		(Yu <i>et al.</i> , 2005)

3.11 qPCR

qPCR was conducted in an Applied Biosystem (Singapore) 7300 Real Time PCR system with SDS software (version 1.3.1) to determine the distribution of the 16S rRNA gene of *Dehalococcoides* sp. and Archaea and the *dsrA* functional gene of sulfate reducing bacteria. For DHC, to each qPCR tube a final concentration of 1X of Maxima® Probe/ROX qPCR master mix (Fermentas Life Sciences Inc.), 0.7 µM each of the 16S rRNA DHC forward and reverse primers (Table 3.1), 0.2 µM of the DHC 16S rRNA gene probe was added, 5 µl of DNA and enough water to make up 25 µl of reaction mixture. The qPCR thermocycler conditions was set as 50°C for 2 minutes, 95°C for 15 minutes, 40 cycles of 95°C for 30 seconds, 58°C for 60 seconds. For Archaea, to each qPCR tube a final concentration of 1X of Power SYBR®Green PCR master mix (Applied Biosystem), 0.7 µM each of the reverse and forward primers (Arch 344F and Arch 806R (Table 3.1), 0.5 mg/ml of Ultrapure™ BSA (Ambion®), 5 µl of DNA and enough PCR grade water to make 25 µl reaction mixture. The thermocycler conditions were: 50°C for 2 minutes; 95°C for 15 minutes; 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds; and 72°C for 30 seconds; followed by 50°C for 5 minutes and a dissociation stage. For sulfate reducing bacteria, to each qPCR tube a final concentration of 1X of Power SYBR®Green PCR master mix (Applied Biosystem), 0.5µM each of the reverse and forward primers (DSR1F+ and DSR-R (Table 3.1), 0.5 mg/ml of Ultrapure™ BSA (Ambion®), 5 µl of DNA and enough water to make a 25 µl reaction mixture. The thermocycler conditions were: 50°C for 2 minutes; 94°C for 15 minutes; 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute; followed by extension of 72°C for 7 minutes and a dissociation stage. Since SYBR green might also bind to nonspecific DNA, a melting curve was performed to assure the specificity of the PCR by analysis of the dissociation curve. For *Methanobacteriales* sp., *Methanococcales* sp., *Methanomicrobiales* sp., and *Methanosarcinales* sp., the SYBR reaction mixture and thermocyclic conditions were same as the Archaea.

3.12 Clone library and qPCR standard curve construction

Plasmids containing the target genes (i.e., 16S rRNA genes of DHC, Archaea and the *dsrA* gene of SRB) were used for qPCR. The plasmid clones were constructed with the TOPO TA cloning®

kit (Invitrogen) according to the manufacturer's instructions. PCR products of DNA extracted from the seed reactor were used to generate plasmids with the cloning kit. Plasmids were extracted from the *E. coli* cells using the TOPO TA® cloning kit (Invitrogen) and Pure Link™ Quick Pasmid Miniprep Kit (Invitrogen).

These plasmids were used to generate standard curves for qPCR by serially diluting the plasmids from 10^{11} to 10^2 gene copies/ul PCR reaction. Duplicate qPCR was performed for each sample and triplicate qPCR for the serially diluted plasmid standard (Figure 3.5). The efficiency of the assay and the coefficient of determination (R^2) of the standard curve were calculated based on the linear regression of the standard curve. Perfect amplification must double the starting DNA in every cycle. In a 10-fold dilution of the standards the number of cycles to increase the DNA 10 times must be about 3.32. Consequently, amplification curves for all standards samples should be evenly separated by 3.32 cycles in order to obtain an efficiency of 100%. The amplification efficiency (E) was calculated from the slope of the standard curve using the formula in Equation 12.

$$Efficiency = -1 + 10^{-1/slope}$$

Equation 12

A

B

C

Figure 3.5. Standard curve for DHC (A), SRB (B) and Archaea (C).

The estimated efficiency for the qPCR reactions were 98.5 % for DHC 16S rRNA genes, 88.6 % for SRB *dsrA* genes, and 71.5 % for Archaea 16S rRNA genes.

3.13 Data analysis

DNA (copy/ μ L) was calculated from the following linear Equations (13, 14 and 15) of standard curve with Ct value analyzed from qPCR:

$$\text{Log 16S rRNA gene copies of DHC/ul} = -0.295 * \text{Ct} + 17.67$$

Equation 13

$$\text{Log } dsrA \text{ gene copies of SRB/ul} = -0.272 * Ct + 14.45 \quad \text{Equation 14}$$

$$\text{Log 16S rRNA gene copies of Archaea/ul} = -0.234 * Ct + 14.17 \quad \text{Equation 15}$$

Then by using the following equation (16) DNA concentration in the original area of the microscope slide of different species was calculated. All gene concentrations from qPCR were calculated as log10.

$$\text{DNA copy/cm}^2 = \frac{\left\{ 10^{\text{Log gene} \frac{\text{copies}}{\text{ul}}} \right\} * \text{Vol of DNA extraction (ul)} * \text{dilution factor}}{\text{Area scrapped cm}^2} \quad \text{Equation 16}$$

Statistical Analysis

All statistical analyses (i.e., linear regressions, Student's t-test) were conducted with Sigma Plot version 11.0 (Systat Software, Inc.).

CHAPTER 4 RESULTS

4.1 Analysis of chemical degradation and culture in seed reactor

The pH in the seed reactor was measured every week and ranged from pH 6 to 6.5. Sulfate reduction was found after a 15 day incubation period (Figure 4.1). The average redox potential of the seed reactor was measured as -56.5 mV (S.D. $\pm$ 45.5) with the range of -20 to -167 mv suggesting that anaerobic reductive dechlorination in the seed reactor occurred in the range of iron reduction to sulfate reduction (Figure 2.4).

Figure 4.1. Sulfate concentrations in the seed reactors over the operational period

The recommended sulfate concentration for enhanced bioremediation is 500 to 1000 mg/L (Henry 2010) although 500 to 5000 mg/L sulfate is the maximum range suitable for reductive dechlorination (Henry 2010). USEPA (1998) indicated that if sulfate exceeds 20 mg/L reductive dechlorination is reduced. High sulfate levels are problematic because of high electron demand (Henry 2010) and may lower the efficiency of anaerobic dechlorination (USEPA 1998). In this study, initial sulfate concentration was 2 g/L and was reduced to 0.3 g/L within 15 days after sulfate addition to the seed reactor.

The culture characteristics of *Dehalococcoides mccartyi* and other microorganism in the seed reactor was analyzed by staining an aliquot of cells on microscope slides with DAPI (Figure 4.2) and comparing the culture characteristics to those published by others. DAPI stains all DNA present in all cells and therefore is an indicator of both live and dead microorganisms in a sample. Additionally, FISH using the Dhe1259t probe specific to DHC was used to verify the presence of DHC in the seed reactor and DHC cells were visualized by epifluorescence microscope (Figure 4.3) and compared to all cells in the culture stained by DAPI. The cells viewed under the microscope were coccoid and small, specifically, 0.1 to 0.5 μm in diameter similar to other published *Dehalococcoides* sp. culture descriptions (Figure 4.2 and Figure 4.3).

DHC was found to be abundant in the reactor based on qPCR analysis in the seed reactor, specifically greater than 1×10^8 to 1×10^{10} DHC 16S rRNA gene copies/ml of reactor and the population was sustained over 255 days of operation. The recommended DHC concentration for natural attenuation in groundwater remediation is near or greater than 1×10^7 gene cells/L (Lu et al 2006a) and in lab scale reactors is greater than 1×10^4 gene cells/ml (Lee *et al.*, 2008, Loeffler 2012). Specifically, concentrations of DHC were measured on 100, 146 and 255 days and were 8.2×10^{14} , 6.7×10^{17} and 2.7×10^{19} DHC 16S rRNA gene copies/ml of reactor, respectively. So it was assumed TCE degradation occurred in the large seed reactors when there were plenty of DHC as many previous studies have shown that DHC only uses organohalogenes as energy sources and thus in presence of active DHC, TCE degradation occurs. Additionally, COD analysis in the seed reactors showed degradation of COD from 3 g/L to 1.6 g/L over 5 days and in type 1 reactor from 671 to 245.4 mg/L over 5 days after addition of sodium lactate and TCE.

Theoretical oxygen demand for the amount of sodium lactate added to the reactors was 857 mg/L.

Figure 4.2. Comparison of culture characteristics of DAPI stained DHC in (A) The KB-1/VC-H₂ culture from literature at 1000X magnification (Duhamel, 2004) and (B) from this study at 100X magnification observed with epifluorescence microscope with emission wavelength of 450-500nm.

Figure 4.3. FISH microscopic images of culture from seed reactor (A) DHC only at magnification of 100X and wavelength 500-570 nm (green) (B) all cells stained with DAPI in same field as A at 450-500 nm wavelength (blue).

4.2 Evaluation of DNA extraction methods by PCR

DNA was extracted from microscope slides by different protocols as discussed in Section 3.8 and amplified by PCR using universal bacterial primers. The most fast and efficient method for DNA extraction was found to be method 3 ii, in which samples were scrapped and cells were resuspended in Tris, then frozen, thawed, boiled and cooled. The resulting DNA concentration, DNA purity and PCR amplification results from the different methods are shown in Table 4.1.

Table 4.1. DNA concentration, purity and amplification results of various DNA extraction methods

DNA extraction method	DNA ng/ul	A260/ 280	Bacterial 16S rRNA PCR amplified?
1: Boil	4.6	2.38	No
2: Phenol:chloroform:isoamyl alcohol treatment	114.4	1.55	No
3i: Freeze-Thaw and Boil-Cool	47.7	1.73	Yes
3ii: Freeze-Thaw and Boil-Cool	34.4	1.47	Yes
4: Freeze- thaw method	NA	NA	No
5 (i): Lysis Method with lysozyme	1063	0.98	No
5 (ii): Lysis Method with SDS	NA	NA	No
6: Proteinase K/Tween-20 lysis method	NA	NA	No
7: DNA extraction using Holmes-Bonner Buffer	6.4	1.12	No
8(i): Phenol chloroform extraction , isopropanol clean up	128.1	1.48	No
8(ii): Phenol chloroform extraction and ethanol cleanup	311.1	1.64	Yes
8(iii): Phenol chloroform extraction and PCR cleanup kit	6.6	1.99	Yes
9: Sonication	194.6	1.24	No
NA = data not available			

DNA concentration from method 3 ii as measured by nanodrop quantification is shown in Figure 4.4. The concentration and purity of DNA is estimated by measuring the amount of ultraviolet radiation absorbed. In the figure, absorbance at the wavelength 260 nm is assumed to be by DNA and the absorbance at 280 nm is assumed to be by proteins. Optimal ratio of absorbance at 260 versus 280 nm is 1.8. In this study the ratio of absorbance at 260 nm to absorbance at 280 nm is 1.8 in type 1 and 2 reactors. Only the DNA extracted from the type 3 reactor had DNA purity below 1.8, however the DNA quality at 1.2 A260/280 still represents DNA of high enough purity not to significantly interfere with the PCR reaction (e.g., Table 4.1). The method 3ii was selected for DNA extraction for qPCR analysis to detect Archaea, DHC and SRB because of higher DNA yields, reduced sample handling requirements, faster overall extractions and lower cost.

A

B

C

Figure 4.4. Nanodrop DNA quantification of: (A) Type 1 reactor (B) Type 2 reactor and (C) Type 3 reactor for method 3 ii.

Amplification of DNA from PCR analysis using different primers is shown in Figure 4.5. The amplification without BSA was not as good as with the addition of BSA (data not shown), thus, BSA was added as an adjuvant during the PCR assay.

Figure 4.5. Detection of (A) bacterial 16S rRNA , (B) *Dehalococcoides* 16S rRNA , (C) *dsrA* genes of sulfate reducers and (D) Archaea 16S rRNA.

As shown in Figure 4.5, amplicons of the expected size targeting the universal bacteria (1502bp), *Dehalococcoides* group (98bp), SRBs (221bp) and Archaea 16S rRNA (462 bp) were obtained in direct PCR with primers. The lengths in the gel of Archaea group is variable which might be due to non specific amplification as the primers were originally developed in 1994 and don't account for current knowledge on 16S rRNA sequences (e.g., NCBI database updates).

4.3 qPCR results

Method 3ii was used for DNA extraction from the microcosms studies conducted to address the main hypotheses in this research. The microorganisms were not found in controls like (i) no cells and (ii) and some DNA were found in growth inhibited control containing sodium azide (Table 4.2). All data is tabulated in the Appendix Table A3.

Table 4.2. Detection of DHC, SRB and Archaea in the type 1, 2 and 3 control reactors

Control Study		Type 1 reactor			Type 2 reactor			Type 3 reactor		
		DHC	SRB	Archaea	DHC	SRB	Archaea	DHC	SRB	Archaea
Growth inhibited (sodium azide)		-	ND	ND	-	ND	ND	-	ND	ND
No cells added		-	ND	ND	-	ND	ND	-	ND	ND
No TCE added		+	+	+	+	+	+	+	+	+

- =cells not present or below detection limits, + = cells present, ND= no data, samples were not analyzed as DHC was not detected by qPCR .

To investigate the relative concentrations of methanogens as compared to the total Archaea population, a subset of samples were tested to determine the relative abundance of methanogens and total Archaea from the seed reactors (Table 4.3). In general *Methanobacteriales* was the most abundant methanogen (i.e., smaller Ct values indicate a larger overall abundance of the particular gene or organism). This *Methanobacteriales* uses H₂ as a substrate (Balch *et al.*, 1979, Zeikus, 1977) and therefore is more likely to compete with DHC for H₂ reducing equivalents generated during degradation of sodium lactate. *Methanococcales* was also abundant in the reactors and primarily uses H₂ as a substrate. The data in Table 4.3 also support the argument that total Archaea 16S rRNA detected by qPCR can be used as a surrogate for methanogens in this study.

Table 4.3. Detection of the different orders of methanogens and total Archaea in the seed reactor

Methanogenic Archaea	Ct
<i>Methanobacteriales</i>	24.2
<i>Methanococcales</i>	24.9
<i>Methanomicrobiales</i>	33
Archaea	24.1

The treatments in the various aquifer model reactors were analysed when compared with control. The relative microbial population as a fraction of the initial concentration for the 6 treatments is presented in Table 4.4. In analyzing these data, it was found that microorganism concentration increased in most of the treatments. However, for some treatments the degradation extent was not statistically significant relative to the control (Appendix Table A3).

Table 4.4. Comparison of the average concentration of microorganisms in all areas after 7 days of incubation with different initial nutrient conditions and TCE concentrations

TCE (mg/L)	Sulfate (mg/L)	Cells	Type 1 reactor			Type 2 reactor			Type 3 reactor		
			DHC	SRB	Archaea	DHC	SRB	Archaea	DHC	SRB	Archaea
100	10	HC	+	++	+++	+	++	+++	+	++	+++
	10	LC	+	++	+++	+	++	+++	++	+	+++
	0.5	HC	++	+	+++	+	+	++	++	+	+++
	0.5	LC	-	+	++	-	+	++	-	+	++
0.1	10	HC	+	++	-	+	++	-	+	-	-
	10	LC	+	++	-	+	++	-	-	-	-
	0.5	HC	-	+	++	-	+	++	-	-	-
	0.5	LC	-	-	-	-	-	+	-	-	-

HC= DHC: 4.6×10^8 gene copies/ μ l, SRB: 1.5×10^8 gene copies/ μ l, Archaea: 2.1×10^{12} gene copies/ μ l for type 1 reactor and DHC: 2×10^8 gene copies/ μ l, SRB: 5.3×10^7 gene copies/ μ l and Archaea: 4.8×10^{11} gene copies/ μ l for type 2 and 3 reactors.

LC= DHC: 2×10^8 gene copies/ μ l, SRB: 5.3×10^7 gene copies/ μ l and Archaea: 4.8×10^{11} gene copies/ μ l for type 1 reactor and 1×10^6 gene copies/ μ l, SRB: 1×10^5 gene copies/ μ l and Archaea: 1×10^9 gene copies/ μ l for type 2 and 3 reactors.

+: 1×10^8 to 1×10^9 gene copies/ μ l

++: 1×10^9 to 1×10^{11} gene copies/ μ l

+++ : $> 1 \times 10^{11}$ gene copies/ μ l

- = Not detected by qPCR

4.3.1 Specific objective 1: To test the hypothesis that increasing TCE concentrations influence the concentration of DHC, SRB and Archaea.

The concentration of DHC, SRBs and Archaea were analyzed in three types of reactors after addition of different concentrations of TCE as an electron acceptor and sodium lactate (which can be degraded to hydrogen) as electron donor, in an agar plug. The three different types of reactors were prepared with different concentrations of TCE (0, 10, 50, 100 mg/L) and incubated for several days. The cells were scrapped after 1 week from three different locations in the reactor and the 16S rRNA gene of DHC and Archaea (a surrogate for methanogens) and *dsrA* gene of SRB were measured by qPCR. Initial concentrations of DHC, SRB and Archaea added to the reactor, were estimated based on the density of cells in the seed reactor and volumes added at each step of the reactor construction. Therefore these initial concentrations of cells may underestimate the total number of cells added to the reactors due to losses during culture handling and washing prior to reactor inoculation.

Concentrations of Archaea were higher in all reactors than the other microorganisms under study and SRBs were always observed to have the lowest concentration (Figure 4.6A, B and C). The average concentration of Archaea in the type 1 reactors ranged from 2.12×10^{12} to 1.33×10^{13} 16S rRNA gene copies/cm², while the DHC concentrations were lower ranging from an average of 4.69×10^{10} to 3.19×10^{11} 16S rRNA gene copies/cm² and SRB concentrations were the lowest ranging from an average of 3.02×10^8 to 3.82×10^9 *dsrA* gene copies/cm². This trend was similar in the type 2 and 3 reactors. In the type 2 reactor; Archaea average concentration ranged from 2.42×10^{13} to 1.37×10^{15} ; DHC ranged from 4.3×10^9 to 9.7×10^9 and SRB from 6.6×10^7 to 7.6×10^8 *dsrA* gene copies/cm². In the type 3 reactor, Archaea average concentration ranged from 1.89×10^{12} to 5.44×10^{14} ; DHC ranged from 2.66×10^{11} to 1.45×10^{12} and SRB from 7.8×10^8 to 1.6×10^{10} *dsrA* gene copies/cm².

In type 1 reactor, the percent increase in average DHC concentration was 25.7% for all TCE concentrations. There was 21.8%, 26%, 24.7%, 28.7% and 30.2% increase in concentration of DHC after seven days in the reactor in the 0.1, 1, 10, 50 and 100 mg/L of TCE treatments, respectively. Hence increasing concentration of TCE increased DHC concentration. In contrast

to the DHC results, with increasing TCE concentration there were no significant changes observed in mean concentrations of Archaea in the type 1. In the type 2 reactor, the percent increase in average DHC was 18% over 7 days, however there was no consistent trend in DHC and TCE concentrations. Specifically, at 10 mg/L TCE the DHC increase was 20%, at 50 mg/L TCE the DHC increase was 13.4% and the DHC increase was 19.58% in the 100 mg/L TCE treatment. There were no significant changes observed in the mean concentration of SRB and Archaea in reactors with different concentrations of TCE. In the type 3 reactor, there was increase of 33 to 42% of DHC over 7 days but the trend was not linear. There was not significant change in concentration of SRB and Archaea population with changes in initial concentrations of TCE.

Figure 4.6. DHC, SRB and Archaea gene concentrations with varying TCE concentrations after 7 days in type 1 reactor (average and standard deviation of duplicates in all areas of reactor), type 2 reactor and type 3 reactor.

The effect of increasing TCE concentrations on the average concentration of DHC, SRB and Archaea in all areas of the reactors, after 7 days of incubation is presented in Figure 4.6. In the type 1 reactor (Figure 4.7A), there was a linear correlation between increasing concentrations of TCE and DHC concentrations (Regression, $F=12.265$, $P=0.025$) and concentrations of TCE and SRB (Regression, $F=14.757$, $P=0.018$). The results suggest a trending relationship between TCE and the concentration of Archaea but at a non significant level (Regression, $F=5.820$, $P=0.073$).

In type 2 reactor, there was no observed correlation between initial concentration of TCE and the concentration of DHC (Regression, $F=0.000201$, $P=0.989$); concentration of TCE and the concentration of Archaea (Regression, $F=1.105$, $P=0.353$) and between concentration of TCE and SRB concentration (Regression, $F=0.0175$, $P=0.901$) (Figure 4.7 B).

Similarly in type 3 reactor, there was not a correlation between the concentration of TCE and the concentration of DHC (Regression, $F=0.0284$, $P=0.869$); the concentration of Archaea (Regression, $F=0.142$, $P=0.714$) and concentration of TCE and SRB concentration (Regression, $F=8.258$, $P=0.017$) (Figure 4.7 C). However, as shown in Figure 4.7C, there is a trending relationship between the DHC, SRB and Archaea with initial TCE concentration.

A

B

C

Figure 4.7. Relationship between DHC, SRB and Archaea gene concentration with respect to different TCE concentration in different reactor types after 7 days; A: type 1 reactor (average of all areas), B: type 2 reactor (average of all areas) and C: type 3 reactor (average of all inside and outside capillary tube). Regression with * is statistically significant ($P < 0.05$).

4.3.2 Specific objective 2: To test the hypothesis that the concentration of DHC, SRB and Archaea decrease with increasing distance from the source of reducing equivalents (i.e., food)

Spatial variability in the distribution of TCE and the distribution of microorganisms with distance from food source in different reactor types compared to different types of natural aquifers was analyzed. Concentration of DHC, SRB and Archaea were quantified in three areas with increasing distance from the location of food and TCE. The concentration of the microorganisms with distance from the food (e.g., agar plug and TCE) source is presented in Figure 4.8, Figure 4.9 and Figure 4.10 below for the type 1, 2 and 3 reactors respectively. Additionally, tests for significant differences between the mean concentrations of microorganisms in all TCE treatment levels in the three areas observed are presented in the Appendix (Appendix Table A4).

In the type 1 reactor (Figure 4.8), there was no statistically significant difference in the mean concentration of DHC, SRB and Archaea observed with distance from the agar plug containing TCE and food. Although DHC concentrations in area 3 in the higher concentration TCE treatments (e.g., 50 and 100 mg/L TCE) did seem to be lower than in areas 1 and 2, these results suggest that DHC concentrations may decline with distance from a TCE source, even in sandy aquifers where significant diffusion of the TCE may occur (i.e., there is less of a concentration gradient). DHC was higher in all areas than control with no TCE or low TCE (0.1 mg/L).

A

B

Figure 4.8. DHC (A), SRB (B) and Archaea (C) gene concentrations with distance from the agar plug and with different TCE concentrations after 7 days in Type 1 reactor (average and standard deviation of duplicates in three different areas of reactor).

In the type 2 reactor, there were strong trends observed in the mean concentration of DHC, SRB and Archaea with distance from the agar plug containing TCE and food. The mean concentration of the DHC in all TCE treatment levels in area 1 compared to area 2 (Student's t-test, $t=2.446$, $P=0.031$) and between area 1 and 3 (Mann Whitney Rank sum $T=135$, $P=0.029$) differ significantly but no significant difference between area 2 and 3. Similarly for Archaea, there was no significant difference between area 1 and 2 but was significant difference between area 2 and 3 (Mann Whitney Rank sum $T=498$, $P=0.018$) and between area 1 and 3 (Mann Whitney Rank sum $T=390$, $P<0.001$) observed in the mean concentration with distance from the agar plug containing TCE and food. There was no statistically significant difference observed in the mean concentration of SRB with distance from the agar plug containing TCE and food. The detailed data are provided in Appendix Table A4.

A

B

Figure 4.9. Concentration of microorganisms with distance from the agar plug and with differing TCE concentrations after 7 days in the type 2 reactor. DHC (A), SRB (B) and Archaea (C).

In type 3 reactors, at higher concentration at 50 and 100 mg/L, DHC growth inside capillary has increased while outside capillary has decreased (Figure 4.10). In presence of trapped TCE in a fracture, the concentration of the DHC, SRB and Archaea between outside and inside tube (i.e., fracture) did not differ significantly (Appendix Table A4). The mean DHC percentage changed inside the tube was 42% and outside was 37.7%; mean SRB percentage changed inside the tube was 20.88% and outside was 21.3%; mean Archaea percentage changed inside the tube was 7.2% and outside was 4.14%.

A

B

C

Figure 4.10. Mean and standard deviations of the log gene concentrations of DHC (A), SRB (B) and Archaea (C) with different concentration of TCE after 7 days in the type 3 reactor.

There is a strongly trending correlation between the initial concentration of TCE added and the concentration of DHC in area 1 (Regression, $F=6.604$, $P=0.062$) and area 2 (Regression, $F=55.47$, $P=0.002$) (Figure 4.11) but not in area 3 in type 1 reactor. There is a correlation between the initial concentration of TCE added and the concentration of SRB in area 2 (Regression, $F=14.341$, $P=0.019$) and in area 3 (Regression, $F=8.736$, $P=0.042$) but not in area 1 (Regression, $F=7.060$, $P=0.057$). There is no relationship between the initial concentration of TCE added and the concentration of Archaea in area 1, in area 2 and area 3. In type 2 reactor, there is no linear correlation between the initial concentration of TCE added and the concentration of DHC in area 1, in area 2 and area 3. Also SRB has no correlation to TCE in area 1, area 2 and area 3. Similarly there is no relationship between the initial concentration of TCE added and the concentration of Archaea in area 1, in area 2 and area 3. In type 3 reactor, there is no correlation between TCE concentration and growth of DHC inside the tube and also outside

the tube. Similarly, the SRB has also no correlation with TCE concentration inside and outside of the tube. There is a correlation of Archaea inside the tube (Regression, $F=14.77$, $P=0.018$) with different concentration of TCE but not outside the tube with different concentration of TCE. The detail data is provided in Appendix Table A4.

Figure 4.11. Relationship between DHC gene concentration with respect to different TCE concentration in type 1 and type 2 reactors after 7 days; A: area 1, B: area 2. Regression with * is statistically significant ($P<0.05$).

Figure 4.12. Relationship between SRB gene concentration in three different areas with respect to different TCE concentration in type 1 reactor after 7 days. Regression with * is statistically significant ($P<0.05$).

4.3.3 Specific objective 3: To test the hypothesis that after bioaugmentation DHC, SRB and Archaea concentrations increase due to microbial growth.

Microorganisms were grown in different types of reactor as described as earlier. A total of 10 mg/L of TCE and 0.5 mg/L of sulfate along with sodium lactate and yeast extract was added in the agar to make the food plug. The reactors were incubated over different period of time (0 day to 7 days) and concentration of DHC, SRB and Archaea were quantified by qPCR to observe microbial growth over time.

The concentration of TCE initially added to the reactor affected the observed concentration of micorganisms, but the result was different in each reactor. The microbial populations increased during the 7 days with some exceptions but this was not a constant trend. In type 1 and type 2 reactors, Archaea have increased and SRB may have increased. There was no change of DHC because the growth or doubling time of DHC is 0.8–3 days (Loeffler 2012) and there was no more growth observed after 3 days (Figure 4.13). *Dehalococcoides* are known to be strict anaerobes (Maymo *et al.*, 1997; He *et al.*, 2003a) and while the microcosms were initially slightly aerobic (as evidenced by the resazurin, DHC did survive in these reactors even though oxygen was present. There was an increase in DHC inside the capillary tube in the type 3 reactors, perhaps due to the more anaerobic conditions expected inside the tubes.

In contrast to the data in Figure 4.13 in the studies for hypothesis 1 there was a significant increase in DHC over the 7 days of study. There was 25.7 % and 18% change in DHC (Figure 4.8) for type 1 reactor and type 2 reactors respectively. This may be due to the higher concentration of DHC used in the previous studies (e.g., 50 and 100 mg/L).

Figure 4.13. Average and standard deviations of the log concentrations of DHC, SRB and Archaea gene copies/cm² in type 1 reactor (A), type 2 reactor (B) and type 3 reactor (C) over time

CHAPTER 5 DISCUSSION

Remediation of chlorinated solvent sites are complex, controversial, expensive and time consuming (Stroo & Ward, 2010) because of DNAPL's likelihood to migrate a large distance and create large dissolved phase groundwater plumes. *In situ* remediation has a wide range of challenges and problems. The major problems associated with the biostimulation and bioremediation studied in this research are:

1. Cometabolic mechanism: Physical, chemical and biological processes that occur during remediation.
2. Spatial variability: The spatial variability in the distribution of microorganisms (DHC, SRB and Archaea) and limited amount of spatial information on the relationship between TCE and microorganisms with distance from food source.
3. Fractured clay or bedrock: The dispersion of TCE over large volume and diffusion of TCE in the inaccessible or low permeability zones such as fractured clay or bedrock where TCE can become trapped posing a difficulty for treatment.

To address the above problems three hypothesis were tested: (1) increasing TCE concentrations influence the concentration of DHC, SRB and Archaea; (2) increasing distance from the source of reducing equivalents decrease the concentration of DHC, SRB and Archaea and (3) DHC, SRB and Archaea concentrations increase due to microbial growth after bioaugmentation.

Three different types of reactors were constructed to model three different types of aquifers. Type 1 reactor was constructed in such a way where dispersion of TCE is the major mechanism of TCE distribution. If it is compared to permeable aquifer which consists of highly permeable layers (sandy soil), bounded by an impermeable clay lens on the bottom of the aquifer and free water surface (Ritzema, 1994). In this type of aquifer DNAPL will tend to pool on the bottom of the aquifer and one major mechanism of distribution of the DNAPL is through diffusion and advection. The type 2 reactor constructed in this study can be compared to silt aquifer types, a poorly or semi-permeable layer (Ritzema, 1994), in which movement of DNAPL is trapped inside the silt soil and migration is possible within some distance through diffusion and less due to advection. The type 3 reactors was constructed with capillary tube with food compacted inside

it. If it is compared to relatively impermeable aquifer such as fractured clay or bedrock, TCE might be trapped in pore space due to geological structures which restricts the free movement of water through it (Ritzema, 1994).

5.1 Cometabolic mechanism

In situ bioremediation challenges include the absence or low concentrations of specific microbes, and toxic or inhibitory conditions which restrict the biological, chemical and physical activities (Simkin & Norris 2010). In nature, anaerobic dechlorination is typically carried out by mixed cultures of dechlorinators (Bradley, 2003). Three different species of anaerobic microcosms were studied in this research and modeled the mixed culture of dechlorinators and other microorganisms found in natural aquifers. In general, in this research the Archaea population was the most abundant and SRBs were the least abundant. The dechlorinator DHC competed successfully with these two types of microbes in the studies presented herein. Reductive dechlorination is favored under sulfate and methanogenic conditions (Chapelle 1996, Vogel *et al.* 1987) because *Dehalococcoides* coexist with sulfate reducing bacteria and methanogens by getting necessary micronutrients (Duhamel et al 2007, Yager 1997). In the type 1 reactor, there was a linear correlation between increasing concentrations of TCE and DHC and SRB concentrations. In type 2 and 3 reactors, there was no observed correlation between initial concentration of TCE with the average concentration of DHC, SRB and Archaea in all areas. The microbial populations increased during the 7 days with some exceptions but this was not a constant trend.

5.2 Spatial variability

One of the problems associated in remediation of chlorinated solvent is the spatial variability and limited amount of spatial information on the distribution of TCE and microorganisms (DHC, SRB and Archaea) with distance from food source. In this study, spatial variability in the distribution of TCE (electron acceptor) and the distribution of microorganisms (DHC, SRB and Archaea) with distance from food source in different reactor types compared to different types of natural aquifers was analyzed. Spatially, the highest concentrations of Archaea were present in

type 1 reactor. DHC was located more near the food source (area 1 and area 2) than the further zones. In type 1 reactor type, TCE might diffuse throughout the reactor and the TCE concentration would probably be the same everywhere because TCE concentration gradient is lower. DHC was found less in area 3 than area 1 and 2 which might be due to less availability of food. However, these organisms still obtain diffused TCE and they attach where the substrate is located (area 1 and 2). Though area 2 has higher growth than area 1, it can be assumed that DHC attached to the food might get poisoned from high concentration of food itself or accumulation of toxic byproducts and pose problems or inhibit microbial growth (Simkin & Norris, 2010). For example, high concentrations of some chemicals such as TCE, are toxic to microbes and have been shown to result in decrease of microbial population (Talley 2006) which might be due to sudden changes in the pH or geochemical conditions or degradation byproducts (Simkin & Norris 2010).

The spatial variability in type 1 reactor representing sandy aquifer is presented Figure 5.1 in which TCE from the DNAPL is diffused throughout the reactor. TCE is present over a large distance. From the study it was observed that in a permeable (sandy) type of aquifer, type 1, DHC, SRB and Archaea concentration did not change significantly with the increasing distance from the food source as shown in Figures 4.8. Movement or flow of groundwater might carry DHC along with it to the zone farther away from the food amendment and there is still possibility of the growth of these microbes in presence of less substrate also. Hence, the spatial distribution of these microorganisms suggests that significant savings are possible with this bioaugmentation approach. In natural environment, bioaugmentation and biostimulation of substrate or reagents can be performed only at a certain point by measuring necessary distances which saves money on reagents as well as on culture. It also helps in designing of injection wells spacing, screen interval and location thus potentially presenting cost savings.

Figure 5.1. Possible location of microorganisms in type 1 reactor in which most microbes are grown near the food amendment competing for electron donors while they still grow farther from food amendment where TCE is present due to diffusion.

In the type 2 reactor, TCE concentration gradients are more significant, thus, there may be lower concentrations of TCE at farther distances from the DNAPL. These types of reactors can be compared to semi-permeable silt aquifers. In this case, the TCE and food amendments gradient decrease together with distance from say a groundwater well injection point and the microbes more directly compete for food than in the type 1 reactor where these components are more diffuse. The possible distribution of microorganisms and food amendment is presented in Figure 5.2. In type 2 reactors, there were decreasing concentrations of these microorganisms or even no microorganisms detected in area 3 or in zones far from the food. It was observed very less and/or no microorganisms at further zones from the food. Hence, in lower permeability aquifers, the maximum concentration of microorganisms is located near the food and TCE source.

Figure 5.2. Possible location of microorganisms in type 2 reactor in which TCE and food amendment are together and the microbes compete for them. At farther zone from food there is less and no growth of those microorganisms.

5.3 Fractured clay or bedrock

Another problem associated in groundwater remediation of TCE is the migration of contamination to the fractured clay or bedrock. The chlorinated solvents present in the fractured clay or bedrock sites are difficult to treat effectively. Due to complex hydro-geologic conditions, remediation is challenging (Simpkin & Norris, 2010). There is difficulty in distributing bioremediation amendments or food in these types of aquifers. Pfiffner et al. (2000) found microbial counts decreased with higher clay content in the sediments. They concluded that the microbial communities can be dependent on the spatial distribution of key physical and chemical properties of the soil matrix. Low permeability or a high degree of heterogeneity limits the distributing of the bioremediation substrate effectively throughout the aquifer (Henry, 2010). The type 3 reactor can be compared to fractures in impermeable layers such as bedrock or lower permeability clay. For effective bioremediation, electron donor and microorganism contact with contaminants is essential. In this study, lower concentrations of microorganisms inside the tube were observed in type 3 reactor (Figure 4.10). Microorganisms might have migrated into the

capillary tube modeling a fracture contaminant DNAPL in a real aquifer due to migration of microorganisms towards the food. Microorganisms added outside the fracture might migrate towards the food present inside the fracture hence competing for TCE and food. In Figure 5.3, the food amendment is inside the fracture or fissure where DNAPL migration took place and the microorganisms were injected outside the capillary tube or fracture. Outside the capillary tube has higher concentrations of these microorganisms and also there was growth observed inside the tube. The microorganisms might get contact with food through small pore volumes or fracture volumes and suspended successfully inside the fractures. Therefore, this study might be important in evaluating site conditions and cost-effective delivery of amendments.

Figure 5.3. Fracture flow microorganisms in type 3 reactor in which TCE and food amendment are together but the space to migrate towards the food is very low. Microbes compete for the TCE located inside the clay as well as water, due to compact structure of clay particle those microbes try to find food.

CHAPTER 6 CONCLUSIONS AND RECOMMENDED FUTURE RESEARCH

6.1 Conclusions

In this research the Archaea population was the most abundant and SRBs were the least abundant. The dechlorinator DHC competed successfully with these two types of microbes in the studies presented herein. Spatially, the highest concentrations of Archaea were present in type 1 reactor. In type 1 reactor, increasing concentration of TCE increased DHC concentration. There was a linear correlation between increasing concentrations of TCE and DHC concentrations and concentrations of TCE and SRB and a trending relationship between TCE and the concentration of Archaea. The percentage change in average DHC was 25.7 %. In type 2 reactors, no observed correlation between initial concentration of TCE and the average concentration of DHC, concentration of TCE and between average concentration of SRB and the average concentration of Archaea. The percentage change in average DHC was 18%. In type 3 reactor, there was increase of 33 to 42%. There was not a correlation between the concentration of TCE and the average concentration of DHC, Archaea and SRB.

DHC was more likely to be located near the food source (area 1 and area 2) than farther from the food. In a permeable (sandy) type of aquifer, type 1, DHC, SRB and Archaea concentration did not change significantly with increasing distance from the food source. In type 2 reactors in this study, there were decreasing concentrations of these microorganisms or even no microorganisms detected in area 3 or in zones far from the food. Hence, in lower permeability aquifers, the distribution of the maximum microorganisms is near the food and TCE source. The type 3 reactor used in this study modeled fractures in impermeable layers such as bedrock or lower permeability clay. Lower concentrations of microorganisms inside the tube were observed in type 3 reactor. Outside the capillary tube were higher concentrations of microorganisms, however growth was observed inside the tube. Growth of microorganisms inside the tube suggests that microorganisms might grow successfully inside the fractures even with very high TCE concentrations.

Hence, it is concluded that various concentrations of TCE and the location of food source affects the population and distribution of DHC, SRB and Archaea. In sandy and silty aquifers, DHC concentrations may decline with distance from a TCE source. In the type 3 reactor (fractured rock), the concentration of the DHC, SRB and Archaea between outside and inside tube did not differ significantly.

6.2 Recommendations for Future Research

Because the set-up of this experiment did not emulate a natural environment, it is thought that the application of these products in the field should be tested before any decision is made but it is believed that this test provides a database for further evaluations. The distance from food source in cms is not practical in the field study. The population of microorganism according to food distance provides useful information to help estimate the amounts of sodium lactate for augmentation in order to obtain removal of TCE and thus it is an important cost component of the treatment system. The concentration of sulfate, TCE and sodium lactate and density of culture are the main factors which help to understand the distribution of DHC, SRB and Archaea. The data found in the laboratory should be matched with field observations or injections of cultures in bench scale soil microcosms. Since primary substrate (electron-donor) injection are among the major cost components of an *in situ* treatment system, methods to grow DHC, SRB and Archaea coexistence are important. The laboratory study suggests that the addition of low sulfate if necessary may help by providing necessary energy without inhibiting TCE degradation.

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APPENDIX

Table A1. Standard Curve for Sulfate Analysis

Sulfate mM	Absorbance at 420 nm
1	0.275
2	0.493
3	0.78
4	1.06
5	1.272

Figure A1. Standard curve for sulfate analysis

Table A2. Standard Curve for COD Analysis

Absorbance at 420 nm	COD mg/L
0.379	0
0.376	0
0.378	0
0.363	10
0.344	20
0.337	20
0.314	30
0.311	30
0.267	50
0.207	75
0.212	75
0.153	100

Figure A2. Standard curve for COD analysis

Table A3. Detailed results for DHC, SRB and Archaea from qPCR analysis in various reactor types and experimental conditions.

Reactor type	TCE concentration mg/L	Sulfate concentration mg/L	Other	Cells added μ l	Area scrapped	DHC (copies/cm ²)	SRB (copies/cm ²)	Archaea (copies/cm ²)
1	100	10		100	1	NA	5.24x10 ¹⁰	1.30x10 ¹⁵
1	100	10		100	2	NA	6.09x10 ¹⁰	1.34x10 ¹⁵
1	100	10		100	3	2.46x10 ⁹	3.22x10 ¹⁰	1.35x10 ¹⁵
1	100	10	Sod. azide	100	1	NA	NR	NR
1	100	10	Sod. azide	100	2	NA	NR	NR
1	100	10	Sod. azide	100	3	NA	NR	NR
1	100	10		10	1	2.18x10 ¹⁰	2.46x10 ¹¹	5.07x10 ¹⁵
1	100	10		10	2	3.25x10 ⁹	2.72x10 ¹¹	6.00x10 ¹⁵
1	100	10		10	3	7.89x10 ⁸	1.49x10 ¹¹	3.86x10 ¹⁵
1	100	0.5		100	1	6.95x10 ¹⁰	1.47x10 ⁹	1.80x10 ¹⁴
1	100	0.5		100	2	7.94x10 ¹⁰	6.88x10 ⁹	3.87x10 ¹³
1	100	0.5		100	3	1.71x10 ¹⁰	8.27x10 ⁹	4.08x10 ¹³
1	100	0.5		10	1	NA	NR	NR
1	100	0.5		10	2	NR	NR	NR
1	100	0.5		10	3	NR	NR	NR
1	100	10		0	1	NA	1.97x10 ¹⁰	7.42x10 ¹³
1	100	10		0	2	NA	1.04x10 ¹⁰	8.59x10 ¹³
1	100	10		0	3	NA	7.20x10 ⁹	4.19x10 ¹³
1	10	0.5		100	1	NA	1.30x10 ¹⁰	3.07x10 ¹³
1	10	0.5		100	2	NA	1.15x10 ¹⁰	2.30x10 ¹³
1	10	0.5		100	3	NA	7.37x10 ⁸	2.13x10 ¹⁴
1	10	0.5		10	1	1.75x10 ¹¹	1.28x10 ¹¹	1.46x10 ¹⁶
1	10	0.5		10	2	1.40x10 ¹¹	1.66x10 ¹¹	1.90x10 ¹⁶
1	10	0.5		10	3	5.17x10 ¹⁰	7.54x10 ¹⁰	3.84x10 ¹⁵
1	10	10		10	1	5.88x10 ⁹	8.59x10 ¹⁰	1.77x10 ¹⁶

Reactor type	TCE concentration mg/L	Sulfate concentration mg/L	Other	Cells added μ l	Area scrapped	DHC (copies/cm ²)	SRB (copies/cm ²)	Archaea (copies/cm ²)
1	10	10		10	2	5.57x10 ⁷	1.07x10 ¹¹	1.32x10 ¹⁶
1	10	10		10	3	NA	NA	NA
1	10	10		100	1	4.51x10 ¹⁰	1.21x10 ¹¹	NA
1	10	10		100	2	8.73x10 ¹⁰	1.63x10 ¹¹	NA
1	10	10		100	3	3.77x10 ⁹	5.80x10 ¹⁰	NA
1	10	10		100	1	1.47x10 ¹¹	3.16x10 ¹¹	NA
1	10	10		100	2	1.36x10 ¹¹	2.27x10 ¹¹	NA
1	10	10		100	3	1.00x10 ¹¹	1.00x10 ¹¹	NA
1	10	10		0	1	2.15x10 ¹⁰	1.94x10 ¹¹	NA
1	10	10		0	2	NA	3.20x10 ¹¹	NA
1	10	10		0	3	NA	1.39x10 ¹¹	NA
1	0.1	10		100	1	NA	NR	NR
1	0.1	10		100	2	NA	NR	NR
1	0.1	10		100	3	NA	NR	NR
1	0.1	10		10	1	NA	5.04x10 ¹⁰	6.64x10 ¹⁴
1	0.1	10		10	2	NA	7.53x10 ¹⁰	7.10x10 ¹⁴
1	0.1	10		10	3	NA	5.05x10 ¹⁰	6.52x10 ¹⁴
1	0.1	10		100	1	NA	NR	NA
1	0.1	10		100	2	NA	NR	NA
1	0.1	10		100	3	NA	NR	NA
1	0.1	0.5		10	1	NA	NR	NR
1	0.1	0.5		10	2	NA	NR	NR
1	0.1	0.5		10	3	NA	NR	NR
1	0.1	0.5		100	1	NA	NR	NR
1	0.1	0.5		100	2	NA	NR	NR
1	0.1	0.5		100	3	NA	NR	NR
1	0.1	10		0	1	NA	NR	NR
1	0.1	10		0	2	NA	NR	NR

Reactor type	TCE concentration mg/L	Sulfate concentration mg/L	Other	Cells added μ l	Area scrapped	DHC (copies/cm ²)	SRB (copies/cm ²)	Archaea (copies/cm ²)
1	0.1	10		0	3	NA	NR	NR
2	100	10		10	1	5.03x10 ⁹	1.20x10 ¹¹	6.86x10 ¹⁵
2	100	10		10	2	NA	1.06x10 ¹¹	6.27x10 ¹⁵
2	100	10		10	3	NA	3.93x10 ¹⁰	3.89x10 ¹⁵
2	100	10	Sod. azide	10	1	5.06x10 ⁷	2.33x10 ⁹	3.80x10 ¹³
2	100	10	Sod. azide	10	2	NA	NR	NR
2	100	10	Sod. azide	10	3	NA	NR	NR
2	100	10		1	1	2.47x10 ⁹	3.41x10 ¹¹	7.50x10 ¹⁵
2	100	10		1	2	1.41x10 ⁹	3.08x10 ¹¹	6.16x10 ¹⁵
2	100	10		1	3	2.37x10 ⁹	2.46x10 ¹¹	8.52x10 ¹³
2	100	0.5		10	1	NA	9.92x10 ⁷	3.20x10 ¹¹
2	100	0.5		10	2	NA	2.65x10 ⁸	2.36x10 ¹⁰
2	100	0.5		10	3	2.23x10 ⁹	4.40x10 ⁷	2.03x10 ¹¹
2	100	0.5		1	1	NA	6.41x10 ⁸	4.52x10 ¹³
2	100	0.5		1	2	NA	NR	NR
2	100	0.5		1	3	NA	NR	NR
2	10	0.5		10	1	NA	3.16x10 ⁸	1.10x10 ¹³
2	10	0.5		10	2	NA	1.30x10 ⁸	1.14x10 ¹⁴
2	10	0.5		10	3	NA	NA	NA
2	10	0.5		1	1	NA	9.94x10 ⁸	1.78x10 ¹²
2	10	0.5		1	2	NA	NA	NA
2	10	0.5		1	3	NA	NA	NA
2	10	10		1	1	4.55x10 ⁹	6.85x10 ¹⁰	2.19x10 ¹⁵
2	10	10		1	2	7.51x10 ⁸	5.40x10 ¹⁰	6.79x10 ¹⁴
2	10	10		1	3	1.90x10 ⁸	3.73x10 ⁹	4.37x10 ¹⁴
2	10	10	Sod Azide	10	1	NA	NR	NR
2	10	10	Sod Azide	10	2	NA	NR	NR
2	10	10	Sod Azide	10	3	NA	NR	NR

Reactor type	TCE concentration mg/L	Sulfate concentration mg/L	Other	Cells added μ l	Area scrapped	DHC (copies/cm ²)	SRB (copies/cm ²)	Archaea (copies/cm ²)
2	10	10		10	1	3.14x10 ⁹	2.59x10 ¹¹	5.59x10 ¹⁵
2	10	10		10	2	1.85x10 ⁹	1.53x10 ¹¹	3.50x10 ¹⁵
2	10	10		10	3	7.41x10 ⁷	1.33x10 ¹¹	NA
2	0.1	10		10	1	1.42x10 ¹⁰	6.12x10 ¹⁰	NA
2	0.1	10		10	2	NA	6.28x10 ¹⁰	NA
2	0.1	10		10	3	3.11x10 ⁷	1.84x10 ¹⁰	NA
2	0.1	10		1	1	NA	2.35x10 ¹⁰	NA
2	0.1	10		1	2	NA	4.98x10 ¹⁰	NA
2	0.1	10		1	3	2.23x10 ⁹	2.58x10 ¹⁰	NA
2	0.1	10	Sod Azide	10	1	NA	NR	NR
2	0.1	10	Sod Azide	10	2	NA	NR	NR
2	0.1	10	Sod Azide	10	3	NA	NR	NR
2	0.1	0.5		1	1	NA	2.37x10 ⁸	5.21x10 ¹¹
2	0.1	0.5		1	2	NA	4.57x10 ⁸	8.36x10 ¹¹
2	0.1	0.5		1	3	NA	4.21x10 ¹⁰	1.01x10 ¹⁵
2	0.1	0.5		10	1	NA	NA	8.62x10 ¹⁵
2	0.1	0.5		10	2	NA	NA	8.42x10 ¹⁴
2	0.1	0.5		10	3	NA	NR	NR
2	100	10		0	1	NA	NR	NR
2	100	10		0	2	NA	NR	NR
2	100	10		0	3	NA	NR	NR
2	10	10		0	1	NA	NR	NR
2	10	10		0	2	NA	NR	NR
2	10	10		0	3	NA	NR	NR
2	0.1	10		0	1	NA	NR	NR
2	0.1	10		0	2	NA	NR	NR
2	0.1	10		0	3	NA	NR	NR
3	100	10		10	out	2.80x10 ⁸	4.07x10 ⁹	1.61x10 ¹⁶

Reactor type	TCE concentration mg/L	Sulfate concentration mg/L	Other	Cells added µl	Area scrapped	DHC (copies/cm ²)	SRB (copies/cm ²)	Archaea (copies/cm ²)
3	100	10		10	tube	3.06x10 ¹⁰	1.61x10 ⁹	2.64x10 ¹²
3	100	10	Sod. azide	10	out	NA	NR	NR
3	100	10	Sod. azide	10	tube	NA	NR	NR
3	100	10		1	tube	7.76x10 ⁹	6.33x10 ⁸	6.08x10 ¹³
3	100	10		1	out	1.18x10 ¹⁰	4.41x10 ⁹	7.50x10 ¹⁴
3	100	0.5		10	out	6.06x10 ¹⁰	2.85x10 ⁹	1.39x10 ¹²
3	100	0.5		10	tube	4.92x10 ⁹	1.83x10 ⁸	NA
3	100	0.5		1	tube	NA	4.88x10 ⁹	1.29x10 ¹²
3	100	0.5		1	out	NA	2.76x10 ⁹	8.54x10 ¹¹
3	10	0.5		10	tube	NA	2.75x10 ⁸	1.73x10 ¹²
3	10	0.5		10	out	NA	9.33x10 ⁸	2.92x10 ¹¹
3	10	0.5		1	tube	NA	2.35x10 ⁸	3.36x10 ¹²
3	10	0.5		1	out	NA	2.92x10 ⁹	4.66x10 ¹¹
3	10	10		1	out	8.13x10 ¹⁰	2.91x10 ⁺¹⁰	1.40x10 ¹⁴
3	10	10		1	tube	7.42x10 ¹⁰	2.07x10 ⁹	NA
3	10	10	Sod Azide	10	out	NA	NR	NR
3	10	10	Sod Azide	10	tube	NA	NR	NR
3	10	10		10	out	8.98x10 ¹⁰	3.25x10 ¹⁰	4.90x10 ¹²
3	10	10		10	tube	9.09x10 ¹⁰	3.61x10 ⁹	1.29x10 ¹²
3	0.1	10		10	out	2.29x10 ¹¹	NA	NA
3	0.1	10		10	tube	NA	NA	NA
3	0.1	10		1	out	NA	NA	NA
3	0.1	10		1	tube	NA	NA	NA
3	0.1	10	Sod Azide	10	out	NA	NR	NR
3	0.1	10	Sod Azide	10	tube	NA	NR	NR
3	0.1	0.5		1	out	NA	NA	NA
3	0.1	0.5		1	tube	NA	NR	NR
3	0.1	0.5		10	out	NA	NR	NA

Reactor type	TCE concentration mg/L	Sulfate concentration mg/L	Other	Cells added μ l	Area scrapped	DHC (copies/cm ²)	SRB (copies/cm ²)	Archaea (copies/cm ²)
3	0.1	0.5		10	tube	NA	NR	NA
3	100	10		0	out	NA	NR	NR
3	100	10		0	tube	NA	NR	NR
3	10	10		0	out	NA	NR	NR
3	10	10		0	tube	NA	NR	NR
3	0.1	10		0	out	NA	NR	NR
3	0.1	10		0	tube	NA	NR	NR
1	0	10		10		7.15x10 ¹⁰	2.60x10 ⁹	7.77x10 ¹²
1	0	10		10		7.71x10 ¹⁰	2.09x10 ⁸	1.43x10 ¹³
1	0	10		10		3.84x10 ¹⁰	1.77x10 ⁹	2.08x10 ¹²
2	0	10		10		1.38x10 ¹⁰	4.69x10 ⁸	4.92x10 ¹³
2	0	10		10		9.09x10 ⁹	4.06x10 ⁸	1.67x10 ¹³
2	0	10		10		6.94x10 ⁸	4.86x10 ⁷	1.40x10 ¹³
3	0	10		10	out	1.39x10 ¹²	2.59x10 ¹⁰	5.34x10 ¹²
3	0	10		10	tube	2.66x10 ¹¹	5.18x10 ⁹	5.53x10 ¹²

NA= Not Amplified

NR= No Run

Table A4. Statistical tests for different reactors and microorganisms

Reactor Type	Microorganisms	Area	Statistical test	Coefficient	P	Remarks
1	DHC	1 vs 2	Student's t-test	t=-0.132	0.896	Not significant
		2 vs 3	Student's t-test	t=1.882	0.089	Not significant
		1 vs 3	Student's t-test	t=1.434	0.168	Not significant
	SRB	1 vs 2	Student's t-test	t=1.218	0.238	Not significant
		2 vs 3	Student's t-test	t=1.313	0.205	Not significant
		1 vs 3	MWRS	T=231	0.967	Not significant
	Archaea	1 vs 2	Student's t-test	t=-0.0182	0.986	Not significant
		2 vs 3	Student's t-test	t=-0.526	0.605	Not significant
		1 vs 3	MWRS	T=40	0.937	Not significant
2	DHC	1 vs 2	Student's t-test	t=2.446	0.031	Significant
		2 vs 3	MWRS	T=137.5	0.827	Not significant
		1 vs 3	MWRS	T=135	0.029	Significant
	SRB	1 vs 2	MWRS	T=398	0.1	Not significant
		2 vs 3	MWRS	T=777	0.427	Not significant
		1 vs 3	MWRS	T=522	0.302	Not significant
	Archaea	1 vs 2	MWRS	T=553	0.461	Not significant
		2 vs 3	MWRS	T=498	0.018	Significant
		1 vs 3	MWRS	T=390	<0.001	Significant
1	DHC	1 vs T ₀	Linear Regression	F=6.604	0.062	Linear correlation
		2 vs T ₀	Linear Regression	F=55.47	0.002	Linear correlation
		3 vs T ₀	Linear Regression	F=1.860	0.244	No linear correlation
	SRB	1 vs T ₀	Linear Regression	F=7.060	0.057	Linear correlation
		2 vs T ₀	Linear Regression	F=14.341	0.019	Linear correlation
		3 vs T ₀	Linear Regression	F=8.736	0.042	Linear correlation
	Archaea	1 vs T ₀	Linear Regression	F=0.0903	0.772	No linear correlation
		2 vs T ₀	Linear Regression	F=3.969	0.117	No linear correlation
		3 vs T ₀	Linear Regression	F=2.459	0.192	No linear correlation
2	DHC	1 vs T ₀	Linear Regression	F=0.775	0.410	No linear correlation
		2 vs T ₀	Linear Regression	F=0.264	0.611	No linear correlation
		3 vs T ₀	Linear Regression	F=0.00255	0.962	No linear correlation

Reactor Type	Microorganisms	Area	Statistical test	Coefficient	P	Remarks
3	SRB	1 vs T ₀	Linear Regression	F=0.338	0.570	No linear correlation
		2 vs T ₀	Linear Regression	F=1.433	0.297	No linear correlation
		3 vs T ₀	Linear Regression	F=0.299	0.673	No linear correlation
	Archaea	1 vs T ₀	Linear Regression	F=0.262	0.630	No linear correlation
		2 vs T ₀	Linear Regression	F=1.292	0.319	No linear correlation
		3 vs T ₀	Linear Regression	F=0.129	0.738	No linear correlation
	DHC	In vs T ₀	Linear Regression	F=0.126	0.777	No linear correlation
		Out vs T ₀	Linear Regression	F=1.502	0.288	No linear correlation
	SRB	In vs T ₀	Linear Regression	F=0.857	0.407	No linear correlation
		Out vs T ₀	Linear Regression	F=0.0357	0.859	No linear correlation
	Archaea	In vs T ₀	Linear Regression	F=14.77	0.018	Linear correlation
		Out vs T ₀	Linear Regression	F=0.611	0.478	No linear correlation
1	DHC	Avg area vs T ₀	Linear Regression	F=12.265	0.025	Linear correlation
	SRB	Avg area vs T ₀	Linear Regression	F=14.756	0.018	Linear correlation
	Archaea	Avg area vs T ₀	Linear Regression	F=5.820	0.073	Linear correlation
2	DHC	Avg area vs T ₀	Linear Regression	F=0.000201	0.989	No linear correlation
	SRB	Avg area vs T ₀	Linear Regression	F=0.0175	0.901	No linear correlation
	Archaea	Avg area vs T ₀	Linear Regression	F=1.105	0.353	No linear correlation
3	DHC	Avg area vs T ₀	Linear Regression	F=0.0284	0.869	No linear correlation
	SRB	Avg area vs T ₀	Linear Regression	F=8.258	0.017	Linear correlation
	Archaea	Avg area vs T ₀	Linear Regression	F=0.142	0.714	No linear correlation
3	DHC	In vs Out	MWRS	T=172	0.214	Not significant
	SRB	In vs Out	Student's t-test	t=-0.140	0.892	Not significant
	Archaea	In vs Out	Student's t-test	T=0.447	0.447	Not significant
1	DHC	Over 7 days	Linear Regression	F=0.104	0.753	No linear correlation
	SRB	Over 7 days	Linear Regression	F=5.755	0.037	Linear correlation
	Archaea	Over 7 days	Linear Regression	F=0.304	0.594	No linear correlation

Reactor Type	Microorganisms	Area	Statistical test	Coefficient	P	Remarks
2	DHC	Over 7 days	Linear Regression	F=0.990	0.376	No linear correlation
	SRB	Over 7 days	Linear Regression	F=2.585	0.183	No linear correlation
	Archaea	Over 7 days	Linear Regression	F=21.253	0.010	Linear correlation
MWRS = Mann Whitney Rank sum						
T ₀ = Initial concentration of TCE

Figure A3. The log concentrations of DHC, SRB and archaea in type 1 and 2 reactor with all distances.

Figure A4. Relative abundance of dechlorinators maintained over the course of 7 days (type 3) reactor. Relative abundance was calculated from qPCR data with genus-specific primer sets.

Figure A5. Archaea cells observed on confocal microscope after FISH stained with DAPI and Arch-647 Alexa probe from the seed reactor

Figure A6. Sulfate reducing bacteria observed on confocal microscope after FISH stained with DAPI and SRB-555 Alexa probe from the seed reactor

Figure A7. Co-distribution of DHC, sulfate reducing bacteria and Archaea in a sludge granule from the seed reactor observed by confocal microscope after FISH staining with DAPI and DHC fluorescein probe, SRB-555 Alexa probe and Arch-647 Alexa probe together (from right to left first is DAPI stained, second DHC, third SRB (upper row), Archaea and last one is all dyes together (lower row)).