Adaptation of a Dechlorinating Culture, KB-1, to Acidic Environments

by

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Abstract

KB-1 is an anaerobic *Dehalococcoides*-containing microbial culture used industrially to bioremediate sites impacted with chlorinated solvents. The culture is typically grown at pH 7. However, lower pH is often encountered and therefore the effect of pH was investigated. Both sudden and stepwise decreases in pH from 7 to 6 and 5.5 were investigated over a period of 450 days. An electron balance was also calculated to look at the flow of electrons for dechlorination. More than 95% of the reducing equivalents went towards methanogenesis and acetogenesis. Select microorganisms were compared by quantitative Polymerase Chain Reaction. It was found that lower rates of dechlorination correspond to low *Dehalococcoides* numbers and that different methanogens were enriched on different electron donors.

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Table of Contents

List of Tables

List of Figures

List of Equations

Abbreviations and Nomenclature

µL	Microliter
µM	Micromolar
cDCE	1, 2-cis-dichloroethene
DGGE	Denaturing gradient gel electrophoresis
DNAPL	Dense non-aqueous phase liquids
H_2	Hydrogen
H_2O	Water
HCl	Hydrochloric acid
L	Liter
MCL	Maximum contaminant level
mg/L	Milligrams per liter
mL	Milliliter
N	Normal
OTU	Operational taxonomic unit
PCE	Tetrachloroethene
pH	Potential of hydrogen
qPCR	Quantitative polymerase chain reaction
TCE	Trichloroethene
USEPA	U.S. Environmental Protection Agency
v/v	Volume by volume
VC	Vinyl chloride

Chapter 1: Introduction

1.1 Chlorinated Ethenes as Groundwater Contaminants

Tetrachloroethene (PCE) and trichloroethene (TCE) are chlorinated organic solvents used frequently in dry cleaning and degreasing operations. Due to unknown health risks and corresponding negligent use of these volatile organics during the 1930s, PCE and TCE are among the most common groundwater pollutants in North America (Moran 2006). According to the U.S. Environmental Protection Agency's (USEPA) toxic release inventory, between the years 1998 and 2001 releases of PCE and TCE totaled 4 and 11 million pounds, respectively. The improper disposal of these volatile organics causes them to leach into soil and groundwater. Due to their low solubility and high density, they form an immiscible layer in the subsurface of the groundwater, known as dense non-aqueous phase liquids (DNAPLs), which can become long-term sources of contamination.

Research has shown that PCE and TCE are potential carcinogens that may affect the central nervous system or liver (Pandey 2009). Due to these health effects, regulations on the disposal and drinking water limits of PCE and TCE have become more stringent. The USEPA has set maximum contaminant levels (MCLs) for the presence of PCE and TCE in drinking water (Pandey 2009). In Canada, TCE is not manufactured but is regulated under the *Canadian Environmental Protection Act, 1999.* Many contaminated sites impact drinking water supplies; thus, there is a need to find more efficient ways to remediate PCE and TCE contaminated sites.

1.2 Bioremediation

Conventional methods for treating contaminated sites involve 'pump-and-treat' technologies, an *ex situ* process where the contaminated groundwater is pumped to the surface and treated by various processes such as air stripping, carbon treatment and/or chemical oxidation (Mackay 1989). Pump-and-treat is often a process that is expensive

and at times ineffective over the long term. Alternatively, *in situ* bioremediation involves the implementation of microbial metabolic capabilities under natural environmental conditions without the need for excavation of the contaminated site (He 2007). Both aerobic and anaerobic microorganisms can be used but anaerobic bioremediation is preferred in contaminated soil and groundwater due to the difficulty of introducing oxygen into these environments. The microorganisms catalyze a reduction-oxidation reaction between an electron donor and electron acceptor to yield energy for cell growth. In particular, chlorinated compounds are anaerobically biodegraded through a process called reductive dechlorination where the chlorinated compound is the electron acceptor and hydrogen is the electron donor as depicted in Figure 1.

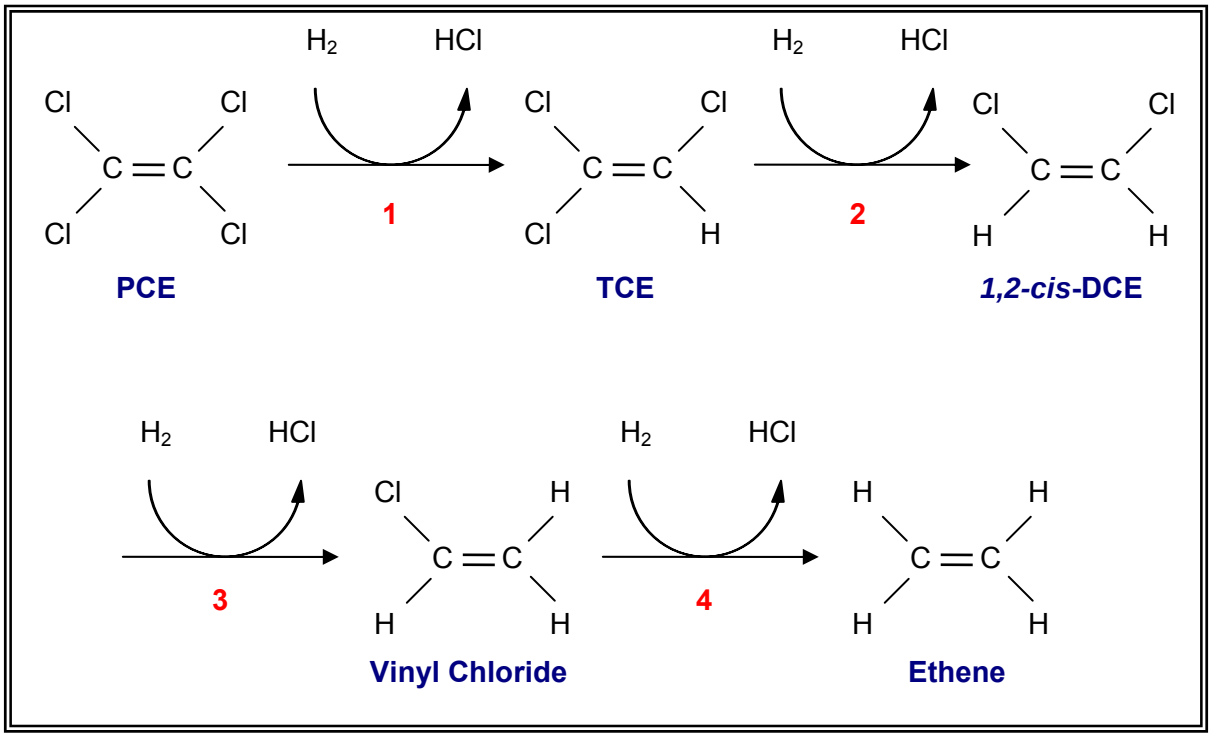

Figure 1: Reductive Dechlorination Pathway of Perchloroethene.
Each dechlorination step requires one hydrogen atom to replace a chlorine atom which then forms hydrochloric acid. The complete dechlorination of one mole of PCE produces four moles of hydrochloric acid. (Duhamel 2005)

The reductive dechlorination of PCE occurs in a step-wise fashion, converting PCE into TCE, 1,2-cis-dichloroethene (cDCE), vinyl chloride (VC), and finally ethene (Figure 1). At each step in the pathway, a chlorine atom is removed and replaced with a

hydrogen atom, producing the product hydrochloric acid (HCl). The complete dechlorination of one mole of PCE produces 4 moles of HCl and 1 mole of ethene. The degradation of PCE to cDCE is a fast process relative to the degradation of VC to ethene. This may lead to accumulation of VC and becomes problematic, since VC is a known human carcinogen and the most toxic of all chlorinated ethenes.

In situ bioremediation can be accomplished with two different approaches: biostimulation and bioaugmentation. Biostimulation involves stimulation of the natural microbial population by the injection of nutrients, electron donors, or a carbon source. Bioaugmentation is the introduction of an exogenous bacterial consortium as well as electron donors into the contaminated site to improve degradation capabilities. Several mixed bacterial consortia capable of reductive dechlorination of PCE (Duhamel 2005; Pant 2010) have been described. Within these cultures, there are several different bacterial genera known to dechlorinate PCE to cDCE but, to date, *Dehalococcoides* is the only genus known to mediate complete dechlorination to ethene (He 2003; Smidt 2004).

1.3 Groundwater Acidification of Chlorinated Ethene Contaminated Sites

Acidification of groundwater at contaminated sites is a frequent problem. A drop in pH can cause a significant reduction in degradation rates or alteration of the solubility of the target chemical for *in situ* bioremediation of chlorinated compounds such as PCE and TCE (Duhamel 2002; McCarty 2007; Brovelli 2012; Lacroix 2012). One of the main causes of groundwater acidification is when bioremediation is applied at sites with high TCE concentrations, leading to the release of substantial levels of hydrochloric acid. Another cause of groundwater acidification is the introduction of organic substrates, which are common electron donors used in biostimulation and bioaugmentation. These substrates are indirect electron donors that need to be fermented into hydrogen, the direct electron donor utilized by the microorganisms involved in dechlorination. Common organic substrates that are fermented into hydrogen include lactate and methanol; the general formula is depicted below:

Equation 1:

$$organic\ substrate + aH_2O = bCH_3COOH + cH_2 + dCO_2 \quad \text{(Lacroix 2012)}$$

The fermentation products are listed on the right and the stoichiometric coefficients (*a, b, c, d*) vary according to the organic substrate used. Fermentation of the organic substrate and the dechlorination process itself result in the production of acetic acid and hydrochloric acid. The production of excess acids released into the groundwater system interferes with the natural buffering capacity of the ambient groundwater and soil causing the pH to drop (McCarty 2007; Robinson 2009; Lacroix 2012). The acidification may also add undesirable organic compounds to the aquifer which can lead to further degradation of water quality through iron, manganese, or sulfate reduction, and methane formation (McCarty 2007).

1.4 Methods for Bioremediation of Low pH Aquifers

Over 50 chlorinated ethene-contaminated sites have been reported with pH ranging between 5.5 and 7, with a few sites lower than 5 (Leeson 2004); almost all of which experienced a drop in pH during bioremediation. These acidic conditions can limit microbial degradation due to the inactivation of anaerobic bacteria at low pH (Lacroix 2012). When the natural buffering capabilities of a site have been exceeded or have alkalinities of less than 100 mg/L, implementation of a pH control system may be necessary (Leeson 2004). During bioremediation, these systems should be carefully evaluated for substrate selection, substrate loading rate, and the addition of buffering reagents.

Common methods to control pH in the field involve circulation of buffer solutions containing dissolved alkaline materials such as sodium or potassium bicarbonate (Robinson 2009). Alternative strategies include the use of silicate minerals (Lacroix 2012) or the use of encapsulated buffers (Rust 2002). For silicate materials, when acidity is produced, the minerals dissolve until a near-neutral pH is reached. This prevents groundwater pH from increasing into the alkaline range. On the other hand,

encapsulated buffers involve a pH-sensitive polymer that has a triggered release of a phosphate buffer (Rust 2002). These capsules are capable of releasing the buffer through pulses that can effectively dissolve over a period of time. Several problems can arise with these methods of pH adjustment: i) shocking of the system, causing reduced performance of methanogens and dechlorinators; ii) difficulties in buffer distribution and diffusion through the soil iii) precipitation of metals and/or salts that could clog well screens and reduce soil permeability and iv) keeping soluble buffering agents in the aquifer so that they are not washed out by groundwater flow (Hickey 2010).

An alternative to pH adjustment is to develop an enrichment culture capable of dechlorination within the low pH environment. Common dehalogenating bacteria have an optimal range between 6.8 – 7.6 (DiStefano 1991; Duhamel 2002; Adamson 2004). There are currently no cultures described in the literature that perform dechlorination outside of this pH range that are currently used in the field. However, Hickey's thesis (2010) describes a low pH enrichment culture, in development, that was derived from a wetland part of a hazardous waste site capable of reductively dechlorinating PCE to ethene at pH levels of approximately 5.9 to 6.1. These enrichment cultures are currently undergoing further studies to see if complete dechlorination can occur at pH lower than 5.9.

1.5 History and Development of KB-1

KB-1 is a bioaugmentation culture, containing *Dehalococcoides* and *Geobacter* spp., which can completely dechlorinate PCE and TCE to ethene. KB-1 was developed through the enrichment of microcosms constructed from the soil and groundwater of a contaminated site in southern Ontario in 1996 (Wehr 2001). This site was contaminated with TCE and methanol, but high levels of ethene were also present and found to have been produced naturally. For several years the KB-1 culture was maintained on TCE and enriched through several transfers into mineral medium. In 1998, the culture was split into four subcultures, each maintained on a different chlorinated ethene: KB-1/PCE, KB-1/TCE, KB-1/cDCE, and KB-1/VC (Duhamel 2002). Many subcultures of these lineages have been maintained on specific electron donor/acceptors throughout the last

several years. The inoculum used in this thesis was from the culture referred to as T3 MP1 which is maintained on TCE and methanol, and is the culture used in this thesis for all KB-1 experiments.

The first successful application of KB-1 was at Kelly Air Force Base, Texas, in 2000 where a bioaugmentation pilot test with KB-1 maintained on TCE achieved nearly complete dechlorination of TCE to ethene within 200 days (Major, McMaster et al. 2002). In 2002, SiREM was created for the purpose of commercializing and marketing KB-1 for bioaugmentation. The KB-1 culture at SiREM is maintained in large 100L bioreactors grown on TCE as the electron acceptor and methanol and ethanol as the electron donors. Since the success of the pilot test, KB-1 has been used to successfully bioaugment over 300 sites throughout North America.

1.6 Research Hypothesis and Objectives

Many chlorinated-ethene contaminated sites are at low pH or experience low pH conditions due to acidification of groundwater. Current methods for pH adjustment involve the use of alkaline materials to buffer the system back to neutral pH. These methods can be expensive and ineffective. An alternative method is to develop a culture capable of complete dechlorination at pH 6 or lower. KB-1 currently has the ability to dechlorinate PCE to ethene at an optimal pH range of 6.8 to 7.6. The most critical detoxification step is the conversion of VC to ethene; therefore this thesis will focus only on the conversion of VC to ethene. Moreover this step targets only *Dehalococcoides* which will also be another main focus in this thesis. The hypothesis of this thesis is that it is possible to acclimate the dechlorinating culture, KB-1, over time to dechlorinate vinyl chloride at a pH below 6.

The objectives of this thesis are to:

1. Determine the average sustained rate of dechlorination in KB-1 cultures grown at pH 7, pH 6, and pH 5.5; Assess if the rate increases with time as the culture adapts to the pH conditions.

2. Determine if the nature of the electron donor (methanol versus hydrogen) impacts the ability of the culture to dechlorinate at decreasing pH. Hydrogen is a direct electron donor to *Dehalococcoides*, while methanol must first be fermented to hydrogen. The objective is to determine if decreasing pH affects fermenting organisms more than the actual dechlorinating bacteria.

3. Analyze changes in the microbial composition of KB-1 as a function of pH and electron donor.

1.7 Thesis Outline

The remainder of this thesis is divided into four additional chapters. Chapter 2 describes experiment materials and methods. Chapter 3 describes the investigation of dechlorination rates at various pH levels. Chapter 4 analyzes the flow of electrons to corresponding reaction pathways involved in KB-1. Chapter 5 contains an in-depth look at the main microorganisms involved in the syntrophic relationships of the KB-1 culture and how they have changed with electron donor and pH.

Chapter 2: Materials and Methods

2.1 Chemical and Analytical Procedures

Standard analytical methods were used to measure specific chemicals and the volatile organic compounds.

2.1.1. Gas Chromatography

Concentrations of VC, ethene and methane in the cultures were measured by sampling the headspace gas (300 µL) and analyzing it by gas chromatography. Samples were taken inside the glovebox where syringes were sterilized with a heating coil immediately before sampling. Samples were then injected into an HP 5890 Series II gas chromatograph (GC) equipped with a GSQ 30 m x 0.53 mm I.D. PLOT column (J&W Scientific) in line with a flame ionization detector. The temperatures were set at 50°C for 1 minute, ramp at 60°C per minute to 150°C then remain at that temperature until 3.17 minutes.

Calibration was performed with external standards prepared gravimetrically with VC, ethene and methane were determined with aqueous standards (See Appendix A for sample calculations and response factors). The gases were added to these standards using a gastight syringe. The VC used was of greater than 97% purity (Sigma-Aldrich). Ethene and methane standards were of greater than 99% purity (Scotty II, Alltech Associates, Inc.)

2.1.2 High Performance Liquid Chromatography

Acetate concentration in the cultures was measured by an Ultimate 3000 High Performance Liquid Chromatography (HPLC) apparatus that included a pump, column, variable wavelength UV detector and Shodex RI-101. Liquid samples of 1.2mL were taken from each of the cultures inside the glovebox anaerobically, 0.6 mL of the sample was used for the void space of the filter (Acrodisc ® Syringe Filter 0.2 µm Super ®

Membrane, low protein binding and non-pyrogenic) and another 0.6mL for the sample itself. Samples were loaded onto a Dionex AS40 automated sampler. The eluent used for the HPLC was 5 mM H_2SO_4 filtered with 0.22 µm GV Millipore Durapore membrane filters and sparged for 30 minutes with helium. Column temperature was 60 °C and the eluent flowrate was 0.6 mL/min for a total run time of 20 minutes.

Standard solutions were prepared at concentrations of 0.2 mM, 1 mM, 2 mM and 5 mM. Standards were also filtered with Millipore paper filters and calibration curves were prepared for each run.

2.1.3 pH Measurement

Liquid samples were taken for pH measurement in 1.5 mL eppendorf tubes inside the glovebox. The pH was measured with an Oakton waterproof big display pH spear. Initial pH of samples was measured in parallel with acetate measurements. The pH of each culture was adjusted accordingly with 5M HCl or 5M NaOH filtered through 0.2 µm syringe filters, and then injected into the culture. The bottle was shaken to disperse the acid/base and 0.3 mL of liquid sample was withdrawn and injected into an eppendorf tube for pH measurement. This process was continued until the necessary pH level was reached. Through trial and error, approximately 1 drop from a BD 22 gauge 1.5 inch needle attached to a 3 mL syringe of acid/base was required to adjust the pH by ±0.1 increments.

2.2 KB-1 Culture Maintenance

All cultures were maintained in 160 mL sterilized serum bottles with a butyl rubber stopper for sampling headspace. All cultures were stored upside down in in the dark at ambient room temperature in an anaerobic glove box to avoid the escape of VOCs and growth of phototrophs (Coy Laboratory Products, Inc. Glasslake, MI). The glovebox atmosphere was a gas mixture containing 10% H_2, 10% CO_2 and 80% N_2 (Praxair) and activated carbon was used to remove VOCs and H_2S in the atmosphere. Palladium catalysts were placed in the glovebox to remove residual oxygen by reaction with H_2 in the atmosphere.

The cultures were analyzed for VOCs through headspace sampling for the GC every week. When VC concentrations dropped below 1 mg/L, the cultures were amended with VC back to 10 mg/L and the corresponding electron donor. At the end of each degradation cycle, samples were taken for acetate analysis, and pH was adjusted accordingly. When the culture concentrations of methane exceeded 15 mM in the gas phase, the bottles were purged for 20-30 minutes with N_2/CO_2 to avoid discrepancies in GC measurements and pressure build up from excess methane and ethene (Day 253, and 358). When culture volume decreased below 80 mL due to sampling, fresh medium was added to bring the volume up to 100 mL and pH was adjusted accordingly.

2.3 DNA Extraction

Genomic DNA was extracted from 5 mL of liquid culture using the UltraClean Soil DNA kit for analysis of microbial composition in KB-1 (Mo Bio Laboratories Inc., Solana Beach, CA). Sterivex filters were used to filter the liquid culture, the filters were then frozen at -80 °C for a minimum of 1 day. In order to extract the DNA, the plastic covering of the filters were cracked and the filter itself cut into relatively small pieces and placed into the bead beating tubes provided in the DNA kit. All other steps were performed in compliance with the manufacturer's instructions. The final product was diluted with 50 uL of purified, DNase-free water. DNA was extracted on Day 140, 245, 360, 426, and 587. Extraction products were then stored at -20 °C to inhibit enzymatic degradation.

2.4 Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reaction (qPCR) was used to measure the number of 16S rRNA gene copies of the major phylotypes in KB-1. Primers (Table 2) were obtained from Sigma-Aldrich and diluted to a 100 µM stock solution using sterile UV treated water and filter tips. Table 1 shows the thermocycling program used for each primer set where T_a is the annealing temperature specific to each organism.

Table 1: Quantitative PCR Standard Protocol, modified from (Zila 2011)

Stage	Time	Temperature	Cycles
Stage 1			
Initial Denaturation	10 min	94°C	1
Stage 2			
Denaturation	30 sec	94°C	45
Annealing	30 sec	T_a*	
Extension	30 sec	72°C	
Stage 3 (melting curve)			
Denaturation		65-95°C	1

* T_a = annealing temperature specific to each primer pair targeting different 16S rRNA sequences (See Table 2 for Annealing Temperatures)

A DNA Engine Opticon 2 Continuous Flow Detector with the program Opticon Monitor version 3.1 was used to analyze *Dehaloccoides* and *Acetobacterium*. The latter organisms were analyzed with a BioRAD CFX96 Real-Time System with a C1000 Thermal Cycler and program BioRAD CFX Manager 2.1. The reason for the switch was due to the fact that the Opticon system malfunctioned after the analysis of the second organism. Fluorescence was measured every 0.5°C during stage 3 of the thermocycling program. Reactions with a total volume of 25 µL was used for each sample, containing 10 µL of SsoFast™ EvaGreen® Supermix (BioRAD), 7 µL of PCR ddH2O, 0.5 µL of the forward and reverse primers (10 µM) each, and 2 µL of DNA template. The forward and reverse primers were added to establish a final concentration of 0.25 µM in a 20 µL volume. Standard curves were prepared with serial dilutions of plasmid containing 16S rDNA sequence for the target organism at concentrations from 10 to 10^8 copies/uL. Previously developed quantitative PCR primer pairs for the operational taxonomic units (OTUs) of interest within KB-1 are shown in Table 2.

Table 2: Primers Used for Quantitative PCR.

Organism	Primer Name	Annealing Temperature (˚C)	Sequence	Reference
Acetobacterium	Aceto 572f	59	5'-GGC TCA ACC GGT GAC ATG CA-3'	(Duhamel 2005)
	Aceto 784r	59	5'-ACT GAG TCT CCC CAA CAC CT-3'	
Dehalococcoides	Dhc 1f	60	5'-GATGAACGCTAGCGGCG-3'	(Duhamel 2005)
	Dhc 264r	60	5′-CCTCTCAGACCAGCTACCGATC-3′	
Methanomicrobiales	Mbiales 471f	59	5'-ACT ATT ACT GGG CTT AAA GC-3'	(Duhamel 2005)
	Mbiales 754r	59	5'-ACC GAT ACA CCT AAC GCG CA-3'	
Methanosarcina	Msarcina 180f	59	5'-ATG CGT AAA ATG GAT TCG TC-3'	(Duhamel 2005)
	Msarcina 511r	59	5'-TAG ACC CAA TAA TCA CGA TC-3'	
General Bacteria	BAC 1055F	55	5'-ATG GYT GTC GTC AGC T-3'	(Dionisi 2003)
	BAC 1392R	55	5'-ACG GGC GGT GTA C-3'	
General Archaea	ARCH-787F	59	5'-ATT AGA TAC CCG BGT AGT CC-3'	(Yu 2005)
	ARCH-1059R	59	5'-GCC ATG CAC CWC CTC T-3'	

Each sample was tested in 1:10 and 1:100 dilutions, in duplicate, to determine whether the effects of inhibition may be minimized through dilution. Results were discarded if the efficiency of the sample was lower than 80%. Overall, the 1:10 diluted samples resulted in higher copy numbers; therefore, this dilution was used to calculate the final copy numbers for each sample. Standard curves of each qPCR run were generated and accepted if the slope was approximately 3.3 ± 0.2.

Chapter 3: Adaptation of KB-1 to Decreasing pH

3.1 Introduction

Enrichment cultures of KB-1 were constructed in duplicates with anaerobic media to evaluate the dechlorinating ability of the culture at different pH levels. Two different electron donors were compared: hydrogen and methanol, with vinyl chloride as the electron acceptor in order to target *Dehalococcoides* specifically. Dechlorination rates were compared between the various amendments at pH 7, 6, and 5.5.

3.2 Experimental Setup of First Transfer: pH 7 vs. pH 6

The first transfer of this experiment consisted of 8 bottles of approximately 33.3% (v/v) culture transferred from T3 MP1 (Figure 2). Two bottles of anaerobic defined mineral medium (Edwards 1994) of 300mL each were prepared. Approximately 0.66 mL of anaerobic, filter sterilized 5N HCl was added to lower the pH of one medium bottle to pH 6. The pH of the other medium was kept neutral at pH 7.

Two sterile centrifuge bottles with O-rings were brought into the glovebox overnight to become anaerobic. The bottles were used to extract 100 mL of T3 MP1 each, then sealed with anaerobic tape and weighed to ensure they were within 0.1 grams of each other in order to be centrifuged. Centrifugation ran at 8000 rpm for 20 minutes at 4°C. The centrifuged bottles were then brought back inside the glovebox and the supernatant disposed of in a waste beaker. One pellet was resuspended with 300 mL of pH 7 medium and the other resuspended with 300 mL of pH 6 medium. The pH 7 and 6 resuspended cultures were transferred into four 120mL serum bottles each containing 75 mL of culture (Figure 2).

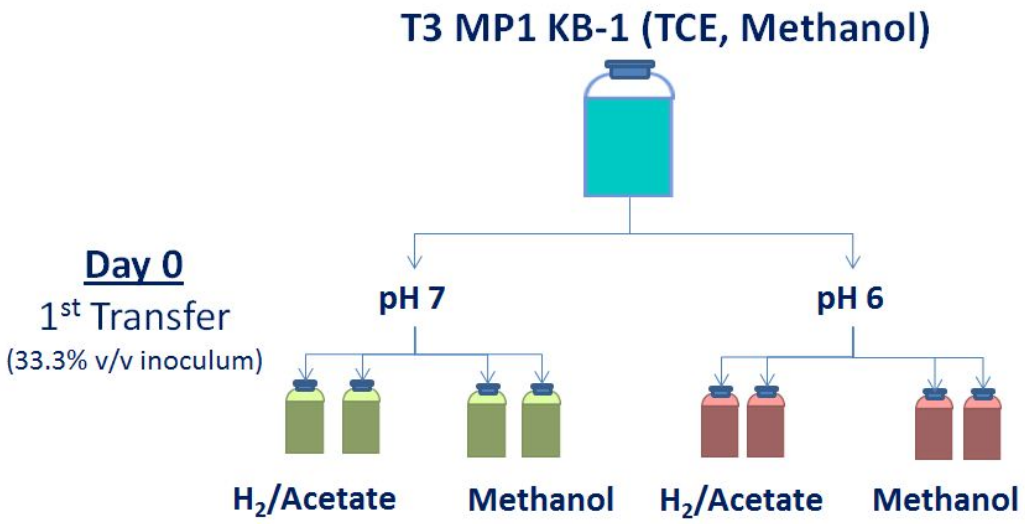

Figure 2: Schematic of the experimental setup of the first transfer.
Duplicate bottles of each culture at each condition were setup for the first transfer from T3 MP1. Green bottles represent cultures at pH 7; red bottles represent cultures at pH 6. VC was amended to all cultures at a target aqueous concentration of 10 mg/L.

The cultures were amended with a target VC concentration in the aqueous phase of 10 mg/L. Hydrogen and methanol were amended at a 5:1 electron donor to electron acceptor equivalence ratio. Sodium acetate was used as the carbon source in the hydrogen-amended cultures with a target concentration of 5 mM in the aqueous phase. During biodegradation, when acetate concentrations ran below 0.05 mM, as measured on the HPLC, acetate was added to bring the concentration to 5 mM again. Cultures were amended with corresponding electron acceptor and donors when the concentration of VC dropped below 1 mg/L. The transfers were kept on VC for approximately 135 days.

3.3 Experimental Setup of Second Transfer: pH 5.5 Conditions

Cultures from the first transfer were maintained on VC and respective electron donors at pH 7 and pH 6 until day 135 at which time a second transfer was made. Fresh anaerobic mineral medium was prepared at pH 7 and pH 6. Each culture bottle was split into two new serum bottles (approximately 30 mL from each culture) and topped up to 100 mL with fresh medium. Each culture bottle was then purged with N_2/CO_2 for

approximately 45 minutes to remove excess methane and ethene. A schematic of the second transfer is shown in Figure 3.

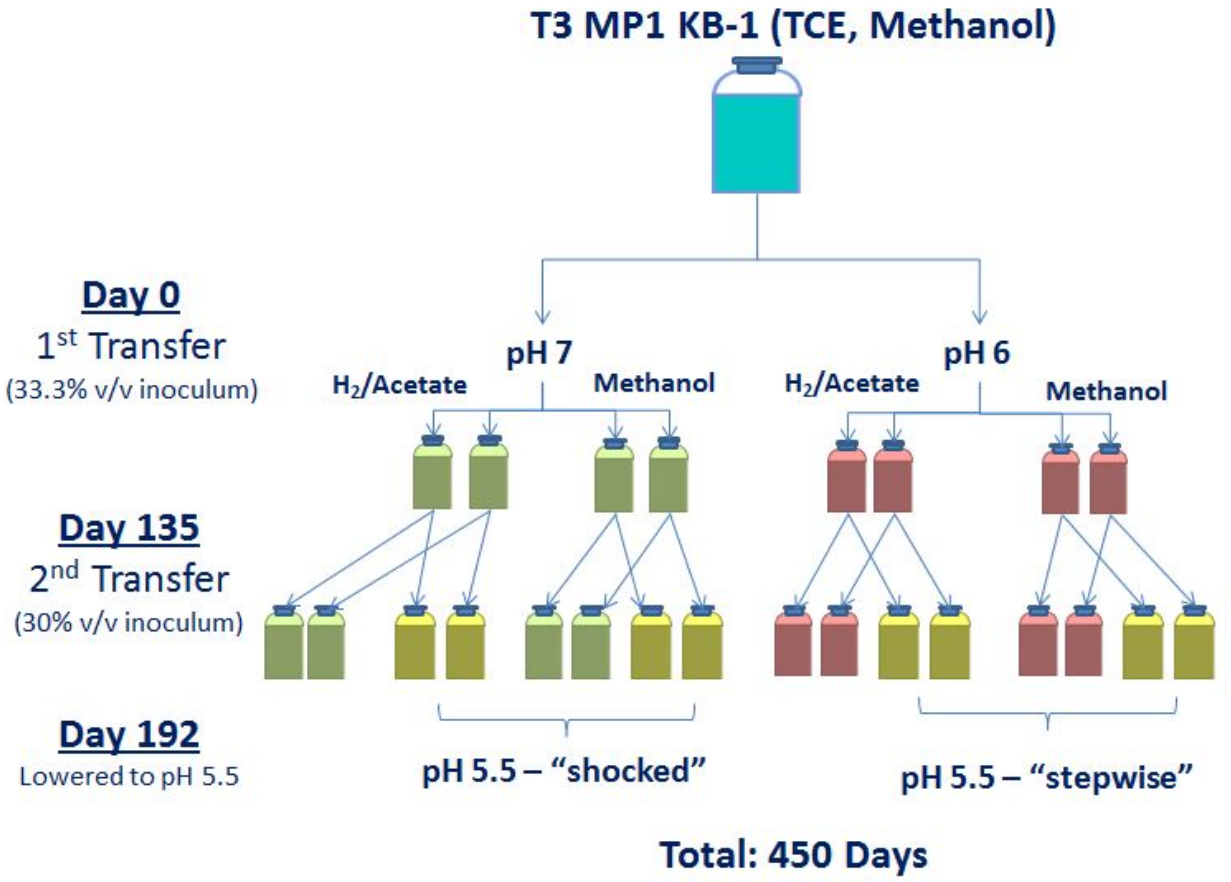

Figure 3: Schematic of the experimental setup of the second transfer.
Each bottle from the first transfer was split and divided into two more bottles. Each new culture was topped up to 100 mL with fresh medium. Green bottles represent pH 7; red bottles represent pH 6 and yellow bottles represent pH 5.5. The experiment ran for a total of 450 days. VC was amended to all cultures at a target aqueous concentration of 10 mg/L.

Cultures were periodically amended with the same amount of electron donor and acceptor as described for the first transfer for 57 days at pH 7 and pH 6 before half of the bottles were lowered to pH 5.5. The cultures that were originally at pH 7 and lowered to pH 5.5 were labeled as “shocked” while the cultures that were originally at pH 6 were labeled as “stepwise” (Figure 3).

3.4 Rate Calculations

The rate of ethene production was used as a measure of dechlorinating activity. Dechlorination rates were calculated as rates of ethene production rather than VC removal because it takes time for VC to equilibrate. Detailed calculations of rate of ethene production can be found in Appendix A.

3.5 Rates of Ethene Production: pH 7 vs. pH 6

The first transfer of the experiment compared the rates of ethene production at pH 7 versus pH 6 for both donors over a period of 135 days (Figure 4). The average rate of ethene production at each pH and electron donor is listed in Table 3.

Table 3: Rate of ethene production for pH 7 and pH 6

	Average Rate of Ethene Production (µmol/day)	**Standard Deviation**	**n***	**%Normalized to pH 7**
pH 7				
Electron donor:				
Hydrogen	1.75	0.45	9	100%
Methanol	1.65	0.32	7	95%
pH 6				
Electron donor:				
Hydrogen	0.86	0.26	6	50%
Methanol	1.09	0.66	5	63%

*n: number of degradation cycles used to calculate average rate of ethene production

The average rates of ethene production were calculated over the entire 135 days. The difference in rates did not appear immediately between pH 7 and pH 6. A slow decline in rate in the pH 6 cultures was observed in the first 50 days of the transfer for both electron donors (Figure 4). KB-1 is a culture that has continuously been

enriched at neutral pH; the slow decline in rate suggests that it took some time for KB-1 to adjust to the new environmental conditions. Nevertheless, the degradation rates did not increase over 135 days at pH 6.

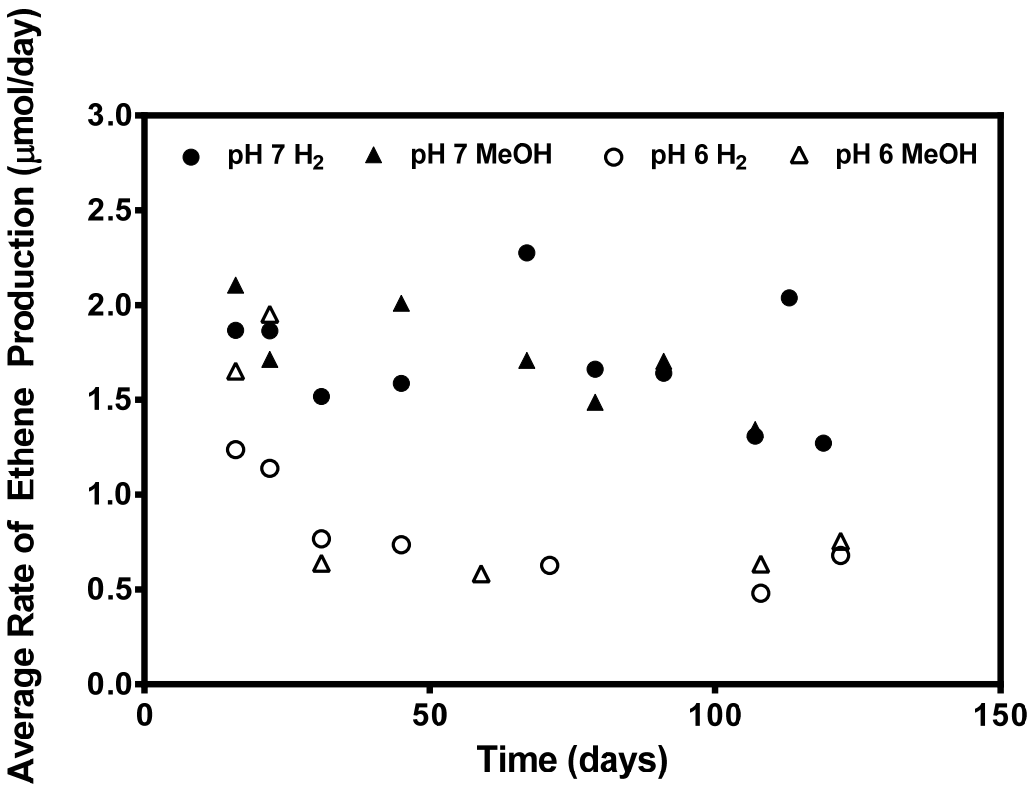

Figure 4: Rate of ethene production in cultures maintained at pH 7 and pH 6
Closed shapes represent average rate of ethene production at pH 7 and open shapes represent average rate of ethene production at pH 6. Triangles represent methanol-amended and circles represent hydrogen amended.

3.6 "Stepwise" vs. "Shocked" pH 5.5 cultures

After the second transfer of the experiment, a comparison between "shocked" and "stepwise" pH conditions was analyzed. The cultures from the previous experiment were transferred and inoculated 30% (v/v) into fresh medium. The set from pH 7 was adjusted to pH 5.5 and named "shocked" to represent an immediate pH change from pH 7, while the set from pH 6 was named "stepwise" to represent a gradual pH change from 6 to 5.5. The purpose of this comparison was to see if acclimation can make a difference in how the rate of VC dechlorination of KB-1 responds to acidic pH. The degradation rates were compared from approximately day 200 to 450 (Figure 5). An

average rate of ethene production for each pH condition and electron donor is listed in Table 4. The “shocked” pH 5.5 cultures experienced a range of rates from little to no dechlorination due to pH fluctuations. It was observed that the hydrogen-amended cultures tend to drift up in pH and the methanol-amended cultures tend to drift down in pH. These fluctuations in pH are discussed later in the thesis.

Table 4: Average rate of ethene production for "Stepwise" and "Shocked" pH 5.5 cultures

	Average Rate of Ethene Production (µmol/day)	**Standard Deviation**	**n***	**%Normalized to pH 7**
“Stepwise” pH 5.5				
Hydrogen	0.314	0.0876	5	18%
Methanol	0.325	0.130	4	19%
“Shocked” pH 5.5				
Hydrogen	0 - 0.207	0.181	5	0-12%
Methanol	0 - 0.101	0.138	5	0-6%

*n: number of degradation cycles used to calculate average rates of ethene production

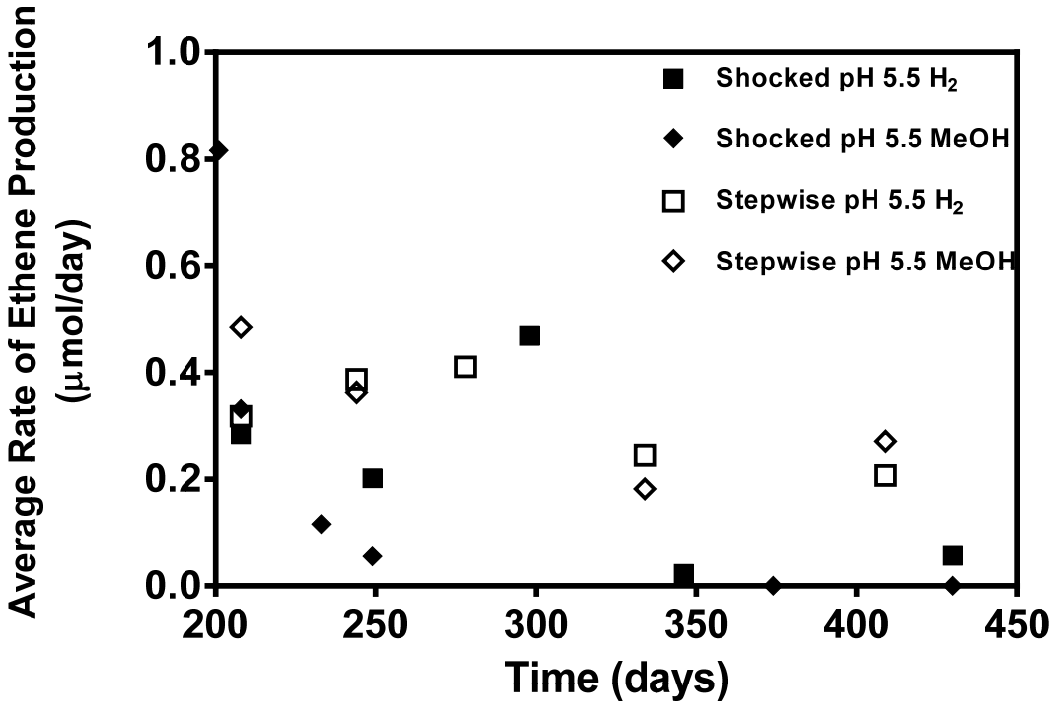

Figure 5: Average rate of ethene production for "stepwise" vs. "shocked" pH 5.5 cultures
Squares represent hydrogen-amended cultures, diamonds represent methanol-amended cultures. Closed shapes represent the "shocked" pH cultures and open shapes represent the "stepwise" pH cultures.

Similar to slow decline in average ethene production rates that occurred at pH 6 after the first transfer, the "shocked" cultures from pH 7 to pH 5.5 experienced a similar slow decline in rate over the first 100 days of the second transfer. The "shocked" pH 5.5 cultures had average rates up to 0.207 µmol/day for the hydrogen-amended and 0.101 µmol/day for the methanol-amended before dechlorination eventually began to stall. This suggests that dechlorination can still occur within a certain time frame (50 days for pH 6 and 100 days for pH 5.5) of exposure to low pH, but is not sustainable.

All of the enrichments at pH 5.5 required at least twice the amount of electron donor during one degradation cycle. Also, the dechlorination of the "shocked" cultures was inconsistent. Degradation would occur immediately after electron donor was added but would stall after some time. The cultures have been maintained in order to determine if they would be able to recover from the initial shock of exposure to low pH.

No increases in rate of ethene production can be observed for both the “shocked” and “stepwise” cultures.

3.6.1 Changes in Acetate Concentrations

Acetate measurements were taken after the second transfer of this experiment starting at day 250. Differences were observed in the methanol-amended enrichments in which the balance between acetate production and acetate consumption changed with pH (Figure 6). At both pH 7 and pH 6 acetate production and consumption are at steady state. At pH 5.5 for both “stepwise” and “shocked” enrichments, the concentration of acetate increased over time indicating that either acetate production increased or acetate consumption decreased; these observations will be discussed later in the thesis.

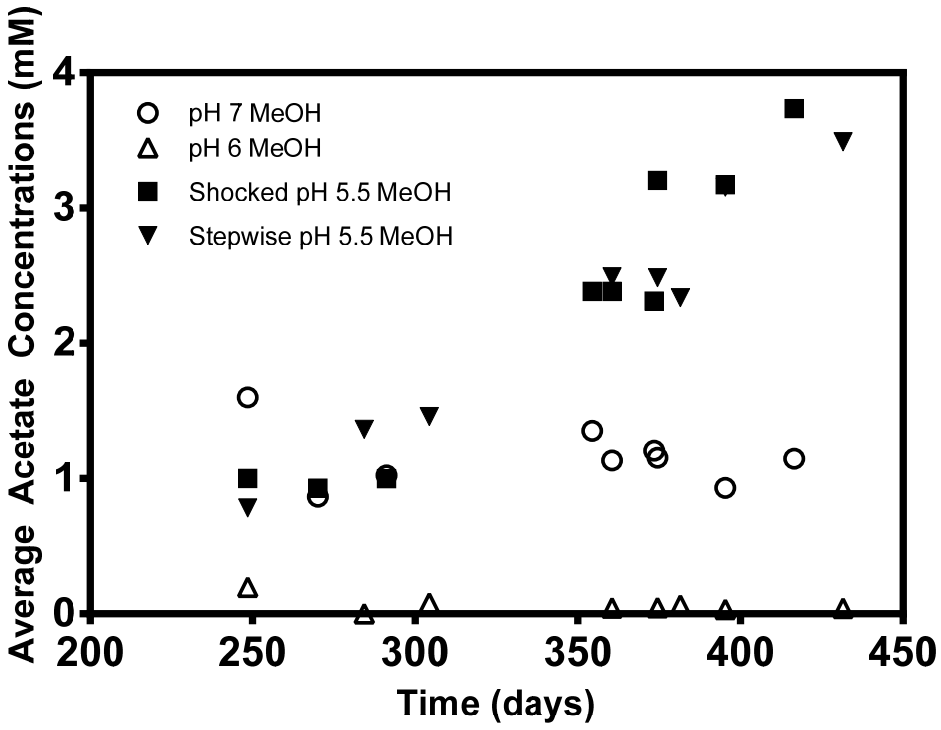

Figure 6: Acetate concentrations of methanol-amended cultures
For the methanol-amended cultures, an increase in acetate concentrations was observed for both pH 5.5 cultures while a steady state concentration level was observed at pH 7 and pH 6.

3.7 Variability in pH

It was discovered after the second transfer that the cultures experienced increases or decreases in pH according to electron donor. It was observed that the H_2-amended cultures had pH increases of approximately 0.3-1.0 pH units after each degradation cycle. After this was discovered the pH was monitored weekly and stabilized at the corresponding pH of 6 or 5.5 for the H_2-amended cultures. The pH of the methanol-amended cultures experienced a decrease of 0.3 to 0.5 pH units. It was expected that the methanol-amended cultures would experience a slight decrease in pH as this is a common occurrence in the field due to fermentation of methanol to acetic acid (Equation 2).

Equation 2:

$$CH_3OH + 0.5CO_2 = 0.75CH_3COO^- + 0.75H^+ + 0.5H_2O$$

Dechlorination continued to occur in the methanol-amended pH 6 cultures even though the pH decreased to approximately 5.7. No further decreases in pH were observed after pH 5.7. The methanol-amended pH 5.5 cultures experienced decreases to pH 5.3 where little to no dechlorination would occur; therefore, pH measurements were taken weekly and pH was adjusted accordingly to be kept at pH 5.5.

There may be two possible explanations for the pH increases in the H_2-amended enrichments. KB-1 contains dechlorinating bacteria, methanogens and acetogens. The methanogens in particular, are capable of utilizing both acetate and hydrogen as electron donors. Acetoclastic methanogens are capable of fermenting acetate into methane via the following equation (Pine 1971; Thauer 1977):

Equation 3:

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$$

This fermentation process produces bicarbonate, a weak base capable of increasing pH. Another possibility of the increase in pH is through hydrogen-utilizing methanogens. These methanogens are very good scavengers of H_2 and can carry out autotrophic methanogenesis according to the following equation (Ziv-El et al., 2012):

Equation 4:

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

This reaction is a proton-consuming reaction, which further suggests that the cause of the pH increase is due to these two methanogenic processes. Chapter 4 and 5 discuss the abundance and role of methanogens and how they are affected by pH.

3.8 Rate of Ethene Production vs. Measured pH

As described in the previous section, pH changes occurred regularly in the cultures, i.e. it was difficult to maintain constant pH. Since pH 7 had the fastest degradation cycle, measurements of pH were taken at the end of each pH 7 degradation cycle for all cultures. Therefore, it was possible to associate a specific rate of ethene production over a period of time with a measured pH. Figure 7 shows that the rate of ethene production decreases with pH.

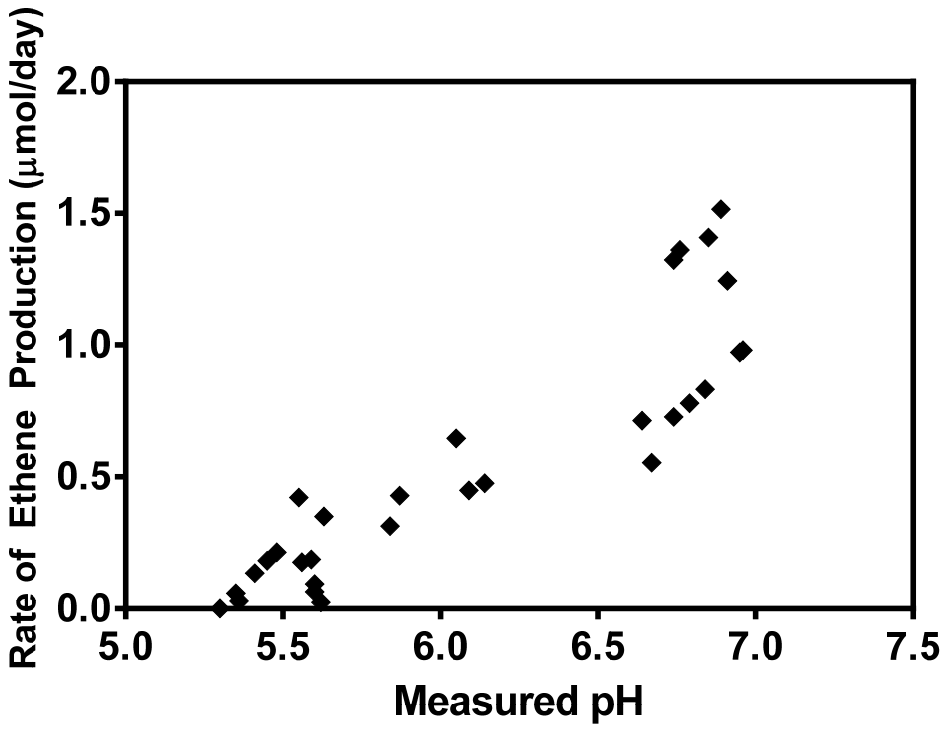

Figure 7: Rate of ethene production vs. measured pH
Filled diamonds represent rates of ethene production from pH 7 and pH 5.5 cultures.

Several papers have looked into the rate of dechlorination at neutral pH. One author, Aulenta *et al.* (Aulenta 2002) reported a dechlorination rate of VC to ethene in sediment microcosms of 6.6-7.6 µmol per L per h with methanol and 7.7-11.8 µmol per L per h with hydrogen. In the current study, the methanol-amended cultures also had higher degradation rates than the hydrogen-amended enrichments at pH 6 (Refer back to Table 3). It is difficult to compare the absolute values of the data, because often the numbers of bacteria involved are unknown. Zhuang *et. al.* (Zhuang 1995) tested the dechlorination of PCE on various pH values: 4,6,7,8 and 9.5. They found that the methane production rate declined more than the dechlorination rate at pH values below 7. As well, at pH 6 there was significant accumulation of cDCE and no observation of VC. The dechlorination rate of PCE at pH 7 was approximately 6 nmol/h and at pH 6 was 2 nmol/h. The studies conducted in this thesis found that VC dechlorination at pH 6 occurred at approximately 50% of the rate at pH 7. The decline in methane production rate did not occur in the KB-1 culture at low pH, in fact the methane production rate either was not affected or increased at lower pH values. The difference in methane production rates between this thesis and Zhuang *et al.* may have been due to the fact that acetate was utilized as the main electron donor for the experiments conducted in Zhuang's paper.

3.9 Indications of Acclimation of KB-1

Bacteria and Archaea live in a variety of niches where survival is dependent on the capacity to sense and adapt to environmental change. Microorganisms have particular mechanisms in which to adapt to acidic conditions, some of which include protecting the cytoplasmic pH (Lowe 1993). For organisms to exist in environments of low pH or experience changes in pH, several survival strategies exist. Either pH conditions inside the cell are maintained as close to homeostasis as possible with the expenditure of cellular energy, or cytoplasmic conditions adjust to adapt to changes in environmental pH, which requires less expenditure of energy (Foster 1999). Studies indicate that intracellular pH of anaerobes is not maintained at constant values (Goodwin and Zeikus 1987; Foster 1995). The research on how anaerobic organisms are affected by pH is still inconclusive. Currently there is no literature on the effects of

extracellular pH specific to dechlorinating organisms. Lowe *et al.* (Lowe 1993) has reviewed several anaerobic microorganisms that have managed to develop adaptive mechanisms to low pH conditions. These mechanisms include the phenomenon of altering carbon and electron flow through the cell to adapt to changing pH conditions as well as changes in cellular morphology, membrane structure, and protein synthesis. One of the objectives of this thesis was to see if KB-1 has the ability to adapt to low pH conditions. The pH 6 cultures were continuously amended with VC to analyze if the degradation rates would improve over time. Figure 8 shows the rate of ethene production (µmol/day) of the pH 6 cultures from day 0 to 550.

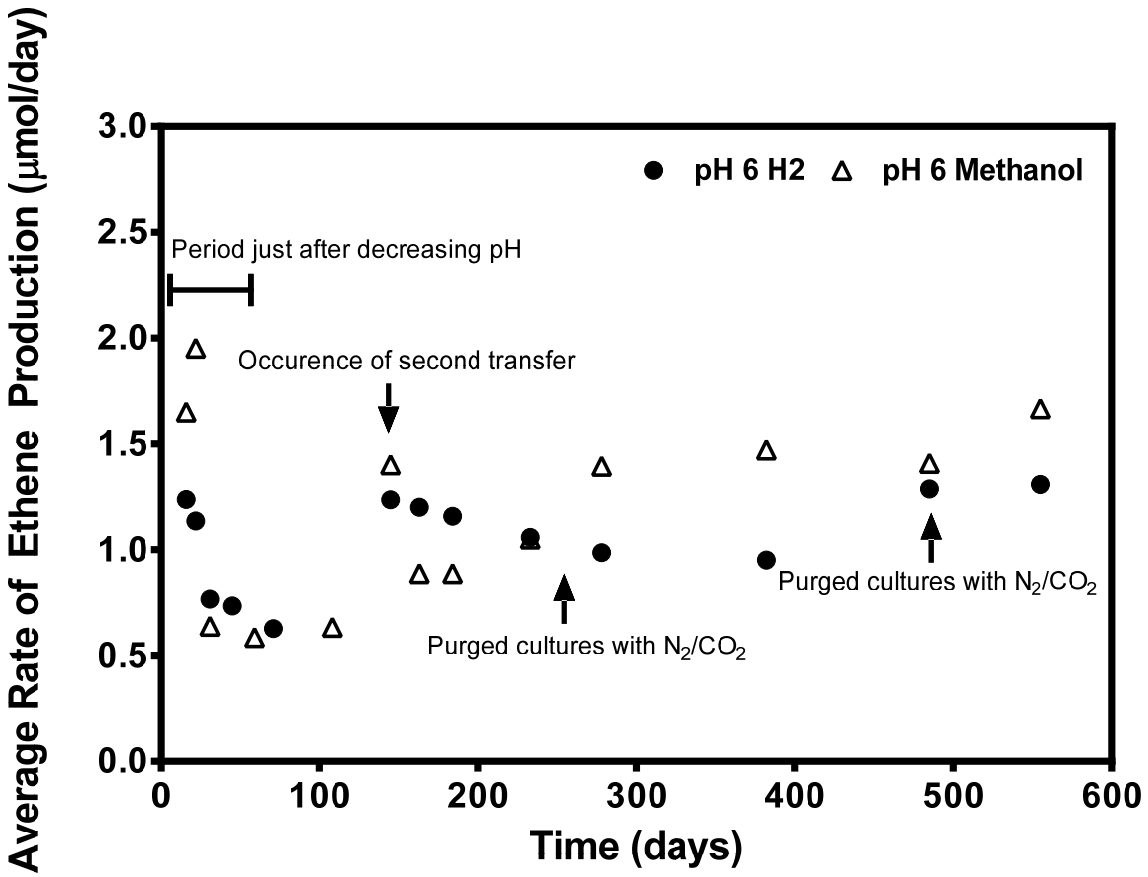

Figure 8: Rate of ethene production in the pH 6 cultures: methanol-amended vs. hydrogen-amended
Average rate of ethene production for pH 6 cultures over course of entire experiment.

After the initial slow decline in rate from day 0-50, no increase in rate was observed for the methanol-amended enrichments between days 50-135 before the first transfer. After the second transfer, the average rate of ethene production increased slightly for the pH 6 hydrogen-amended cultures but continued to slowly decline between days 200-400. No further transfers were made and the cultures were allowed to adapt to the new conditions. Between days 200-500 there was improvement in the rate of the methanol-amended cultures from approximately 0.5 µmol/day to 1.5 µmol/day. No increase in rates was observed for the hydrogen-amended enrichments until recently at day 500. Further measurements for the hydrogen-amended cultures are needed to determine if acclimation occurred in these enrichments.

One of the research objectives of this thesis is to asses if the rate of ethene production would improve over time in the low pH cultures. The motivation behind this objective was to develop a culture that is capable of dechlorinating at low pH for the purpose of bioaugmentation of chlorinated ethene contaminated sites. Since there are indications of acclimation of the KB-1 culture to pH 6, a microcosm study would be ideal to determine if the culture can be used for bioaugmentation. An initial microcosm study was done on November 25th, 2011 in collaboration with SiREM to test low pH KB-1 cultures on soil and groundwater from a low pH contaminated site, details can be found in Appendix E. Only dechlorination from TCE to cDCE was achieved in the microcosm study. This may have been due to the fact that the pH 6 cultures used to inoculate the microcosms were enriched only up until day 277 before any indications of acclimation occurred. Since the rate of ethene production in the pH 6 methanol-amended cultures has improved since day 277 it would be ideal to conduct another microcosm study to test the ability of these cultures to bioaugment chlorinated ethene contaminated sites. The pH 6 cultures enriched from the experiments conducted for this thesis will continue to be maintained on VC and further measurements will be made to determine if the rate of ethene production will continue to improve.

Chapter 4: Electron Balance

4.1 Introduction

To further understand the effect of pH on the microbial community structure of KB-1, an electron balance was conducted on the pH 7 and pH 6 cultures, in order to account for all inputs and outputs and perhaps to correlate trends between electron flow and the relative abundance of dechlorinators and other microorganisms. The electron donors were methanol and hydrogen and the electron acceptors were VC and carbon dioxide. This electron balance analysis was done on the major microbial processes of dechlorination, methanogenesis, and acetogenesis. A reasonable electron balance was achieved for the cultures.

4.2 Methods of Calculations

An overall electron balance was calculated for each pH 7 and pH 6 culture. The electron balance is based on the electron equivalents of each reactant and product. It was assumed that the use of dead biomass as an electron donor and biomass synthesis can be ignored in order to simplify the electron balance analysis. The half reactions and electron equivalents for each measured reactant and product involved are listed in Table 5.

Table 5: Half reactions of reactants and products taken from McCarty 2001

Component	Half Reaction (As reductions)
Acetate	$CO_2 + HCO_3^- + 8H^+ + 8e^- = CH3COO^- + 3H_2O$
H_2	$2H^+ + 2e^- = H_2$
Methanol	$CO_2 + 6H^+ + 6e^- = CH_3OH + H_2O$
Methane	$CO_2 + 8H^+ + 8e^- = CH_4 + 2H_2O$
VC	$C_2H_3Cl + 2H^+ + 2e^- = C_2H_4 + Cl^-$

In order to calculate the electron balance, an initial and final time need to be established. In this case, the initial and final time was established as the start and end of a degradation cycle, respectively. The initial concentrations of electron donors were calculated from the 5:1 electron donor to electron acceptor ratio of electron equivalents. The final concentrations of hydrogen and methanol were not measured. However, they are typically rapidly consumed, so were assumed to be zero at the final time. Acetate concentrations were measured by HPLC while methane, VC and ethene were measured by GC as described in Chapter 2. The end of the degradation cycle was designated when the concentration of VC was less than 1 mg/L (i.e. more than 90% degraded).

Aqueous concentrations of each compound measured by GC were calculated via Henry's Law, which states that at a constant temperature the amount of a given gas that dissolves in a given volume of liquid is directly proportional to the concentration of that gas in equilibrium with that liquid. Henry's law is expressed by the equation:

Equation 5:

$$k_H = \frac{c_{gas}}{c_{aq}}$$

Where

k_h = dimensionless Henry's constant

c_{aq} = concentration of compound in aqueous solution (mol/L)

c_{gas} = concentration of compound in gas phase (mol/L)

A summary of the dimensionless Henry's Law constants at 298K are shown in the table below for each compound:

Table 6: Henry's law constants for gaseous components

Component	Henry's Law Constant	Reference
Methane	31.4	(MacKay 1992)
VC	0.93	(Yaws 1992)
Ethene	8.7	(MacKay 1992)

Since the Henry's law constant of methane is particularly high, indicating that the vast majority of methane is in the gaseous phase, it was assumed that the amount of methane dissolved in the liquid phase was negligible.

In the case of ethene and VC, the response factors from the GC were used to calculate the concentration of ethene in the gas phase and the concentration of VC in the liquid phase, respectively. Henry's law was then used to calculate the total moles of VC and ethene in each culture bottle as shown in Appendix A. Then, in order to calculate the number of electrons, the millimoles in each bottle were calculated first. Assumptions for millimole calculations are as follows:

- The volume of liquid in each bottle was assumed to be 0.1 L
- The volume of methanol amended to the culture was 7 μL
- Methane was assumed to be all in the gaseous phase with a volume of 0.06 L

Only certain degradation cycles were chosen due to the fact that some lacked measurements of acetate concentrations at the beginning or end of a cycle. Balances were deemed reliable if the total number of electrons between the initial and final time were within 10% of each other.

4.3 Results and Discussion

An analysis of the flow of electrons in the KB-1 culture can help elucidate the relative abundance of microorganisms involved in dechlorination versus acetogenesis and methanogenesis. The electron balance was calculated for each degradation cycle but not all were used to calculate the average reducing equivalents. The requirements for representable degradation cycles included accurate acetate concentrations at the beginning and end of the degradation cycle, an electron balance within 10% of the total electrons at the initial and final times, and complete dechlorination of VC. The degradation cycles used to calculate the average reducing equivalents can be found in Appendix C. Hydrogen was in excess to the system (5:1 electron donor to electron

acceptor equivalents ratio) and therefore it is important to note that methanogenesis likely continued to occur even when dechlorination was complete. It was assumed for the final time that when the VC concentration in the liquid phase approached 0.1 µM, the dechlorination was complete.

Acetogenesis also occurred occasionally in the methanol-amended cultures. The values calculated for acetate generation and consumption in the electron balance of the methanol-amended cultures occurred episodically and did not follow a visible trend; therefore the electron equivalents that went towards acetogenesis were added with methanogenesis for easier comparison between the different donors. Electron balances were calculated for each of the cultures and a total average was taken between each duplicate culture for the selected degradation cycles. The reducing equivalents were calculated for the pH 7 and pH 6 enrichments for both donors. Table 7 summarizes the average values of reducing equivalents for each condition.

Table 7: Total Average Reducing Equivalents for each electron donor at pH 7 and pH 6

	Total Average Reducing Equivalents	**n**
pH 7 – H_2 Amended		11
%Methanogenesis	96.5%	
% Dechlorination	3.5%	
pH 7 – Methanol Amended		10
%Methanogenesis and Acetogenesis	95.7%	
% Dechlorination	4.3%	
pH 6 – H_2 Amended		10
% Methanogenesis	98.3%	
%Dechlorination	1.7%	
pH 6 – Methanol Amended		11
% Methanogenesis and Acetogenesis	96.1%	
%Dechlorination	3.9%	

*n = number of degradation cycles used to calculate average reducing equivalents

In order to determine if the difference between conditions was statistically significant a t-test was performed. Sample calculations can be found in Appendix F. Two comparisons were made to determine if there was statistical significance between the donors at the same pH and between the different pH values with the same donor. The research and null hypotheses for each comparison are stated as:

Comparison 1

a) Research Hypothesis: The average reducing equivalents for dechlorination (with the same electron donor) is higher at pH 7 than the average reducing equivalents at pH 6.
b) Null Hypothesis: There is no difference in the average reducing equivalents for dechlorination between pH 7 and pH 6.

Comparison 2

a) Research Hypothesis: The average reducing equivalents for dechlorination at the same pH is higher with methanol as the electron donor than hydrogen.
b) Null Hypothesis: The average reducing equivalents for dechlorination at the same pH is the same between the methanol and hydrogen as the electron donors.

The table below lists the final p-values for each condition:

Table 8: t-test results to determine statistical significance between different pH and electron donors

	p-value	**Statistically Significant?**
H2-Amended pH 7 vs. pH 6	0.0215	Yes
MeOH Amended pH 7 vs. pH 6	0.466	No
pH 7 H_2 vs. MeOH	0.0527	No
pH 6 H_2 vs. MeOH	0.0066	Yes

Several authors with similar cultures to KB-1 have established that the reducing equivalents available from electron donor consumption were mainly channeled to side reactions such as acetogenesis or methanogenesis rather than dechlorination. All of these cultures were maintained at neutral pH. In the experiments of Adamson *et al.* (Adamson 2004) the level of methane production indicated that hydrogen and acetate were being utilized more by the methanogens than the dechlorinators. Dehalorespiration in these experiments accounted only for 5-15% of the electron equivalents utilized for PCE dechlorination. Similar electron equivalents were found in this study for VC dechlorination. In the batch systems of Carr *et al.* (Carr 1998), it was calculated that less than 1% of the H_2 equivalents consumed was utilized for dechlorination and that 69% of the H_2 equivalents was accounted for by methane production. The remaining 30% was presumably shunted to cell growth and/or acetogenesis. These electron equivalents were calculated for PCE dechlorination with hydrogen as the electron donor. Acetogenesis was not observed in the hydrogen-amended cultures of this study since acetate was being added as a carbon source to the cultures. Aulenta *et al.* (Aulenta 2002) achieved similar results where the calculated distributions of electrons favored methanogenesis rather than dechlorination (approximately 80% of the electrons were shuttled towards methanogenesis). Finally Ma *et al.* (Ma 2003) also found that methanogenesis accounted for the major fraction of the H_2 equivalents at approximately 94% and only 5% went towards dechlorination, which is the closest to what was found in the cultures of this study.

The proportion of electron equivalents towards methanogenesis in the hydrogen-amended pH 6 cultures was greater than that in the pH 7 cultures, which indicates that certain methanogens are capable of metabolic activity in low pH conditions. This is reflected in the electron equivalents distribution between the H_2-amended pH 7 and pH 6 electron equivalents. The t-test proved the comparison of electron flow between these two conditions to be statistically significant. There is an increase in methanogenesis and decrease in dechlorination at pH 6 when hydrogen is the electron donor. This differs in comparison to other studies of hydrogen and methanol as electron donors. One such study, Aulenta *et al.* (Aulenta 2002) showed that the highest methane formation rates were observed in the presence of methanol at neutral pH due to methylotrophic

microorganisms rather than to acetoclastic and hydrogenophilic microorganisms. Methane production was higher in the hydrogen-amended cultures of this study compared to the methanol-amended cultures.

Since dechlorination was slower at lower pH, the methanogens that are not as affected by low pH are able to outcompete the dechlorinators for the excess hydrogen present in the system. Methanogens that are capable of thriving in low pH environments have been reviewed in the literature (Sizova 2003; Brauer 2004). One example, Brauer *et. al.* (Bräuer 2006; Brauer 2011) was able to isolate a methanogen from an acidic peat bog that belongs to an uncultured family-level clade in the *Methanomicrobiales* order and concluded that a mildly acidophilic community of hydrogenotrophic methanogens is present in acidic peat soil that has a pH optimum near 5. *Methanomicrobiales* is known to be one of the main methanogens in the KB-1 culture and so therefore it is possible that acid-tolerant methanogens are present in the KB-1 culture. More evidence indicating the presence of acid-tolerant methanogens found in the low pH cultures is discussed in Chapter 6.

There was no statistical significance between the relative proportions of electron equivalents of the methanol-amended cultures at pH 7 and pH 6. An indication that the flow of electrons is similar for these conditions which further suggests acclimation may be possible at pH 6 for the methanol-amended cultures. Although there was no statistical significance between the two electron donors at neutral pH, the p-value of 0.0527 was fairly close to the 95% confidence interval. This suggests that there may be a comparable difference between the electron donor hydrogen and methanol at pH 7 as well. Further data measurements would be required to make this conclusion. Finally, since the hydrogen-amended displayed the least amount of electron equivalents towards dechlorination, there was a statistically significant difference at pH 6 between the two electron donors.

The relative comparison of rates of ethene production and flow of electrons between the electron donors at pH 6 indicates that methanol-amended cultures perform better than hydrogen-amended cultures. Many authors have addressed the issue of competition for H_2 between dechlorinators and methanogens in contaminated

subsurface environments (Smatlak 1996; Ballapragada 1997; Yang 1998; Cupples 2004). Literature suggests that dechlorinating microorganisms compete better than previously thought when hydrogen is limiting (Cupples 2004). Hydrogen was added to the cultures in this study in excess (5:1 electron donor to electron acceptor ratio). This suggests that hydrogen concentrations may have a higher effect at low pH for the dechlorinators.

In contrast, the fermentation of certain alcohols is thermodynamically favourable under higher hydrogen partial pressures, provided that in an alcohol-amended culture a high number of H_2-producing, fermentative bacteria are present compared with the number of H_2-utilising microorganisms. (Aulenta 2006). In a previous study, methanol fermentation resulted in H_2 liquid concentrations higher than 400 nmol/L and PCE dechlorination to ethene proceeded at its maximum rate and was not H_2 rate-limited (Aulenta 2002). Although the concentrations of hydrogen were not measured in the cultures of this study, the literature suggests that it is possible for methanol-amended cultures to perform better than hydrogen-amended cultures.

Overall, the electron balance conducted in this chapter suggests that methanol may be the more proficient electron donor at pH 6. The hydrogen-amended pH 6 cultures had the lowest proportion of electron equivalents shuttled towards dechlorination (1.7%) while the methanol-amended pH 6 cultures had proportions of electron equivalents towards dechlorination close to that of pH 7 (3.9%). Although no statistical significance was found between the hydrogen and methanol-amended pH 7 cultures, the p-value was relatively close to the alpha value (0.05) chosen. Further measurements of more degradation cycles would determine if the electron donor also affects the distribution of electron equivalents at pH 7.

Chapter 5: Microbial Community Analysis

5.1 Introduction

The main organisms and processes involved in dechlorination of KB-1 have been extensively characterized throughout the years (Duhamel 2004; Duhamel 2006; Waller 2009; Chan 2010; Hug 2012). Techniques used to analyze KB-1 include denaturing gradient gel electrophoresis (DGGE) and quantitative PCR (qPCR) targeting the 16S rRNA gene. These authors have also studied the growth yields of the dechlorinating organisms, rates of dechlorination, effects of inhibitors, optimal electron donors and temperature conditions. The robustness of KB-1 is due to the syntrophic relationships between the various organisms and redundancy in organisms carrying out similar processes in the culture. Complete dechlorination of PCE and TCE to ethene has been observed in microcosms and enrichment cultures of KB-1. The role of other populations involved in KB-1 such as acetogens, methanogens and other fermenters have also been established, as illustrated in Figure 9. The main dechlorinating organisms in KB-1 are two strains of *Dehalococcoides* and one *Geobacter* species (Duhamel 2004; Duhamel 2006). Complete dechlorination of PCE to ethene requires both these organisms where *Geobacter* first reduces PCE to cDCE and *Dehalococcoides* completes the final dechlorination steps from cDCE to ethene. It is known that VC often accumulates because VC to ethene dechlorination is frequently the rate limiting step (Aulenta 2002; Cupples 2004; Heimann 2006). Therefore, for the simplification of the experiments conducted in this study and to target the primary dechlorinator *Dehalococcoides,* VC was used as the main electron acceptor.

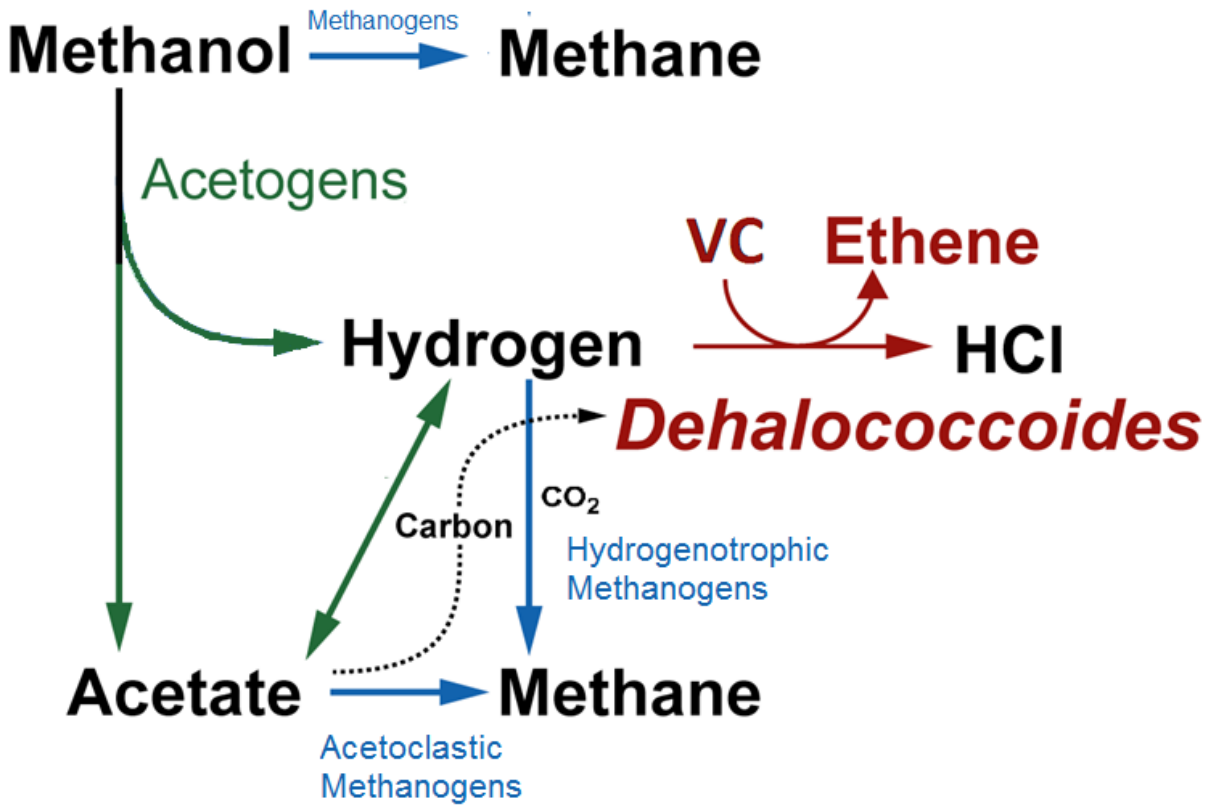

Figure 9: General processes in the KB-1 culture
This is a general representation of the known processes and main microorganisms involved in the KB-1 culture. Modified from Hug (2012). Green arrows represent acetogenesis. Blue arrows represent methanogenesis. Red arrows represent dechlorination.

5.2 Selection of Representative Microorganisms

Previous analyses of the composition of the KB-1 culture T3 MP1 has helped determine which target organisms to examine in the pH experiments. KB-1 is a culture composed mainly of dechlorinators, acetogens and methanogens; thus the main order of microorganism from each category was chosen. From these previous analyses it was determined that the following predominant microorganisms would be examined: *Dehalococcoides, Methanosarcina, Methanomicrobiales,* and *Acetobacterium*, total Bacteria and total Archaea were determined as well. Figures 10 is a previous quantitative PCR characterization of various KB-1 cultures including the T3 MP1 culture used for the experiments in this. Waller (Waller 2009) conducted an investigation on the

dynamics of the microbial population of T3MP1 over a five year time span (May 2003-February 2008). It was confirmed that major shifts occurred in the bacterial populations, where within a three month period the dominant acetogen *Sporomusa* disappeared and was replaced by another putative acetogen *Acetobacterium*. Therefore, in the current pH experiments only *Acetobacterium* was analyzed. Two different types of methanogens were also measured in the pH cultures: the acetoclastic methanogen, *Methanosarcina* and the mainly hydrogenotrophic methanogen, *Methanomicrobiales.*

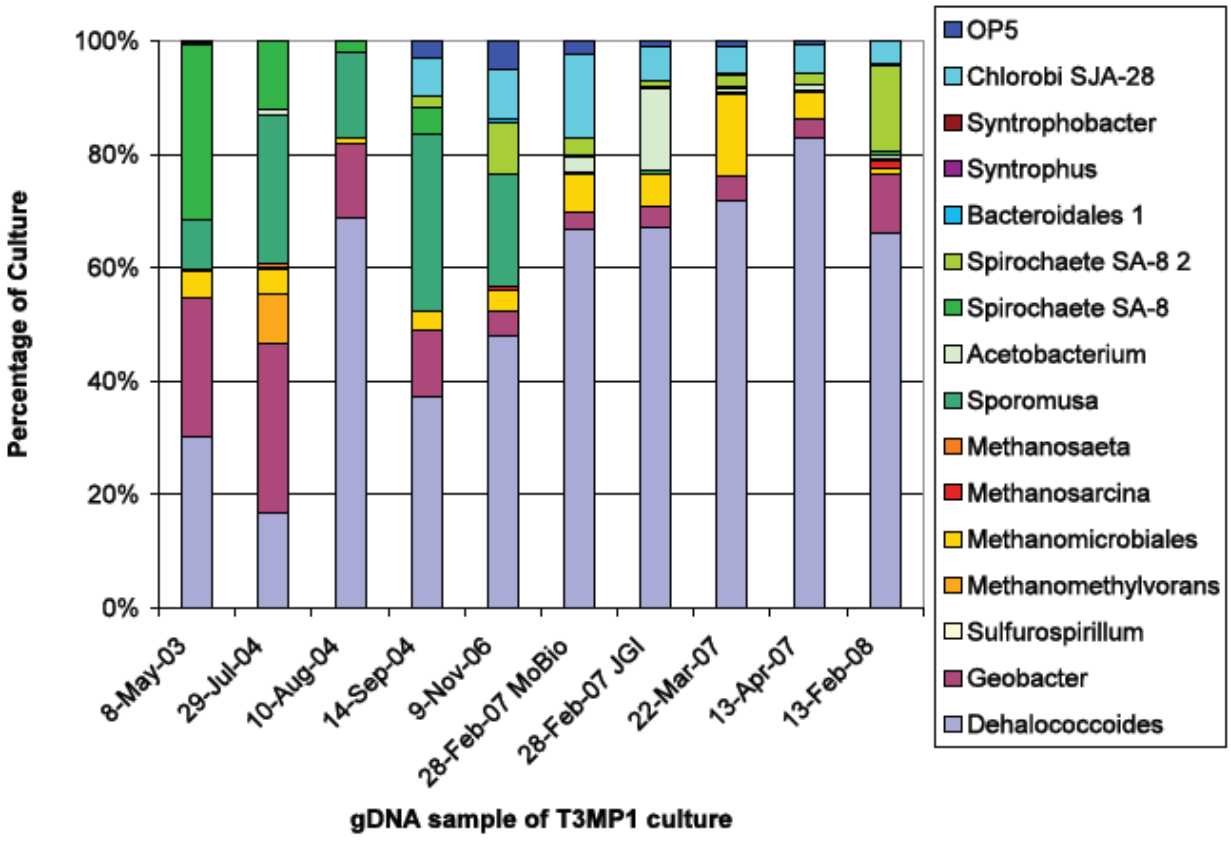

Figure 10: Quantitative PCR analysis of the 16S gene for different OTUs in the T3MP1 culture from May 2003-February 2008 (Waller 2009).
Each OTU is represented by a different colour and the stacked bars illustrate the percentage that each OTU occupies of the culture.

5.3 Results and Discussion

KB-1 is a culture that relies on the syntrophic relationships between dechlorinators, methanogens and acetogens. Many other dechlorinating cultures have similar combinations of microorganisms (Dennis 2003; Gu 2004; Macbeth 2004; Freeborn 2005; Hug 2012). The competition for the electron donors and the capabilities of the other organisms to survive at low pH conditions can affect dechlorination. In this study, the effects of two major electron donors were looked at: methanol and hydrogen. Methanogens and acetogens both compete for available methanol while it is also known that there is competition for hydrogen by hydrogenophilic methanogens and homoacetogens. In the midst of the competition between these organisms, *Dehaloccoides* must also compete in order to survive. Several microorganisms were examined in the pH cultures that originated from a TCE/methanol KB-1 culture known as T3 MP1. The qPCR results were analyzed from DNA extracted on day 360 of the experiment, sample calculations can be found in Appendix D that show the conversion of raw data to 16S rDNA copies/mL of culture. The primers targeting Archaea and Bacteria were used to estimate the abundance of these total populations. By subtracting the abundance of all measured specific phylotypes within each domain, the abundance of Archaea and Bacteria that were not targeted by the specific primers could be estimated in this study. These were labelled as “other Archaea” and “other Bacteria”.

The pH 7 cultures were compared with the KB-1 culture where the inoculums were first obtained from, T3 MP1. The most recent complete phylotype analysis of T3 MP1 was done by Hug (2012) in 2008. Figure 11 compares the pH 7 cultures, amended with VC as the electron acceptor and hydrogen or methanol as the electron donor, with data obtained from Hug (2012) of the T3 MP1 KB-1 culture, amended with TCE as the electron acceptor and methanol as the electron donor.

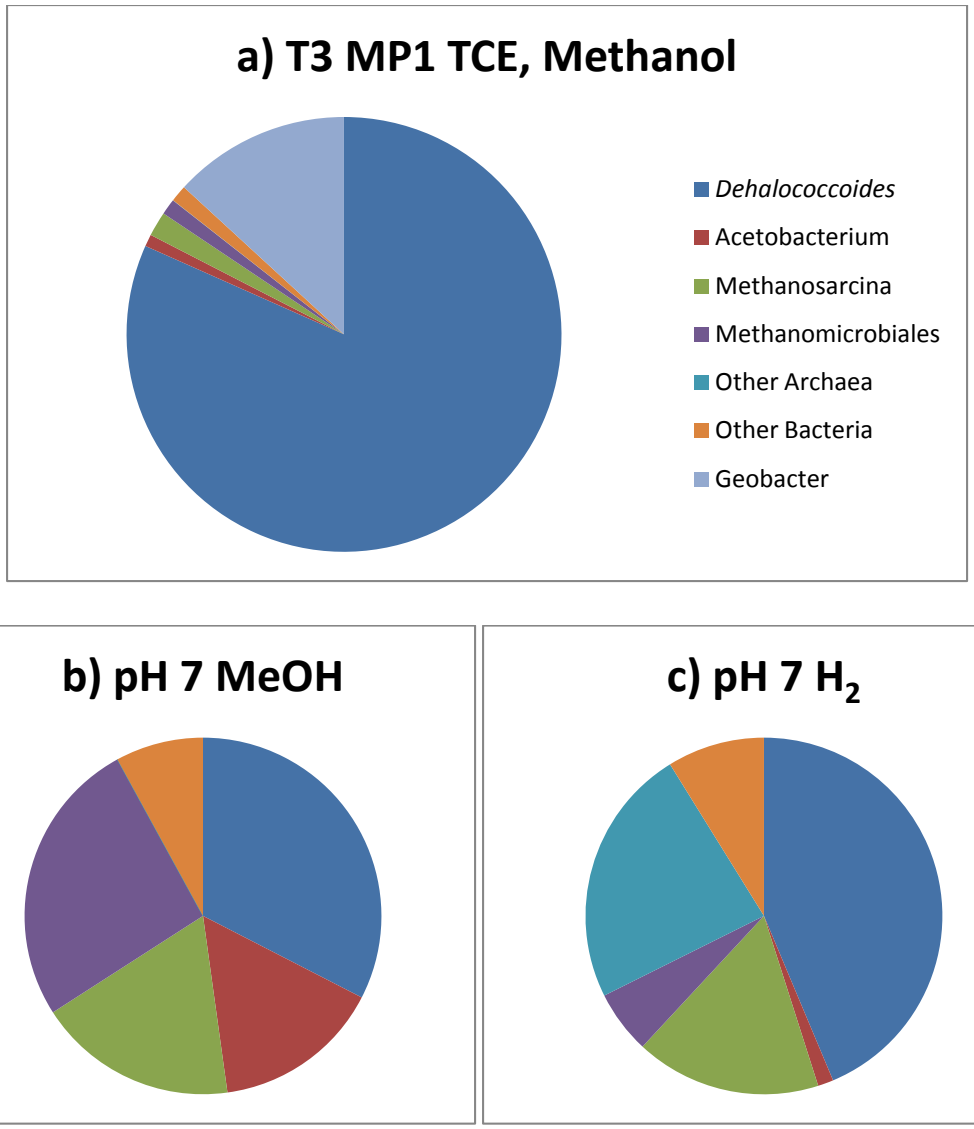

Figure 11: Proportional comparison of 16S rDNA copies/mL from qPCR analysis of KB-1 cultures at pH 7
Select microorganisms were measured for their 16S rDNA copies/mL. a) Data for the T3 MP1 KB-1 culture was measured in 2008 modified from Hug 2012. b) and c) represent the proportions of microorganisms in the pH 7 methanol-amended and hydrogen-amended cultures, respectively.

A relative comparison of the pH 7 cultures to the T3 MP1 KB-1 culture shows that *Dehalococcoides* accounts for approximately 1/3 of the microorganisms in the pH 7 VC-amended cultures. This may be due to the fact that VC provides 1/3 of the electron equivalents that TCE does as an electron acceptor. Geobacter was not measured in the cultures for the experiments in this thesis due to the fact that this organism is only capable of dechlorinating PCE and TCE to cDCE and not VC to ethene. A comparison between the methanol-amended and hydrogen-amended pH 7 cultures shows that

there are higher proportions of *Methanomicrobiales* and *Acetobacterium* in the methanol-amended cultures since fermentation of methanol is required before dechlorination occurs. A higher proportion of other Archaea present in the hydrogen-amended cultures suggest that other hydrogenotrophic methanogens other than *Methanomicrobiales* are dominant in these cultures. The proportions of other Bacteria and *Methanosarcina* did not change between the electron donors. Figure 12 compares the concentrations of the 16S rDNA gene for each target OTU within each pH culture.

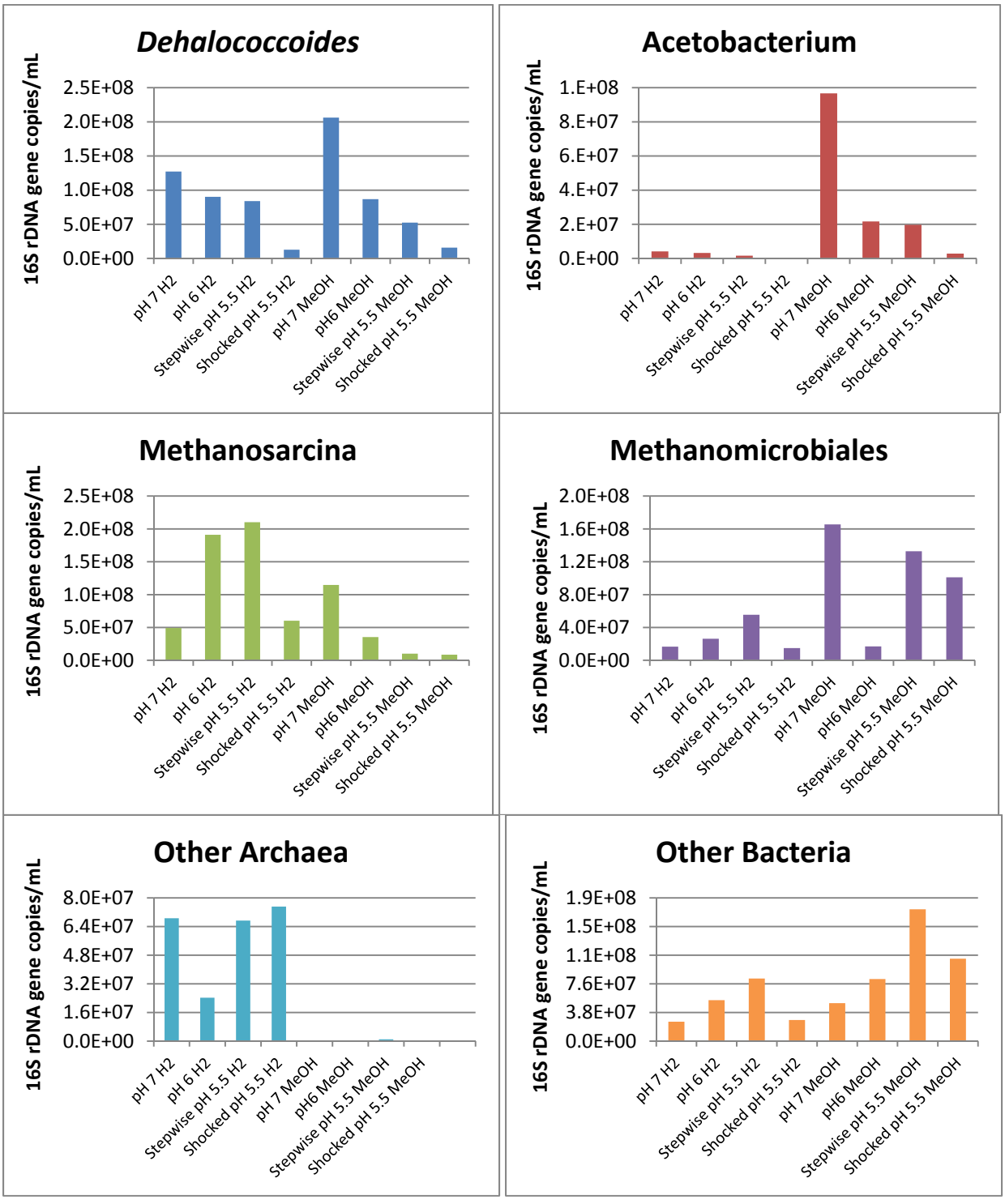

Figure 12: Quantitative PCR analysis for select OTUs in the pH cultures (May 2012 – Day 360)
Each graph represents a specific OTU. The bars represent each culture under different pH and electron donors.

The 16S rDNA copies/mL of *Dehalococcoides* decreased with decreasing pH. The concentration of *Dehalococcoides* for the "stepwise" cultures was higher than the "shocked" cultures for both electron donors. A brief thermodynamic analysis was conducted on the dechlorination of VC to ethene with hydrogen as the electron donor where free energies of reaction were calculated according to pH (Appendix B). Changes in free energy of reaction as a function of pH were found to be insignificant and therefore no further analysis was made. At pH 7, there was slightly greater 16S DNA copies/mL of *Dehalococcoides* for the methanol-amended cultures than the hydrogen-amended cultures. The effect of switching the electron donor from methanol to hydrogen caused a decrease in *Methanomicrobiales* and *Acetobacterium.* Previous analyses (Figure 11) of T3MP1 and its subcultures displayed similar results. Since hydrogen is the direct electron donor for *Dehalococcoides* there is less dependence on fermentation by acetogens. A lesser presence of the hydrogen-utilizing methanogen, *Methanomicrobiales, (*compared to the methanol-amended culture) indicates that *Dehalococcoides* was able to outcompete this particular methanogen for hydrogen.

As for the other microorganisms in the culture, the two different donors resulted in growth of different methanogens. These results correlate with the electron balance findings in which approximately 95-98% of the H_2 equivalents were channeled towards methanogenesis (and acetogenesis). Because of the slower rate of dechlorination at low pH, the methanogens were able to outcompete the dechlorinators for H_2 and therefore were in higher abundance compared to the pH 7 cultures.

The methanol-amended cultures also experienced a decrease in *Dehalococcoides* as a function of pH. The acetogen, *Acetobacterium* is only present in the methanol-amended cultures due to the fact that *Acetobacterium* can ferment methanol to hydrogen and acetate. This corresponds with the electron balance results found in the previous chapter that had some indications of acetogenesis occurring in the methanol-amended cultures and not the hydrogen-amended cultures. As the pH decreased the 16S rDNA copies/mL of both *Methanosarcina* and *Acetobacterium* also decreased but there was an increase in other bacteria that were not targeted by the qPCR primers chosen in the low pH cultures. Section 3.6.1 discussed the increase in

acetate concentrations at pH 5.5 for both “shocked” and “stepwise” conditions suggesting that either acetate consumption or acetate production has been disturbed. The increase in bacteria may suggest that more acetate is produced than can be consumed by methanogens. It suggests a combination of an acetogen able to produce acetate at low pH and an acetoclastic methanogen unable to grow at low pH.

Growth of *Methanomicrobiales* is consistent with the literature and past enrichments of KB-1 on methanol at pH 7(Duhamel 2007). This particular order of methanogens utilize hydrogen and formate as electron donors (Castro 2004). The methanogens is present in all of the culture with a most significant presence in the pH 7, and 5.5 methanol-amended cultures, which suggests this methanogen may be acid-tolerant. Recently, an acidophilic methanogen, *Methanoregula boonei*, was isolated with growth between pH 4.5-5.5 and an optimum pH near 5.1 (Brauer 2011). The author also describes how several representatives from mixed cultures, including KB-1, contain methanogens that share 98-99% identity with this specific strain.

The microorganisms that were mainly affected by pH in the hydrogen-amended cultures were *Dehalococcoides* and *Methanosarcina*. The analysis in Figure 12 shows a decrease in *Dehalococcoides* and increase in *Methanosarcina* as a function of pH. The enrichment of the acetoclastic methanogen *Methanosarcina* in the H_2-amended cultures may be due to the fact that acetate was also provided as a carbon source for the dechlorinators. Acetate was added only to the hydrogen-amended cultures with an initial target concentration of 5 mM. The culture seemed to perform dechlorination better at the beginning of the addition of acetate. When the acetate concentrations dropped below 1-2 mM, dechlorination would slow down. Heimann *et al.* suggests that *Methanosarcina* may be a driver of VC dechlorination (Heimann 2006). He suggested that *Methanosarcina*, while cleaving acetate to methane, simultaneously oxidizes acetate to CO_2 and H_2 which drives dechlorination of VC to ethene by *Dehalococcoides.* The electron balance indicated the electron equivalents favoring methanogenesis instead of dechlorination for both pH conditions with H_2 as the electron donor but qPCR results indicate no enrichment of hydrogenotrophic methanogens. From the electron balance there was increased consumption of acetate at pH 6 which correlates with the

results of the qPCR indicating that growth of *Methanosarcina* continued even for the stepwise pH 5.5 and pH 6. With fewer electrons shifting towards dechlorination at low pH, the available electrons from acetate favored methanogenesis which resulted in *Methanosarcina* to increase at pH 6 and pH 5.5. As for the other organisms analyzed in the hydrogen-amended cultures, the small presence of *Methanomicrobiales* and other Bacteria did increase slightly but not significantly compared to its abundance in the methanol-amended cultures.

Since both the population of *Dehalococcoides* and the average rate of ethene production decreased as a function of pH, a correlation can be made between the population and the rate. A linear model was created from the calculated average rates of ethene production at each pH excluding the "shocked" pH rates (Figure 13). The concentration of *Dehalococcoides* was converted to 16S rDNA copies/L and the rate of ethene production was converted to µmol/L/hr.

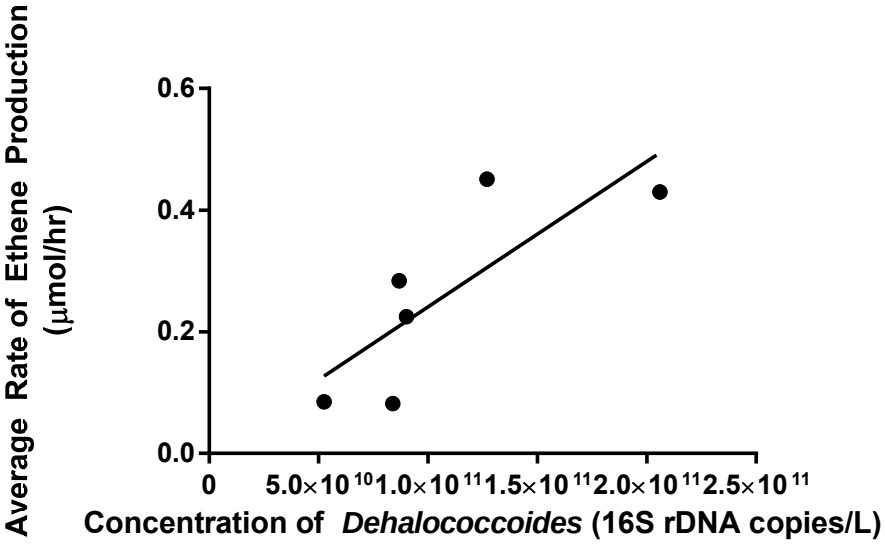

Figure 13: Linear model of population of *Dehalococcoides* and average rate of ethene production

The model creates a relationship between the population of *Dehalococcoides* and an average rate of ethene production (µmol/hour). The linear equation was determined to be:

Equation 6:

$$\text{Rate of Ethene Production } \left(\frac{\mu\text{mol/L}}{\text{hr}}\right) = 2.4 \times 10^{-12} \times \textit{Dehalococcoides} \text{ Population } \left(16\text{S rDNA}\frac{\text{copies}}{\text{L}}\right) + 1.6 \times 10^{-3}$$

Simmonds (2007) also found a correlation between the population of *Dehalococcoides* and rate of VC dechlorination. She reported the relationship between VC dechlorination rates in µmol/L/hr was related to the number of *Dehalococcoides* 16S rDNA gene copies/L via the following equation:

Equation 7:

$$\text{VC dechlorination rate } \left(\frac{\frac{\mu\text{mols}}{\text{L}}}{\text{hr}}\right) = 1 \times 10^{-11} \times \text{Number of } \textit{Dehalococcoides } \left(16\text{S rDNA gene}\frac{\text{copies}}{\text{L}}\right) + 2.9$$

The slope of the equation found in Simmond's thesis is relatively larger than the slope for this thesis. The KB-1 culture used in the dechlorination assay for Simmond's thesis was obtained from SiREM that has been enriched on TCE for several years and has relatively different organisms from the original KB-1 culture, T3 MP1, used in this thesis (Waller, 2009). This relationship had a reported R^2 value of 0.97, which is significantly higher than the R^2 value of 0.64 reported for this thesis. The discrepancy may be due to the fact that the population of microorganisms in KB-1 was a function of pH while the relationship found in Simmond's thesis was from growth of *Dehalococcoides* in KB-1 cultures obtained from SiREM. As well, the model for this thesis was determined through changes in the population dynamics of the microorganisms in the KB-1 culture at one point in time. It may be possible to correlate a more accurate R^2 value with qPCR analysis of *Dehalococcoides* at various time points during the course of the experiment as well as a function of pH. The linear correlation between rate of ethene production and the population of *Dehalococcoides* suggests that changes in dechlorination rates are subject to changes in the culture composition under certain conditions. Therefore, in this case, the effect of decreasing pH corresponds to a relationship between rate of ethene production and number of *Dehalococcoides* that can be modeled linearly.

Chapter 6: Conclusions and Engineering Significance

6.1 Summary

Acidification of groundwater at chlorinated ethene contaminated sites is a common problem where the acid build-up can cause a decrease in pH and associated reduction in dechlorination rates. Common methods to prevent acidification include circulation of buffer solutions containing dissolved alkaline materials such as sodium or potassium bicarbonate. The use of these materials can be quite costly depending on the acidity of the site and, at times, are ineffective. An alternative solution is to develop an enrichment culture capable of dechlorinating at low pH environments.

The objectives of this thesis were to compare the rates of dechlorination in KB-1 cultures grown at pH 7, pH 6 and pH 5.5 with two different electron donors: methanol and hydrogen. Rates of dechlorination at pH 6 were approximately half of those at pH 7 but continuous enrichment of the pH 6 cultures resulted in a slow improvement of rate over 500 days, suggesting acclimation to this pH. The KB-1 culture performs better when exposed to a lower pH in increments rather than instantaneously. In addition, the exposure of KB-1 to pH 6 and 5.5 does not result in an immediate drop of the rate of ethene production; rather the rate of ethene production declines slowly over time and multiple degradation cycles. All of the cultures are still currently maintained in order to investigate if the rate of dechlorination at pH 6 or pH 5.5 for either of the electron donors will improve with further incubation. The relative proportions of methanogenesis, acetogenesis and dechlorination at each of the pH conditions was also determined. The majority of electrons from donors were used for methanogenesis and/or acetogenesis rather than dechlorination at all pH conditions. The abundance of specific microorganisms known to be present in KB-1 was measured by qPCR as a function of pH and electron donor. In addition, total Bacteria and total Archaea were also measured to account for other organisms not targeted by specific qPCR primers. It was found that the population of *Dehalococcoides* decreased with decreasing pH and that acid-tolerant methanogens may be present in the KB-1 cultures.

6.2 Conclusions

- Sustained dechlorination is possible in cultures maintained at pH 6 and is approximately half the rate of cultures maintained at pH 7 for both electron donors.
- The KB-1 culture performs better when exposed to a lower pH in increments rather than instantaneously.
- The pH fluctuated during the course of the experiment and required constant observation and correction in order to obtain set point values. In the hydrogen-amended cultures the pH tended to increase and in the methanol-amended cultures the pH tended to decrease in accordance with acid-consuming and acid-generating reactions.
- Improvements in the average rate of ethene production in the methanol-amended pH 6 cultures suggest acclimation of the KB-1 culture may be possible through exposure to low pH over an extended period of time.
- More than 95% of the total electron equivalents were used towards methanogenesis and acetogenesis. There was a statistically significant difference between the pH 6 hydrogen-amended and methanol-amended cultures, where the pH 6 methanol-amended cultures had higher electron equivalents shuttled towards dechlorination. This further suggests that methanol may be a more proficient electron donor than hydrogen at low pH.
- Lower rates of dechlorination correspond to low *Dehalococcoides* numbers except between the pH 7 and pH 6 methanol-amended cultures, where similar proportions of *Dehaloccoccoides* between the cultures were observed.
- Different methanogens were enriched between the hydrogen-amended and methanol-amended cultures, but both methanogens proved to be acid-tolerant in the cultures. *Methanosarcina* was dominant in the hydrogen-amended cultures while *Methanomicrobiales* was dominant in the pH 5.5 methanol-amended cultures.
- A linear relationship was found between the population of *Dehalococcoides* and the rate of ethene production that corresponds to a slope of 2.4×10^{-12} µmol/hr/16S rDNA gene copies and an R^2 value of 0.64.

6.3 Engineering Significance

Bioaugmentation of chlorinated ethenes at contaminated sites has been very successful over the years as a remediation technique. These contaminated sites are often at near neutral pH but an increasing number of sites become acidic during remediation by the production of HCl and acetic acid. The current solution is to disperse various buffer solutions containing alkaline or bicarbonate materials into the contaminated site to raise the pH. Since KB-1 is a widely used successful bioaugmentation culture that stemmed from several years of enrichments, it may be a possibility to expand the range of KB-1's dechlorinating abilities to include dechlorination at pH 6 or lower. This thesis has shown preliminary results that adaptation of a dechlorinating culture, such as KB-1, may be possible by exposure to lower pH conditions for an extended period of time. Examining how the distribution of electrons and microbial composition can be affected by pH also helps further decipher microbial community interactions in KB-1 to improve understanding of the dynamics of the system. The experimental studies done in this thesis have expanded the general knowledge of KB-1 and explored the outer boundaries of what KB-1 is capable of in the bioremediation industry.

6.4 Future Work

This thesis involved the development of an enrichment culture of KB-1, branched off from the main culture T3 MP1, capable of dechlorinating at pH 6 or lower. Currently the methanol-amended enrichments are capable of complete VC dechlorination at pH 5.7 with relatively rapid dechlorination (approximately 60% of pH 7). Some future work with these cultures may be:

- Continue maintenance and observation of the low pH cultures to determine if further improvement in rates can occur.
- Scale-up of enrichments to reactor sizes of 1 L.

- Further decreasing the pH to acclimate the cultures to lower pH.
- McCarty *et al.* (2007) suggested that formate as an electron donor may be a solution to low pH issues in bioaugmentation. Formate as an electron donor produces bicarbonate as a product that can neutralize acid produced during dechlorination.
- A microcosm study can be conducted on acidic contaminated sites using the enrichment culture currently developed that is capable of dechlorinating at pH 6.
- A further in-depth overall analysis of the unknown Archaea and Bacteria that changes in microbial composition using a recently developed technique known as pyrotag sequencing that provides more depth of coverage of microbial composition (currently in progress).
- Samples were extracted for DNA at certain time points over the course of the experiment (Day 140, 245, 360, 426, 587). Further quantification of microbial composition can be conducted with qPCR on these samples to observe changes over a period of time.

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Appendix A: Calculation for Determining Dechlorination Rates

This section will describe how the rate of ethene production was calculated from the GC raw data. The concentrations of VC will be calculated.

Calibration curves for VC, methane and ethene were generated by injecting different known volumes of each compound into 250 mL mininert bottles filled with 200 mL of water to achieve a target concentration. The volume of each compound to add was calculated as follows using VC as an example:

Target aqueous concentration of VC added to mini-inert bottle = **0.01 mM**

Using Henry's law, the total number of moles can be calculated as follows:

First the concentration in the gas phase must be calculated using Henry's law:

$$K_H = \frac{c_{gas}}{c_{aq}}$$

$$c_{gas} = 0.01 \times 0.9289 = 0.009289\ mM$$

Then the total number of moles can be calculated knowing the volume of each phase:

$$total\ \#\ of\ moles = (c_{gas} \times V_{gas}) + (c_{aq} \times V_{aq})$$

$$total\ \#\ of\ moles = (0.009289mM \times 0.05L) + (0.01mM \times 0.2L) = \mathbf{0.00246\ mmoles}$$

Once the total number of moles was established, the volume to inject into the bottle was calculated from the ideal gas law:

$$PV = nRT$$

$$V = \frac{0.00246 \times 10^{-3}\ moles \times 8.314\ \frac{L\ kPa}{K\ mol} \times 298.15K}{101.3\ kPa} \times \frac{10^6 \mu L}{L} = 60\ \mu L$$

Once each standard was made with target concentrations of VC, headspace of each bottle was measured on the GC in triplicates. A sample calibration curve is shown in Figure 1. The slope of the calibration curve was 4.76×10^{-8} mM (aqueous phase)/Peak Area.

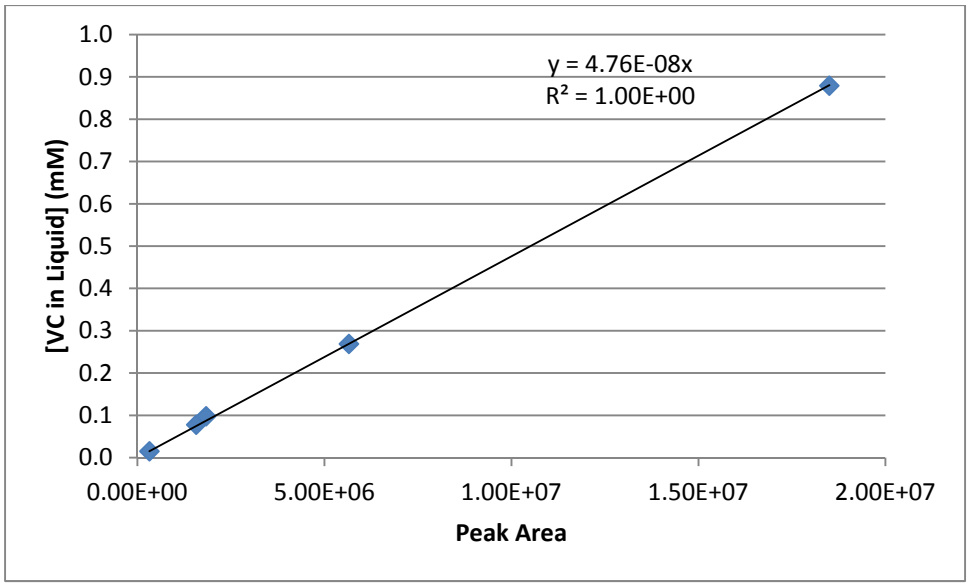

Figure A-1: Sample GC calibration curve for VC

Therefore, the concentration in mM of VC in the liquid phase can be determined from the slope of the calibration curve.

Response factors for all measured compounds are listed in the table below:

Table A-1: Response factors for measured VOCs on the GC

Compound	Response Factor (mM/peak area)
Vinyl Chloride (liquid phase)	4.73×10^{-8}
Ethene (gas phase)	4.77×10^{-8}
Methane (gas phase)	6.74×10^{-8}

An example of the area counts from one VC degradation cycle of the pH 7 cultures (in duplicates) is shown in Table A-2.

Table A-2: Sample area counts for one degradation cycle in the pH 7 methanol-amended cultures from the second transfer

	Area count measured on GC			
	pH 7 methanol-amended			
Day	**Ethene**		**VC**	
	Replicate 1	Replicate 2	Replicate 1	Replicate 2
203	1.97E+07	2.17E+07	3.26E+06	3.15E+06
210	2.76E+07	3.33E+07	1.59E+05	1.40E+03
219	2.80E+07	3.38E+07	0.00E+00	0.00E+00

At day 219 the VC area counts have reached 0 and the ethene area counts can be used to calculate concentrations and a rate of ethene production of this specific degradation cycle.

Using Henry's law a mole balance is first calculated in order to determine if the degradation cycle is feasible for rate calculations. The area counts are first converted into mM in the aqueous phase for VC and mM in the gas phase for ethene (calibration curves were generated for ethene using the same method as described previously for VC). Table A-3 shows the converted concentration values.

Table A-3: Concentrations of ethene and VC for pH 7 methanol-amended cultures

	Concentrations			
	pH 7 methanol-amended			
Day	**Ethene (mM in gas phase)**		**VC (mM in aqueous phase)**	
	Replicate 1	Replicate 2	Replicate 1	Replicate 2
203	0.9422	1.0366	0.1548	0.1498
210	1.3155	1.5871	0.0075	0.0001
219	1.3391	1.6148	0.0000	0.0000

Henry's law is used to convert the concentrations into either gas phase or liquid phase concentrations and the following equation is used again to calculate the total number of moles.

$$total\ \#\ of\ moles = (c_{gas} \times V_{gas}) + (c_{aq} \times V_{aq})$$

The total number of moles of VC and ethene are tabulated in Table 3. The mole balance is based on the assumption that the liquid volume in the bottles does not change. The volumes are assumed to be 100 mL of liquid and 60 mL of headspace. Once a reasonable mole balance has been achieved, the rate of ethene production can be calculated from the concentrations.

Table A-4: Mole balance for sample degradation cycle

	Total number of moles (mmoles)			
	pH 7 methanol-amended			
Day	**Ethene**		**VC**	
	Replicate 1	Replicate 2	Replicate 1	Replicate 2
203	0.0673	0.0741	0.0241	0.0233
210	0.0940	0.113	0.0117	1.56×10^{-5}
219	0.0957	0.115	0	0

The rate of ethene production for this degradation cycle was calculated as follows:

$$Rate\ of\ ethene\ production = 1000 \times \frac{(0.0957 - 0.0673) + (0.115 - 0.0741)}{2} \div (219 - 203)$$

$$= \mathbf{2.16}\ \mu\boldsymbol{mol/day}$$

Appendix B: Thermodynamic Sample Calculations

A brief thermodynamic analysis was conducted to determine if changes in rate or population of *Dehalococcoides* was due to thermodynamic limitations. The delta G of reaction, ΔG_r was calculated for the VC → ethene reaction.

Assumptions

- A midway VC and ethene concentration was used; all other compounds were at standard conditions.
    - Electron Acceptor [VC] = $5.92e^{-5}$ mol/L
    - Electron Donor [H_2] = $1e^{-4}$ atm *Estimation based on thesis (Duhamel 2005)
- Temperature: 298.15 K
- Cell formula: $C_5H_7O_2N$ (113 g/mol)
- Nitrogen source: NH_4^+; ΔG_{pc} = 18.8 KJ/eeq
- Assume ϵ = 0.6 (based on experimental data from McCarty 1969)

Sample Calculations

Electron acceptor: VC → Ethene at pH 7

Electron donor: Hydrogen

Carbon source: Acetate

Balanced half-reaction for electron acceptor:

$$\frac{1}{2}C_2H_3Cl + H^+ + e^- = \frac{1}{2}C_2H_4 + \frac{1}{2}Cl^-$$

The ∆G for this electron acceptor half-reaction can be calculated as follows:

$$\Delta G^{0\prime} = \frac{1}{2}\Delta G^{0\prime}_{ethene} + \frac{1}{2}\Delta G^{0\prime}_{Cl} - (\frac{1}{2}\Delta G^{0\prime}_{VC} + \frac{1}{2}\Delta G^{0\prime}_{H+})$$

$$\Delta G^{0\prime} = \frac{1}{2}(81.43) + \frac{1}{2}(-131.3) - \left[\frac{1}{2}(59.65) + \frac{1}{2}(-39.93)\right] = -34.79\ kJ/eeq$$

Since these values are under standard conditions of pH 7, 1 atm and 1 M concentrations the following equation can be used to reflect new free energy values under actual experimental conditions:

$$\Delta G = \Delta G^{0\prime} + RTlnK \quad where\ K = [products]/[reactants]$$

Therefore for VC → ethene at pH 6 and the following experimental conditions:

[VC] = 5.92×10^{-5} mol/L

[H_2] = 1×10^{-4} atm *Estimation based on thesis (Duhamel 2005)

[Acetate] = 0.004 mol/L

The ΔG_d becomes:

$$\Delta G_d = -34.79 + 0.000831 \times 298.15 \times \ln\left(\frac{(5.92\times10^{-5})^{0.5}\times10^{0.5}}{(5.92\times10^{-5})^{0.5}\times(1\times10^{-4})}\right) = -37.65\ kJ/eeq$$

The same method is used to calculate the electron donor half-reaction (ΔG_a). The final ΔG_r can then be calculated as follows:

$$\Delta G_r = \Delta G_d - \Delta G_a$$

The ΔG_r at each pH for VC to ethene with hydrogen as the electron donor is depicted in the table below:

Table B-1: Comparison of thermodynamic free energy changes at experimental conditions for various pH values

pH	**7**	**6**	**5.5**
ΔG_r (kJ/e^- eq)	-63.3	-60.5	-59.0

Table B-2: Thermodynamic properties and experimental conditions

Compound				H_2 Amended			MeOH Amended			Concentration Units	Reference
Name	Formula	Std activity	$\Delta G^{0,}$ (kJ/mol)	7	6	5.5	7	6	5.5		
VC(aq)	C_2H_3Cl	1	59.65	5.92E-05	5.92E-05	5.92E-05	5.92E-05	5.92E-05	5.92E-05	mol/L	Dolfing and Janssen 1994
Ethene(aq)	C_2H_4	1	81.43	5.92E-05	5.92E-05	5.92E-05	5.92E-05	5.92E-05	5.92E-05	Partial pressure (atm)	Dolfing and Janssen 1994
Hydrogen ion	H^+	1.00E-07	-39.93	1.00	10.00	31.62	1.00	10.00	31.62	mol/L	Thauer et al. 1977
Hydrogen	H_2	1	0	1.00E-04	1.00E-04	1.00E-04	1.00E-04	1.00E-04	1.00E-04	Partial pressure	Brock Biology of Microorganisms
Carbon Dioxide	CO_2	1	-394.38	1	1	1	1	1	1	Partial pressure	Brock Biology of Microorganisms
Methane (aq)	CH4 (aq)	1	-34.74	1.09	1.09	1.09	1.09	1.09	1.09	Partial pressure	Brock Biology of Microorganisms
CO2 (aq)	CO2 (aq)	N/A	-394.4	1	1	1	1	1	1	Partial pressure	Brock Biology of Microorganisms
Methanol	CH3OH	1	-175.39	1	1	1	3.23E-04	3.23E-04	3.23E-04	mol/L	Brock Biology of Microorganisms
water (l)	water (l)	1	-237.17	1	1	1	1	1	1	mol fraction	Brock Biology of Microorganisms
Pyruvate	CH3COCOO-	1	-474.63	1	1	1	1	1	1	mol/L	Brock Biology of Microorganisms

Appendix C: Electron Balance Calculations

Table C-1: Electron balance results of hydrogen-amended pH 7 duplicate cultures

H_2 amended												
	pH 7 (G2-1)					pH 7 (G1-1)						
Date at end of each degradation cycle	Sept-6-11	Oct-17-11	Dec-20-11	Apr-10-12	May-16-12	Sept-6-11	Sept-19-11	Oct-17-11	Dec-20-11	Mar-21-12	June-12-12	
Total # of Electrons:												
Time = 0	7.20	6.32	5.42	11.02	2.69	7.78	7.54	7.52	5.27	5.63	7.90	
Time = Final	6.85	5.88	4.63	11.46	2.09	7.26	7.09	7.19	4.11	5.54	6.79	Total Var
ΔAcetate	-0.768	-0.984	-0.592	-1.043	-0.147	-1.720	-0.880	-0.400	-1.528	-0.667	-0.926	4.21E-04
ΔH2	-0.424	-0.576	-0.576	-0.576	-0.576	-0.576	-0.288	-0.576	-0.576	-0.576	-0.576	
ΔMethane	0.701	1.123	0.384	1.430	0.144	1.776	0.715	0.653	0.936	1.157	0.432	Total Stdev
ΔVC	-0.025	-0.025	-0.037	-0.045	-0.052	-0.012	-0.007	-0.020	-0.046	-0.034	-0.041	0.021
ΔEthene	0.023	0.023	0.030	0.052	0.030	0.015	0.005	0.017	0.051	0.038	0.049	
												Total Avg
%methanogenesis	96.8%	98.0%	92.8%	96.5%	82.7%	99.2%	99.3%	97.5%	94.9%	96.8%	89.8%	96.1%
%dechlorination	3.2%	2.0%	7.2%	3.5%	17.3%	0.84%	0.69%	2.5%	5.1%	3.2%	10.2%	3.9%

Table C-2: Electron balance results of hydrogen-amended pH 6 duplicate cultures

H_2 Amended												
	pH 6 (Y2-1)					pH 6 (Y1-1)						
Date at end of feeding cycle	Sept-9-11	Sept-19-11	Oct-17-11	Feb-28-12	June-12-12	Sep-9-11	Sept-30-11	Oct-17-11	Jan-24-12	Feb-28-12	Apr-26-12	
Total # of Electrons:												
Time = 0	3.23	7.32	8.12	11.48	1.79	3.06	9.38	8.11	8.62	9.99	0.77	
Time = Final	3.32	8.13	7.66	11.31	2.64	3.29	6.93	7.88	7.72	9.65	1.20	Total Var
ΔAcetate	-1.000	-0.248	-3.592	-3.374	-0.299	-0.240	-3.880	-0.320	-4.000	-3.618	-0.149	9.73E-05
ΔH2	-0.288	-0.288	-0.288	-0.576	-0.576	-0.288	-0.288	-1.152	-1.152	-0.576	-0.576	
ΔMethane	0.494	1.056	2.828	3.787	1.738	0.744	1.718	1.253	4.248	3.845	1.152	Total Stdev
ΔVC	-0.132	-0.011	-0.005	-0.048	-0.054	-0.011	-0.011	-0.035	-0.043	-0.040	-0.041	0.0099
ΔEthene	0.011	0.010	0.020	0.048	0.044	0.021	0.011	0.024	0.042	0.048	0.045	
												Total Avg
%methanogenesis	97.9%	99.0%	99.3%	98.8%	97.5%	97.3%	99.4%	98.1%	99.0%	98.8%	96.2%	98.3%
%dechlorination	2.10%	0.96%	0.70%	1.24%	2.46%	2.75%	0.64%	1.90%	0.97%	1.24%	3.76%	1.70%

Table C-3: Electron balance results of methanol-amended pH 7 duplicate cultures

Methanol-Amended															
	pH 7 (G3-1)						pH 7 (G4-1)								
Date at end of feeding cycle	**Sept-9-11**	**Sept-19-11**	**Oct-17-11**	**Nov-7-11**	**Dec-21-11**	**Feb-28-12**	**Sept-9-11**	**Sept-19-11**	**Nov-7-11**	**Dec-21-11**	**Jan-19-11**	**Mar-7-12**	**Apr-24-12**	**June-12-12**	
Total # of Electrons:															
Time = 0	**3.39**	**3.56**	**3.69**	**4.65**	**2.51**	**4.27**	**4.40**	**4.77**	**5.25**	**2.66**	**3.54**	**1.48**	**2.04**	**2.19**	
Time = Final	**3.12**	**3.24**	**3.73**	**4.25**	**2.23**	**3.60**	**4.34**	**4.42**	**4.97**	**2.63**	**3.56**	**1.39**	**1.26**	**1.62**	**Total Var**
ΔAcetate	0.016	-0.200	-0.168	0.056	0.192	-0.222	0.160	-0.048	-0.784	0.056	-0.040	0.125	0.070	0.033	**3.04E-04**
ΔMethanol	-0.905	-0.452	-0.452	-0.905	-0.905	-0.905	-0.905	-0.452	-0.904	-0.904	-0.904	-0.904	-0.904	-0.904	
ΔMethane	0.629	0.331	0.653	0.456	0.437	0.456	0.686	0.158	1.411	0.811	0.965	0.666	0.062	0.307	**Total Stdev**
ΔVC	-0.012	-0.013	-0.012	-0.038	-0.044	-0.048	-0.032	-0.013	-0.032	-0.043	-0.053	-0.032	-0.041	-0.061	**0.017**
ΔEthene	0.018	0.012	0.024	0.031	0.040	0.057	0.034	0.008	0.026	0.047	0.010	0.055	0.029	0.045	
															Total Avg
%methanogenesis & acetogenesis	97.2%	96.6%	96.5%	94.3%	94.0%	88.9%	96.2%	95.3%	98.2%	94.9%	99.0%	93.5%	82.2%	88.2%	**96.0%**
%dechlorination	2.77%	3.38%	3.49%	5.67%	6.01%	11.08%	3.82%	4.69%	1.81%	5.12%	1.05%	6.48%	17.81%	11.78%	**4.03%**

Table C-4: Electron balance results of methanol-amended pH 6 duplicate cultures

Methanol-Amended													
	pH 6 (Y3-1)					pH 6 (Y4-1)							
Date at end of feeding cycle	**Sept-9-11**	**Sept-22-11**	**Oct-17-11**	**Mar-7-12**	**Jun-12-12**	**Sept-6-11**	**Sept-19-11**	**Sept-30-11**	**Oct-17-11**	**Nov-2-11**	**Apr-10-12**	**June-12-12**	
Total # of Electrons:													
Time = 0	**2.62**	**2.72**	**4.02**	**3.52**	**1.82**	**2.59**	**2.22**	**2.95**	**4.10**	**4.21**	**1.34**	**1.97**	
Time = Final	**2.48**	**2.71**	**3.90**	**3.47**	**1.65**	**2.66**	**2.52**	**3.19**	**3.30**	**3.33**	**1.34**	**2.12**	**Total Var**
ΔAcetate	-0.400	0.000	-0.016	0.005	-0.001	-0.144	-0.112	0.008	0.040	-0.088	-0.035	0.040	**5.12E-04**
ΔMethanol	-0.452	-0.452	-0.905	-0.905	-0.905	-0.452	-0.452	-0.452	-0.904	-0.904	-0.904	-0.904	
ΔMethane	0.715	0.432	0.816	0.840	0.730	0.677	0.840	0.672	0.067	0.125	1.296	1.018	**Total Stdev**
ΔVC	-0.015	-0.014	-0.032	-0.046	-0.057	-0.018	-0.009	-0.011	-0.031	-0.035	-0.402	-0.058	**0.023**
ΔEthene	0.009	0.025	0.022	0.057	0.066	0.011	0.028	0.017	0.024	0.018	0.041	0.056	
													Total Avg
%methanogenesis & acetogenesis	98.8%	94.6%	97.4%	93.7%	91.7%	98.3%	96.8%	97.5%	81.5%	87.4%	97.0%	94.7%	**96.1%**
%dechlorination	1.22%	5.39%	2.65%	6.34%	8.27%	1.66%	3.18%	2.49%	18.54%	12.61%	3.04%	5.25%	**3.95%**

Appendix D: Quantitative Real-time PCR Calculations

This section will show the sample calculations for the determination of 16S rDNA copies/mL of culture quantified by qPCR assays. A sample calculation for the quantification of *Dehalococcoides* will be shown here.

Plasmids for calibration were made using the method described in Zila (2012) and Duhamel (2005). Plasmid concentrations were measured in triplicate using a Nanodrop spectrophotometer.

Concentration of *Dehalococcoides* 16S rDNA plasmid = $\frac{32.6+33.4+33.4}{3} = \boldsymbol{33.13\ ng/\mu L}$

The concentration of 16S rDNA copies/µL was calculated based on an average molecular mass of 660 g/mol and Avogradro's number using the following equation:

$$Concentration\ \left(\frac{copies}{uL}\right) = \frac{6.02 \times 10^{23} bp/mol \times concentration(\frac{ng}{\mu L})}{Plasmid\ size(\frac{bp}{plasmid}) \times \frac{660g}{mol} bp \times 10^{9} ng/g}$$

$$= \frac{6.02 \times 10^{23} \times 33.13}{5450 \times 660 \times 10^{9}} = \boldsymbol{5.54 \times 10^{9}\ 16S\ rDNA\ copies/\mu L}$$

Calibration standards were made by serial dilution of plasmid in UltraPure™ DNase/RNase-Free Distilled Water from 10^{-1} to 10^{-8} times the original concentration. A sample plate template is shown in Table 1. All organisms were assayed with a 1/10 and 1/100 dilution in duplicate. In general the 1/10 dilutions produced better efficiencies and was therefore used to calculate concentrations instead. Calibration curves for each microorganism analyzed in qPCR are shown in the figures below. The equation of the standard curve for *Dehalococcoides* (r^2 = 0.999) was as follows:

$$y = -3.621x + 35.13$$

$$\mathrm{C(T)} = -3.621 \times \log\left(Dehaloccoides\ 16S\ rDNA \frac{copies}{\mu L}\right) + 35.13$$

C(T) represents the cycle number at which fluorescence in the sample passed the threshold fluorescence. The equation of the standard curve is then used to calculate the concentration of

Dehalococcoides copies. For example, sample G1-1 (1/10 dilution) from April 21st produced a C(T) value of 15.63 for *Dehalococcoides,* therefore:

$$Dehalococcoides\,\frac{copies}{\mu L} = 10^{\frac{15.63-35.13}{-3.621}} = \mathbf{2.42 \times 10^5}$$

In order to calculate the final copies per template volume in the culture the volume of culture from which the DNA was extracted and volume of extraction must be known. In this case, the DNA volume extracted was 5 mL from a 100 mL culture.

$$16S\ rDNA\,\frac{copies}{mL}\,culture = \frac{\frac{copies}{\mu L}\,template \times extraction\ volume(\mu L)}{culture\ volume\ (mL)} \times dilution\ factor$$

$$= \frac{2.42 \times 10^5 \times 5000}{100} \times 10 = \mathbf{1.21 \times 10^7 copies/mL}$$

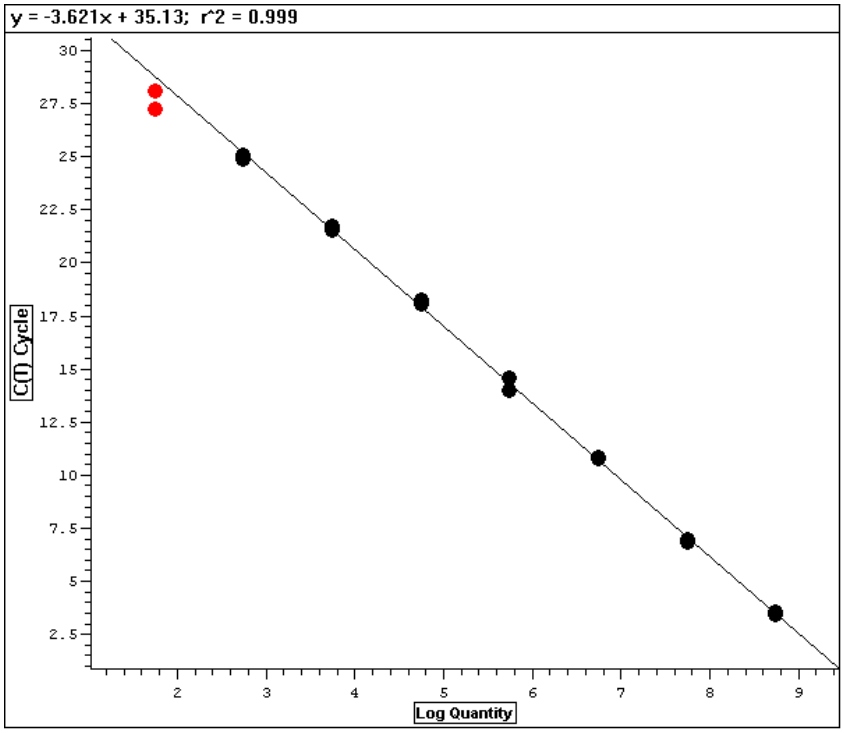

Figure D-1: Quantitative PCR Standard Curve for *Dehaloccoides*

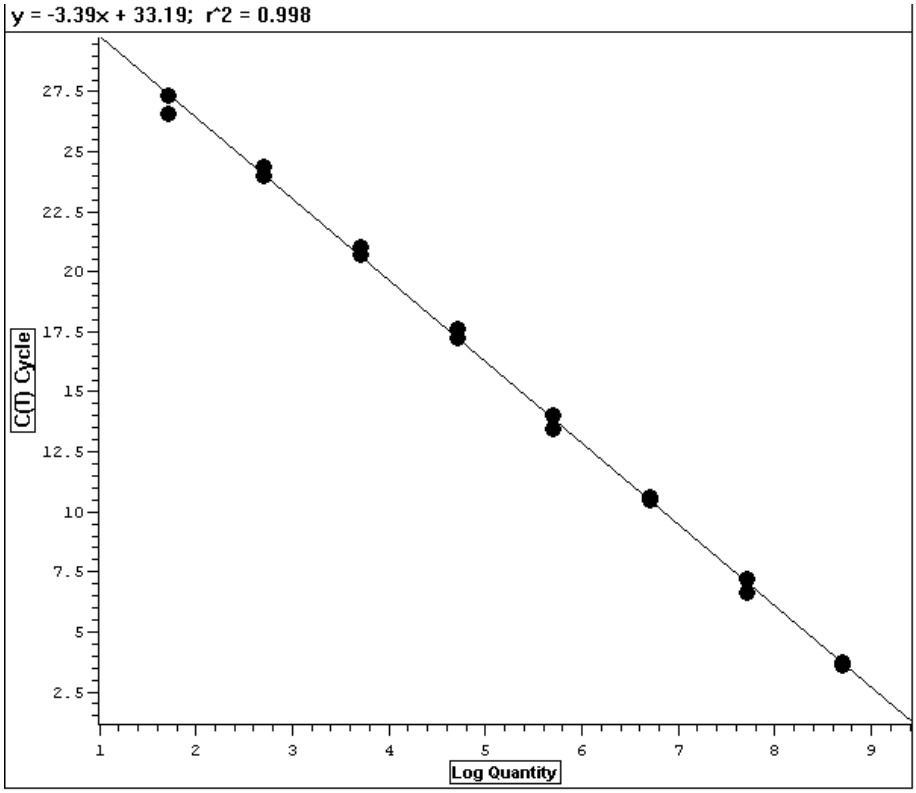

Figure D-2: Quantitative PCR Standard Curve for *Acetobacterium*

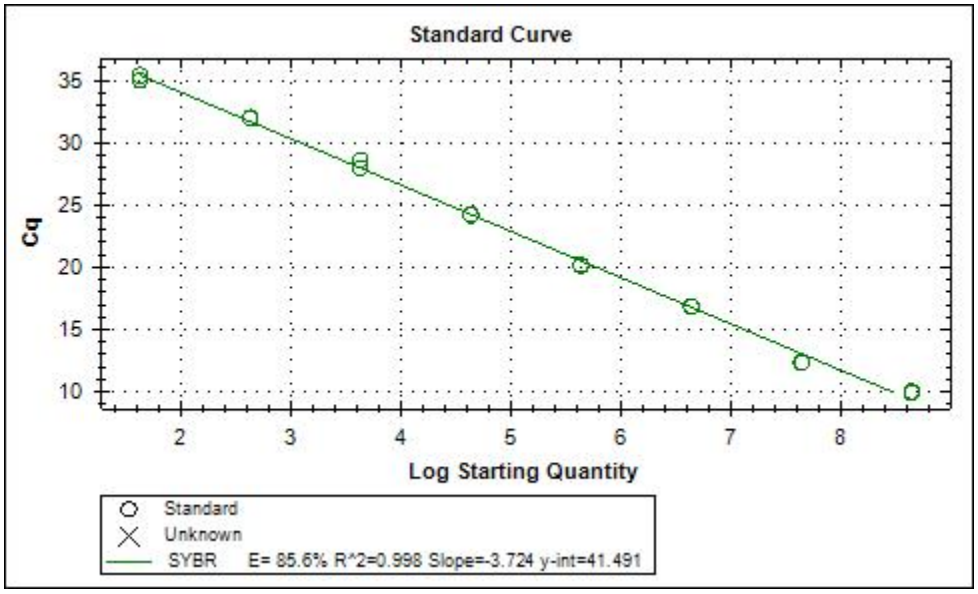

Figure D-3: Quantitative PCR Standard Curve for General Bacteria

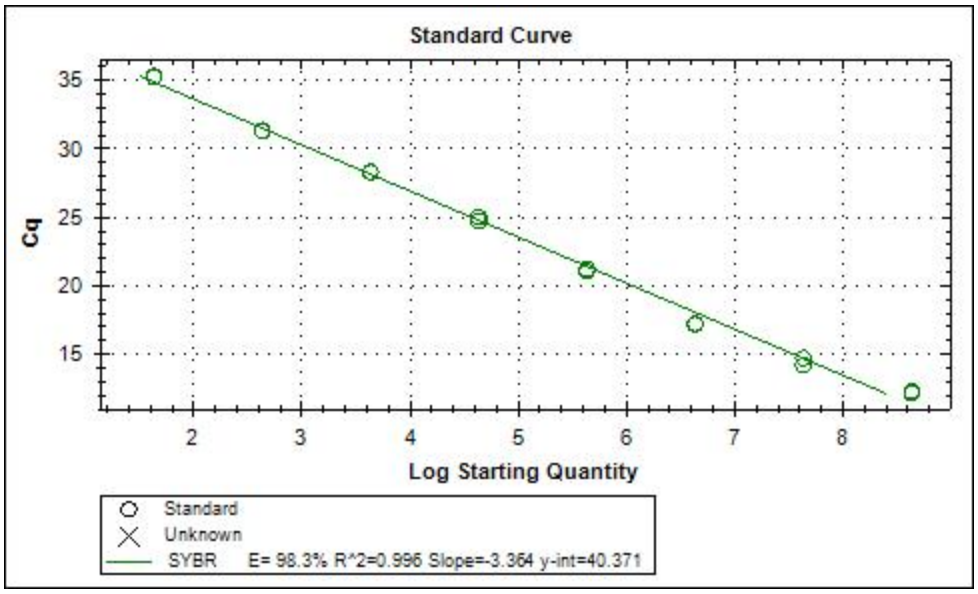

Figure D-4: Quantitative PCR Standard Curve for *Methanomicrobiales*

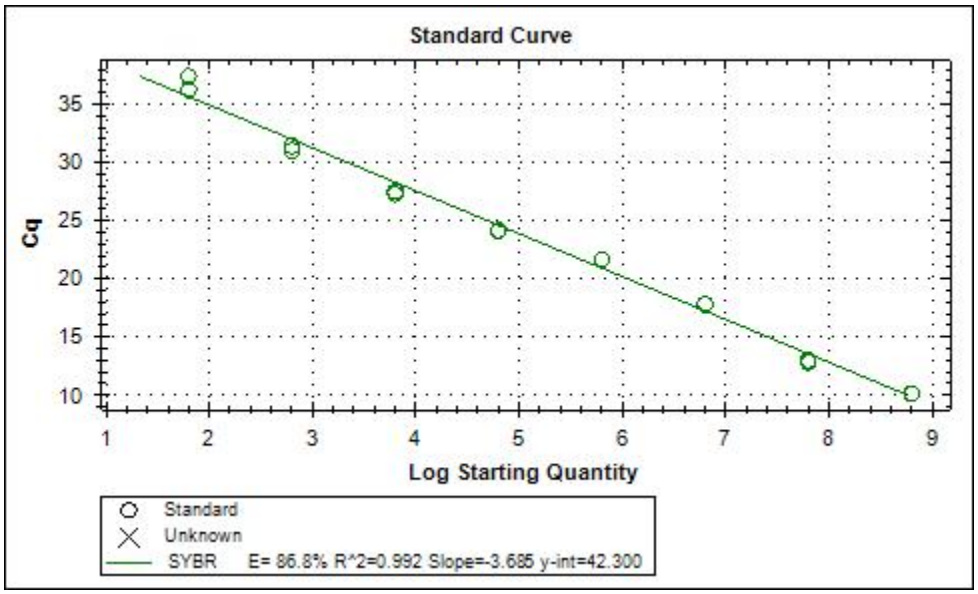

Figure D-5: Quantitative PCR Standard Curve for *Methanosarcina*

TableD-1: Quantitative PCR data for all OTUs for each pH culture

Units (16S rDNA gene copies/mL)	Shocked pH 5.5 MeOH	Stepwise pH 5.5 MeOH	pH6 MeOH	pH 7 MeOH	Shocked pH 5.5 H2	Stepwise pH 5.5 H2	pH 6 H2	pH 7 H2
Dehalococcoides	1.59E+07	5.25E+07	8.69E+07	2.06E+08	1.27E+07	8.39E+07	9.02E+07	1.27E+08
Acetobacterium	2.81E+06	1.97E+07	2.16E+07	9.67E+07	3.31E+05	1.62E+06	3.22E+06	4.11E+06
Methanosarcina	8.59E+06	1.02E+07	3.55E+07	1.15E+08	6.04E+07	2.10E+08	1.91E+08	4.91E+07
Methanomicrobiales	1.01E+08	1.33E+08	1.70E+07	1.65E+08	1.49E+07	5.55E+07	2.62E+07	1.66E+07
Total Archaea	2.08E+07	2.88E+07	9.38E+06	5.55E+07	3.01E+07	6.66E+07	4.83E+07	2.69E+07
Total Bacteria	2.56E+07	4.94E+07	3.82E+07	7.06E+07	8.24E+06	3.37E+07	2.95E+07	3.14E+07
Other Archaea*	- 5.35E+06	9.77E+05	- 5.61E+06	- 2.59E+06	7.52E+07	6.74E+07	2.43E+07	6.86E+07
Other Bacteria**	1.09E+08	1.75E+08	8.25E+07	5.04E+07	2.81E+07	8.31E+07	5.43E+07	2.57E+07

*The concentration for other Archaea was calculated from the Total Archaea concentration analyzed on qPCR. i.e. (copies/mL) Total Archaea – *Methanosarcina* – *Methanomicrobiales.* Negative values of other Archaea indicate no other Archaea are present in the culture.
**The concentration for other Bacteria was calculated from the total Bacteria concentration analyzed on qPCR, i.e. (copies/mL) Total Bacteria – *Dehalococcoides* - *Acetobacterium*

Appendix E: Discussion of Microcosm Study at low pH

An experiment was conducted where the low pH cultures developed in this thesis and cultures from SiREM were tested in a microcosm study with soil and groundwater obtained from a low pH contaminated site. SiREM also began development of a low pH culture amended with TCE as the electron acceptor and methanol as the electron donor in concurrence with the experiments done in this thesis. The microcosm study began on November 25th, 2011 approximately 7 months after the start of the development of the low pH culture in this thesis. A description of each treatment is described in Table E-1. The materials and methods for this experiment were as follows:

- Microcosms were constructed from the soil and groundwater obtained from the confidential Florida site.
- An Agilent GC System 7890A with G1888 Network headspace sampler was used to measure VOCs. Liquid samples of 1 mL were obtained from each microcosm every two weeks. Each sample was then injected into a vial with 5 mL of 0.012 M acidified water to stop any further reactions from occurring. The vials were immediately covered and crimped to avoid the escape of VOCs.
- The target concentration in the aqueous phase for TCE was 20 mg/L. A mixture of methanol, ethanol and lactate (MEL) at an electron donor to electron acceptor ratio of 3:3:3:1 was amended to each microcosm according to Table E-1.
- AquaBupH was used to adjust pH of the site representative control (approximately 4 mL was added to each triplicate microcosm to bring the pH to 7±0.1. 5 M sodium hydroxide was used to adjust the pH of the positive control to pH 7.
- Quantitative PCR was conducted on *Dehalococcoides* for the SiREM and UofT cultures to determine volume required for bioaugmentation. The copies/L for each culture was approximately 10^8 copies/L, therefore 0.5 mL of each culture was injected into the corresponding microcosms according to Table E-1.

Dechlorination up to only cDCE occurred in all of the cultures. The positive control achieved complete dechlorination at approximately 60 days after the start of the experiment. The site representative control was able to achieve dechlorination up to VC with some completion to ethene. None of the microcosms bioaugmented with pH 7 and pH 6 KB-1 cultures completed dechlorination from TCE to ethene. Resazurin was added to one of each of the triplicates of each treatment in order to indicate anaerobic conditions have been achieved in the microcosms. This resulted in each of the resazurin-amended microcosms to lag in dechlorination from TCE to

cDCE. The pH 6 cultures developed in this thesis that were used to inoculate the microcosms of this study were enriched up until day 277, before any signs of acclimation occurred. Now that there has been evidence of acclimation in the pH 6 cultures it may be possible to conduct another microcosm study to produce better results.

Table E-1: Treatment table summarizing conditions for each microcosm used in the study

Treatment/Control	Assigned Bottle Number	Sediment	Groundwater	Headspace	Electron donor	pH	SiREM KB-1	SiREM Low pH Culture	U of T Acclimated Culture
ANSC	1 to 3	60	200	20	None	Site 6			
ANAC	4 to 6	60	200	20	None	Site 6			
Positive Control w/ SiREM KB-1	7 to 9	60	200	20	MEL	7.0	1 mL		
Site Representitive Control w/ SiREM KB-1	10 to 12	60	200	20	EOS/AquaBupH	7.0	0.5 mL	0.5 mL	
SiREM Low pH culture	13 to 15	60	200	20	MEL	Site 6		1 mL	
SiREM normal KB-1	16 to 18	60	200	20	MEL	Site 6	1 mL		
U of T Acclimated MeOH Culture 6.0	19 to 21	60	200	20	MEL	Site 6			1 mL
U of T Normal Culture 7.0	22 to 24	60	200	20	MEL	Site 6			1 mL

ANSC – geologic materials autoclaved and groundwater poisoned with mercuric chloride and sodium azide
ANAC – intrinsic control, microcosms amended with electron acceptor only
MEL – methanol, ethanol and lactate

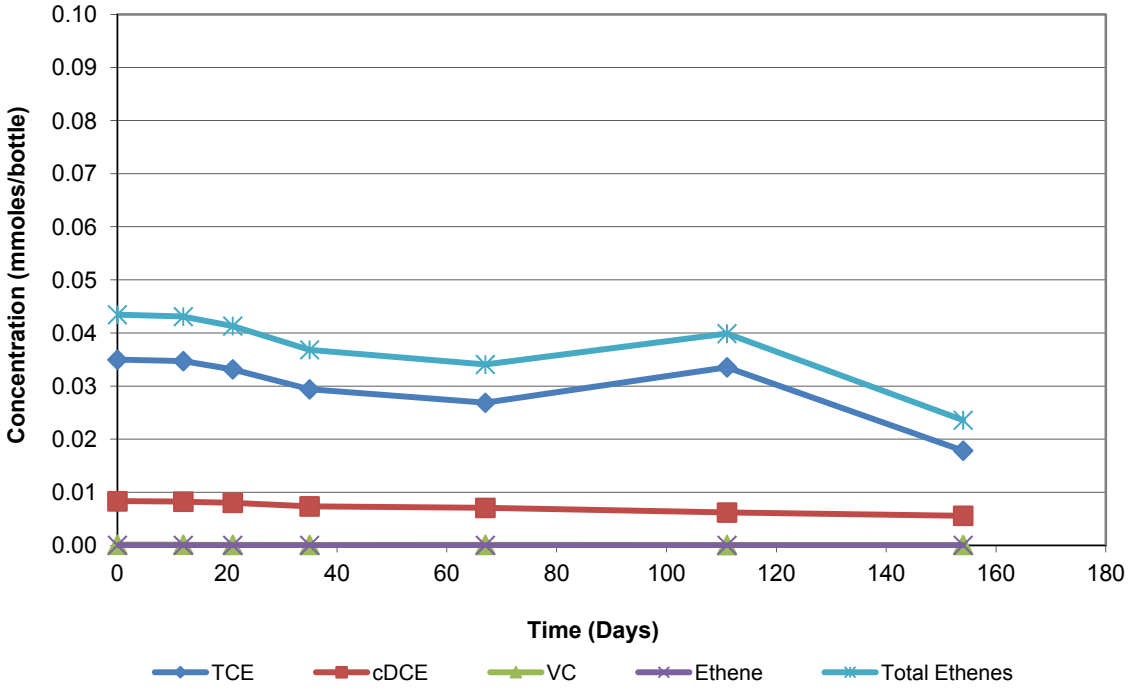

Figure E-1: Chlorinated Ethene Concentration Trends in Anaerobic Sterile Control Microcosms at pH 6 (ANAC)

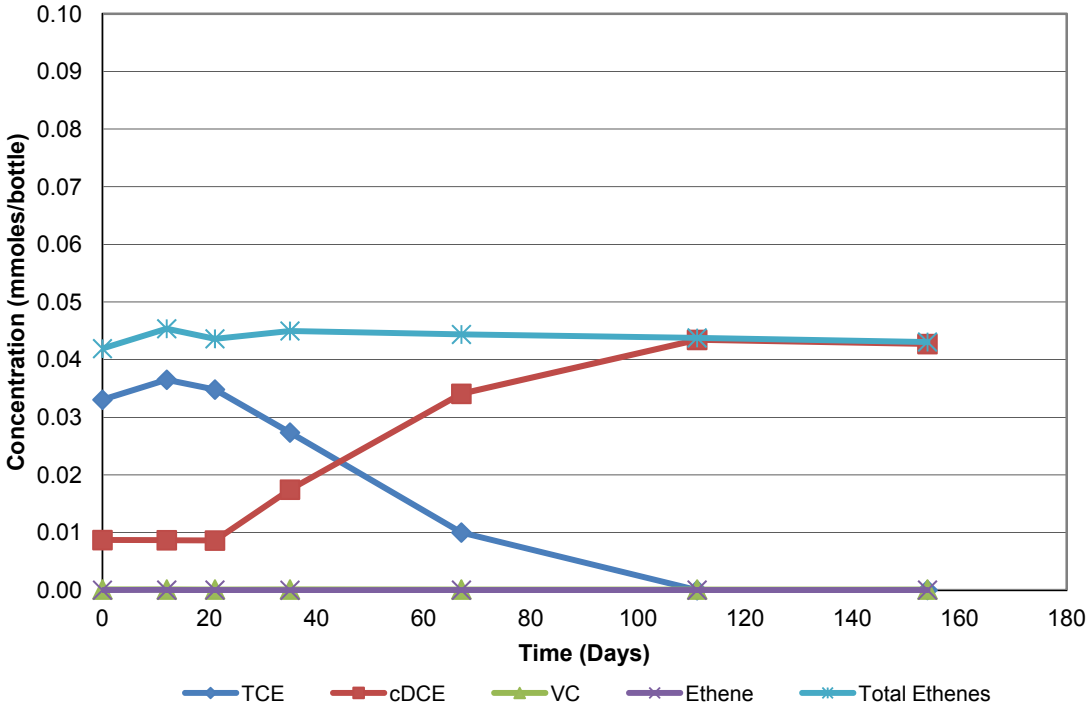

Figure E-2: Chlorinated Ethene Concentration Trends in Anaerobic Active Control Microcosms at pH 6 (ANAC)

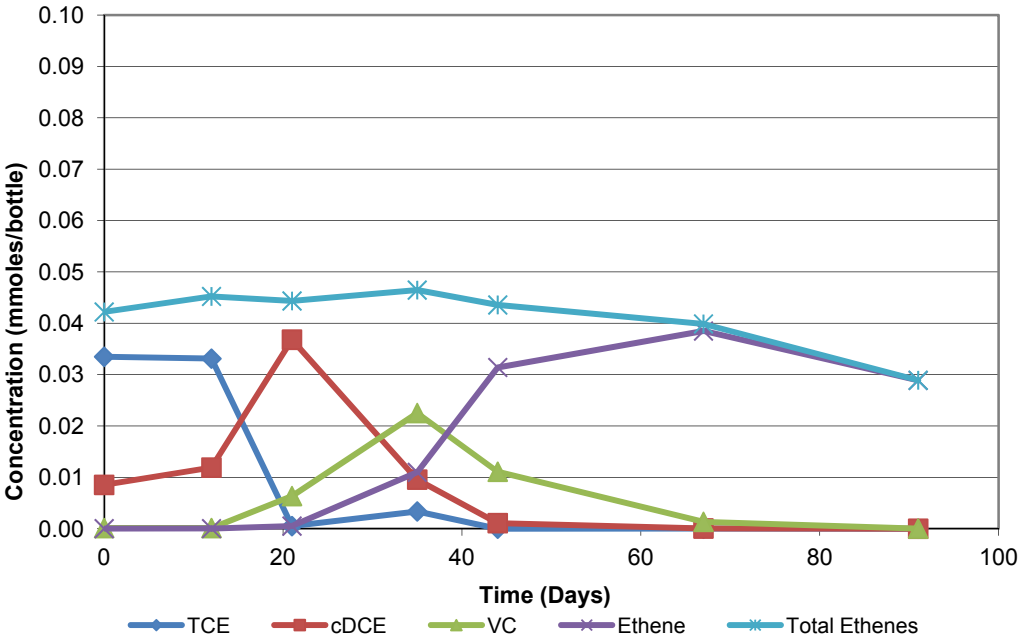

Figure E-3: Chlorinated Ethene Concentration Trends in Positive Control at pH 7

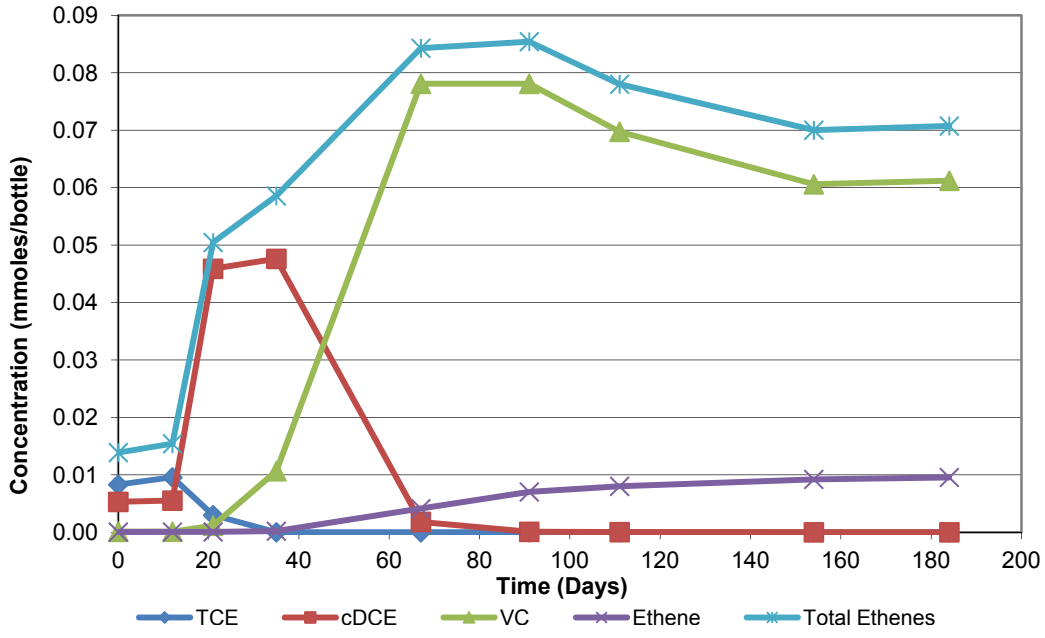

Figure E-4: Chlorinated Ethene Concentration Trends in Site Representative Control at pH 6

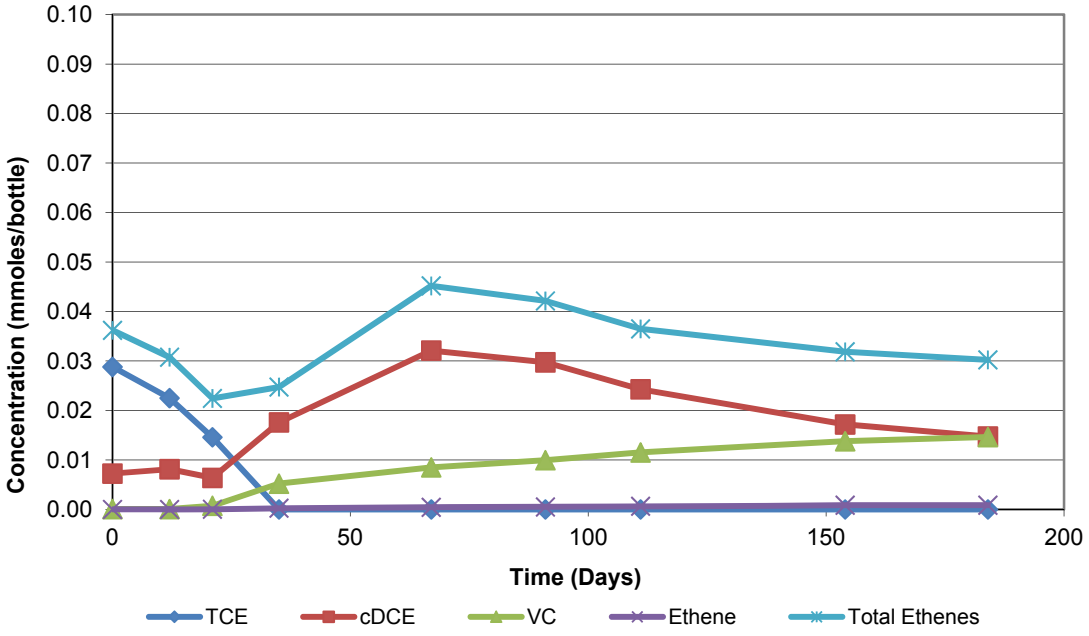

Figure E-5: Chlorinated Ethene and Concentration Trends in SiREM KB-1 pH 7 Microcosms

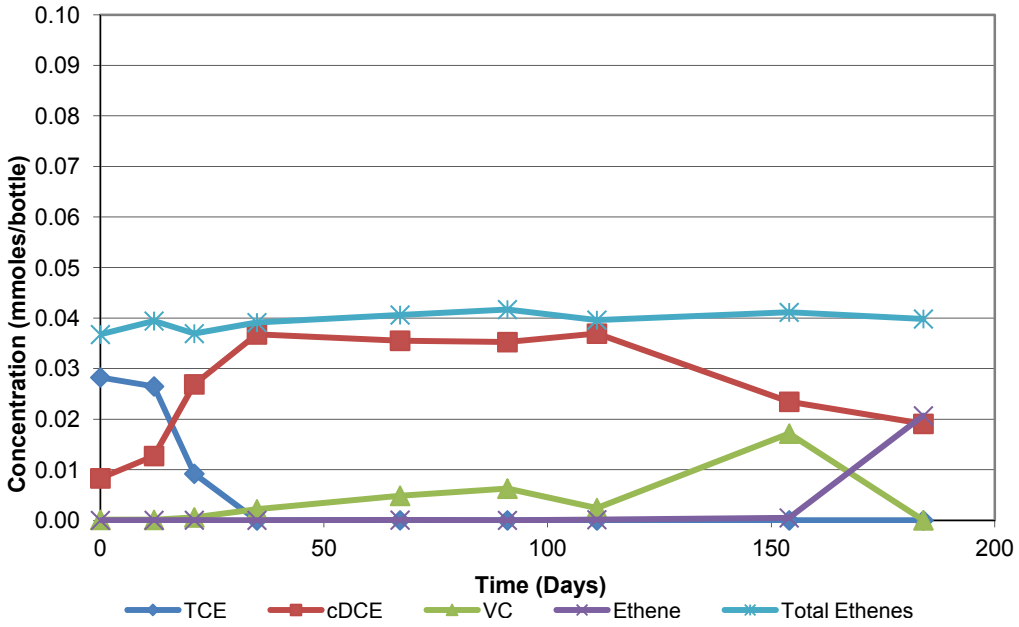

Figure E-6: Chlorinated Ethene Concentration Trends in SiREM KB-1 low pH culture

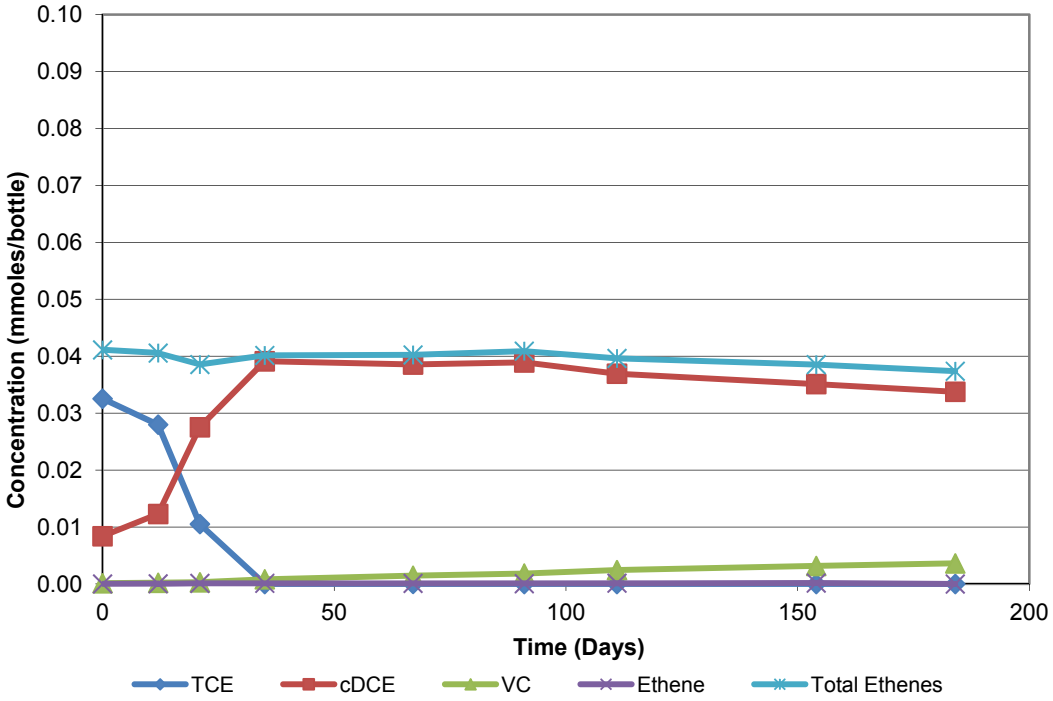

Figure E-7: Chlorinated Ethene Concentration Trends in UofT KB-1 pH 7 Microcosms

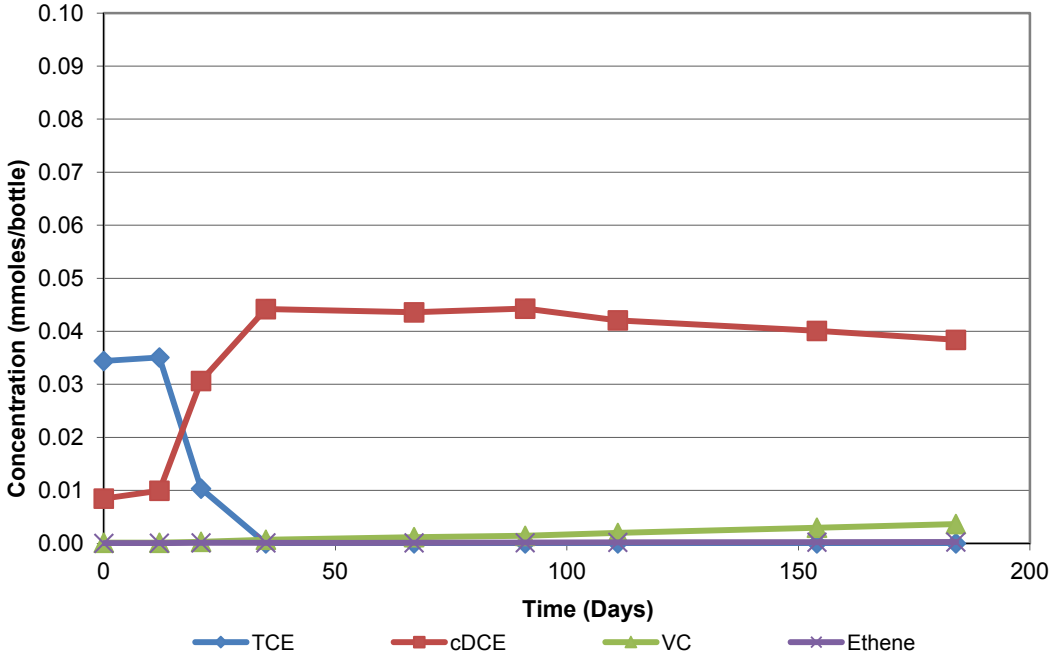

Figure E-8: Chlorinated Ethene Concentration Trends in UofT KB-1 pH 6 Microcosms

Appendix F: Standard deviation and t-test

This section will detail the sample calculations for the standard deviation calculated for the rate of ethene production at pH 7, 6 and 5.5 and the t-test for the electron balance calculations used in Chapters 4, 5 and 6.

Calculation for Standard Deviation

The average rate of ethene production calculated for each degradation cycle was explained in Appendix A. An average rate was calculated for each pH condition. The standard deviation of the sample was calculated according to the following equation:

$$\sigma = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(x_i - \bar{x})^2}$$

Where

σ = standard deviation of sample

N = number of samples

x_i = observed values of sample items

$\bar{x}$ = mean value of sample items

Calculation for t-test

In order to determine significant differences between samples, a t-test was performed on the electron balance.

The following assumptions were made for the t-test (Welch's t-test):

i) One-tailed distribution

ii) Alpha = 0.05

iii) Unpaired, unequal standard deviation

The t statistic was calculated as follows:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{s_{\overline{x_1} - \overline{x_2}}}$$

Where

$\bar{X}_1$ = mean of sample 1

$\bar{X}_2$ = mean of sample 2

$$s_{\overline{x_1} - \overline{x_2}} = \sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}$$

Where

s = standard deviation of sample 1 or 2

n = total number of samples in each group

Appendix G: Dechlorination Data

Table G-1: Nomenclature for each culture from the first transfer according to substrate and pH

Substrate and pH	Culture Nomenclature
pH 7	
Hydrogen	Green-1
	Green-2
Methanol	Green-3
	Green-4
pH 6	
Hydrogen	Yellow-1
	Yellow-2
Methanol	Yellow-3
	Yellow-4

Table G-2: Nomenclature for each culture from the second transfer according to substrate and pH

Culture Nomenclature	Electron Donor	pH
Green 1-1	Hydrogen	7
Green 1-2		5.5
Green 2-1	Methanol	7
Green 2-2		5.5
Yellow 1-1	Hydrogen	6
Yellow 1-2		5.5
Yellow 2-1	Methanol	6
Yellow 2-2		5.5

Table G-3: Rate of ethene production for pH 6 cultures

	Average Rate of Ethene Production (µmol/day)	
Time (days)	H_2-amended	Methanol-amended
0		
16	1.24	1.65
22	1.14	1.95
31	0.77	0.64
45	0.73	
59		0.58
71	0.63	
108		0.63
145	1.24	1.40
163	1.20	0.89
184	1.16	0.89
233	1.06	1.05
278	0.99	1.39
382	0.95	1.47
485	1.29	1.41
555	1.31	1.67

Table G-4: Rate of ethene production for pH 7 vs. pH 6

	Average Rate of Ethene Production (µmol/day)			
Time (days)	pH 7 H_2	pH 7 MeOH	pH 6 H_2	pH 6 MeOH
0				
16	1.87	2.11	1.24	1.65
22	1.86	1.71	1.14	1.95
31	1.52		0.77	0.64
45	1.10	1.33	0.74	
59				0.58
67		1.58		
71			0.63	
76				
79	2.29	1.45		
87				
91	2.32	2.05		
107	1.31	1.34		
108				0.63
113	2.04			
119	1.27			
122			0.68	

Table G-5: Rate of ethene production at pH 5.5 for "shocked" vs. "stepwise" cultures

	Average Rate of Ethene Production (μmol/day)			
Time (days)	**Shocked pH 5.5 H_2**	**Shocked pH 5.5 MeOH**	**Stepwise pH 5.5 H_2**	**Stepwise pH 5.5 MeOH**
201				
213	0.284	0.332	0.318	0.485
233		0.116		
244			0.387	0.363
249	0.202	0.056		
278			0.410	
298	0.469			
334			0.245	0.182
346	0.023			
374		0.000		
409			0.207	0.271
430	0.057	0.000		

Table G-6: Tabulated data for measured pH versus rate of ethene production

Rate of Ethene Production (µmol/day)								
pH	pH 7 H2/Acetate	pH 7 MeOH	Shocked pH 5.5 H2	Shocked pH 5.5 MeOH	pH 6 H2	pH 6 MeOH	Stepwise pH 5.5 H2	Stepwise pH 5.5 MeOH
5.30				0.000				
5.35								0.057
5.36								0.029
5.41				0.134				
5.45			0.181					
5.48				0.213				
5.55								0.422
5.56			0.175					
5.59							0.186	
5.60							0.063	0.092
5.62							0.024	
5.63								0.350
5.75						1.473		
5.76						1.244		
5.84			0.312			1.394		
5.87							0.429	
6.00								
6.02						1.637		
6.03					1.042			
6.05			0.646		0.834			
6.09							0.448	
6.12					1.072			
6.14				0.475				
6.15					1.001			
6.64		0.713						
6.67	0.554							
6.74	0.727	1.323						
6.76		1.361						
6.79	0.779							
6.84	0.833							
6.85		1.409						
6.89	1.516							
6.91	1.244							
6.95	0.972							
6.96	0.980							

6.99	1.759
7.00	2.595

Table G-7: Average acetate concentrations available only after second transfer

Time (days)	pH 7 MeOH	Shocked pH 5.5 MeOH	pH 6 MeOH	Stepwise pH 5.5 MeOH
249	1.600	1.001	0.198	0.782
249				
253				
260				
270	0.866	0.929		
277				
284			0.000	1.364
291	1.024	0.999		
297				
304			0.081	1.458
305				
318				
333				
339				
345				
354	1.352	2.383		
355				
361	1.135	2.385	0.043	2.492
374	1.205	2.312		
375	1.154	3.204	0.047	2.486
382			0.064	2.337
395	0.932	3.173	0.033	3.155
408				
135			0.043	3.048
417	1.147	3.735		
429				
432			0.043	3.489