Reductive Dechlorination Sustained by Microbial Chain Elongation

by

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ABSTRACT

Trichloroethene (TCE) is a ubiquitous soil and groundwater contaminant. The most common bioremediation approach for TCE relies on the process of reductive dechlorination by *Dehalococcoides mccartyi*. *D. mccartyi* use TCE, dichloroethene, and vinyl chloride as electron acceptors and hydrogen as an electron donor. At contaminated sites, reductive dechlorination is typically promoted by adding a fermentable substrate, which is broken down to short chain fatty acids, simple alcohols, and hydrogen. This study explored microbial chain elongation (MCE), instead of fermentation, to promote TCE reductive dechlorination. In MCE, microbes use simple substrates (e.g., acetate, ethanol) to build medium chain fatty acids and also produce hydrogen during this process. Soil microcosm using TCE and acetate and ethanol as MCE substrates were established under anaerobic conditions. In soil microcosms with synthetic groundwater and natural groundwater, ethene was the main product from TCE reductive dechlorination and butyrate and hydrogen were the main products from MCE. Transfer microcosms using TCE and either acetate and ethanol, ethanol, or acetate were also established. The transfers with TCE and ethanol showed the faster rates of reductive dechlorination and produced more elongated products (i.e., hexanoate). The microbial groups enriched in the soil microcosms likely responsible for chain elongation were most similar to *Clostridium* genus. These investigations showed the potential for synergistic microbial chain elongation and reductive dechlorination of chlorinated ethenes.

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CHAPTER 1

INTRODUCTION

The historical extensive use of chlorinated solvents as industrial solvents for degreasing or as intermediates in the manufacturing of chemicals has resulted in significant groundwater contamination.[1] Trichloroethene (TCE), a chlorinated solvent released from industries, military, agriculture, and households, is among the most ubiquitous groundwater contaminants. TCE is highly toxic and a known human carcinogen; it ranks at number 16 on the Substance Priority List for the Agency of Toxic Substances and Disease Registry (ATSDR). A known eco-friendly method for bioremediation of TCE involves reductively dechlorinating bacteria that sequentially reduces TCE to ethene, a non-toxic gas, via daughter products, *cis*-dichloroethene (DCE) and vinyl chloride (VC) (Figure 1).[2] Many phylogenetically diverse microbes have the ability to reduce TCE to DCE, but to date, only the anaerobes *Dehalococcoides mccartyi* have been shown to completely reduce TCE to ethene.[3,4,5]

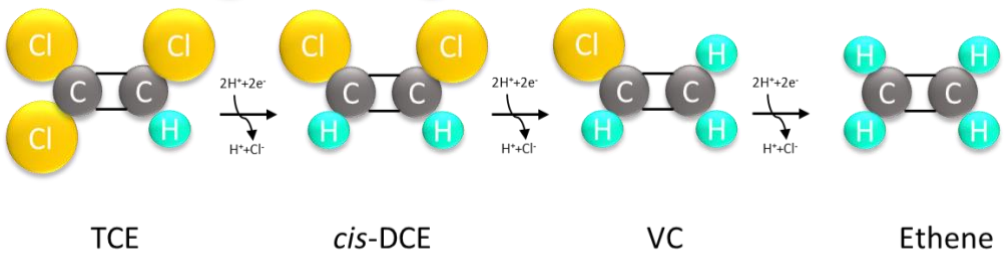

Figure 1. Schematic of TCE reduction pathway by *D. mccartyi*

D. mccartyi use TCE, DCE, and VC as electron acceptors, hydrogen (H_2) as the electron donor, and acetate as the carbon source.[5] Conventional *in situ* bioremediation of TCE or growth of *D. mccartyi* bioaugmentation cultures rely on fermentation of organic

substrates such as methanol, ethanol, lactate, and emulsified vegetable oil for the production of H_2.[6,7,8] In fermentation, fermenting microorganisms break down more complex, organic substrates to simpler, smaller organic substrates to produce H_2.[5] For example, lactate could be fermented to acetate, the carbon source and H_2, the electron donor for *D. mccartyi* (Table 1).[6] When H_2 is limiting for reductive dechlorination, fermentable substrates are re-added in lab experiments and or during *in situ* treatment of groundwater.[1,9,10]

Table 1: Examples of fermentation reactions using organic substrates to yield acetate and hydrogen and their corresponding Gibbs free energies.

Fermentation reaction	$\Delta G_f^{0'}$ (kJ/reaction)
$2\ CH_3OH \rightarrow C_2H_3O_2^- + 2H_2 + H^+$	-81.09
$C_2H_6O + H_2O \rightarrow C_2H_3O_2^- + H^+ + 2H_2$	-12.94
$C_3H_5O_3^- + 2H_2O \rightarrow C_2H_3O_2^- + HCO_3^- + 2H_2 + H^+$	-26.55

The standard free enthalpies of formation ($\Delta G_f^{o'}$) are reported at 1 M, pH 7, and 25°C.[11]

A lesser studied process that produces H_2 is microbial chain elongation (MCE). In MCE, microorganisms, use simple acids and alcohols (e.g., acetate, ethanol) to build more complex, medium chain fatty acids (e.g., butyrate, hexanoate) and produce H_2 as a by-product.[12,13,14,15,16] For instance, 3 moles of acetate and 5 moles of ethanol will produce 4 moles of butyrate and 2 moles of H_2 (Table 2).[15,17]

Table 2: Examples of chain elongating reaction using fatty acids and Gibbs free energies.

MCE Reaction	$\Delta G_f^{0'}$ (kJ/reaction)
$3C_2H_3O_2^- + 5C_2H_6O \rightarrow 4C_4H_7O_2^- + 3H_2O + H^+ + 2H_2$	-177.85
$2\ C_2H_6O \rightarrow C_4H_7O_2^- + H^+ + 2H_2$	-54.17
$C_2H_6O + C_4H_7O_2^- \rightarrow C_6H_{11}O_2^- + H_2O$	-13.58

The standard free enthalpies of formation ($\Delta G_f^{o'}$) are reported at 1 M, pH 7, and 25°C.[11]

Recent work has shown that soils readily contain microorganisms with chain-elongating capabilities.[13] In this work with soils, a high concentration of H_2 accumulated when 90

mM ethanol and 88 mM acetate were used for chain elongation.[13] H_2 production from chain elongation has also been mentioned in studies with municipal solid waste or acetate and ethanol as MCE substrates, but H_2 concentrations were not reported.[14,15] Furthermore, low methanogenesis activity (an electron sink in TCE bioremediation) compared to typical fermentation has been observed in chain elongation studies.[15,16,17] These lines of evidence suggest that chain elongation could be used for groundwater bioremediation. This study specifically aimed to explore the synergy between microbial chain elongation (MCE), and TCE reductive dechlorination in soil and transfer microcosms when supplied with MCE substrates for bioremediation of TCE.

CHAPTER 2

MATERIALS AND METHODS

2.1 Soil, groundwater, and microbial inocula

Soil and groundwater were obtained from a Superfund site in Arizona. Soil cores with a pH of 7.6 ± 0.4 from 0-170 ft below ground surface were homogenized in an anaerobic glovebox and used in microcosm experiments.[18] In a previous study, this soil was found to have a low concentration of chain elongating microorganisms.[13] The soil had the following characteristics: sandy clayey soil, conductivity of 180 ± 10 μS cm^{-1}, total dissolved solids of 130 ± 20 ppm, salinity of 3.5 ± 0.9 ppm, chemical oxygen demand of 3.5 ± 0.9 mg g^{-1} and total organic carbon of 5700 ± 300 mg kg^{-1}.[13]

Two dechlorinating microbial enrichment cultures, ZARA-10 and SDC-9, were used to setup soil microcosms in this study ZARA-10 is grown at Arizona State University in a continuously stirred tank reactor (CSTR) using TCE, lactate, and methanol. ZARA-10 contains *D. mccartyi* strains most similar to 195, GT, and VS.[19] SDC-9 is a commercially available culture grown in batch reactors using PCE, lactate, and yeast extract. SDC-9 contains four *D. mccartyi* strains but their identity has not been published (CB&I, Woodlands, TX 30).[20] The MCE microbial inocula were enrichment cultures grown on acetate and ethanol.[13,21] The MCE inocula primarily consisted of *Clostridiales* within the phylum *Firmicutes*.[13]

2.2 Experimental setup of soil microcosms and transfers

Soil microcosms were established in an anaerobic chamber in 120 mL glass serum bottles closed with butyl rubber stoppers and sealed with aluminum crimps. The

microcosms contained 10 g of homogenized soil, 80 mL of reduced anaerobic mineral medium or natural groundwater, 2.5 mL of ZARA-10 culture, 2.5 mL of SDC- 9 culture, and 5 mL of chain-elongating enrichment culture. Reduced anaerobic mineral medium was prepared containing the following reagents per liter: 1 g NaCl, 0.06 g $MgCl_2 \times 6H_2O$, 0.2 g KH_2PO_4, 0.3 g NH_4Cl, 0.3 g KCl, 0.005 g $CaCl_2 \times 2H_2O$, 1 mL of Trace A, and 1 mL of Trace B solutions. Trace element stock solution A contained per liter: HCl (25% solution, w/w), 10 mL; $FeCl_2 \times 4H_2O$, 1.5 g; $CoCl_2 \times 6H_2O$, 0.19 g; $MnCl_2 \times 4H_2O$, 0.1 g; $ZnCl_2$, 70 mg; H_3BO_3, 6 mg; $Na_2MoO_4 \times 2H_2O$, 36 mg; $NiCl_2 \times 6H_2O$, 24 mg; $CuCl_2 \times 2H_2O$, 2 mg. Trace element stock solution B contained per liter: $Na_2SeO_3 \times 5H_2O$, 6 mg; $Na_2WO_4 \times 2H_2O$, 8 mg; NaOH, 0.5 g. The medium was buffered with 10 mM phosphate (initial pH = 7.5), purged with ultra-high purity (UHP) N_2 gas for 20 min, and reduced with 0.2 mM Na_2S and 0.4 mM L-cysteine.[22] The soil microcosm conditions are described in Table 1. Triplicate microcosms were established for each condition.

Table 3. Experimental conditions for reductive dechlorination of TCE and chain elongation in soil microcosms.

Condition	Acetate (mM)	Ethanol (mM)	TCE (mM)	Medium	Cultures
Natural Groundwater	50 ± 0.5	50 ± 9	0.07, 0.6, 1.63	Aerobic natural groundwater	Yes
Synthetic Groundwater	57 ± 8	53 ± 10	0.06, 0.57, 1.22	Reduced anaerobic medium	Yes
Synthetic Groundwater w/out TCE	53 ± 1	54 ± 4	0	Reduced anaerobic medium	Yes
Synthetic Groundwater w/out Bioaugmentation	54 ± 0.6	52 ± 8	0.08	Reduced anaerobic medium	No

Soil microcosm transfers were established in 160 mL glass serum bottles. The experimental conditions were 50 mM Acetate + 50 mM Ethanol, 100 mM Ethanol, 100 mM Acetate and TCE at 0.55 ± 0.04 mM, 0.51 ± 0.02 mM, 0.51 ± 0.01 mM was added to each condition, respectively. 2.5% v/v supernatant from "Synthetic Groundwater" soil microcosms was transferred to fresh 95 mL reduced anaerobic mineral medium. All soil microcosms and transfers were incubated in the dark at 31℃ and shaken on a platform shaker at 150 RPM.

2.3 Analytical methods for the composition of gas and liquid

The concentrations of chlorinated ethenes, ethene, and methane were measured by injecting a 200 Ul gas sample from headspace into a gas chromatography (Shimadzu GC-2010; Columbia, MD) equipped with a flame ionization detector (FID), and an Rt-QS-BOND capillary column 30m X 0.32 mm ID, 1.8 µm df (Restek; Bellefonte, PA). H_2 was the carrier gas. The oven temperature was maintained at 110℃ for 1 min, followed by a temperature increase of 50℃ min^{-1} to 200℃. Then, the temperature ramp was further raised to 240℃ with a 15℃ min^{-1} gradient and held for 1.5 mins. The temperature of the FID and injector were 240℃. Calibrations of chlorinated ethenes, ethene, and methane were created using 160 mL bottles with 100 mL of liquid volume in a range of 0.05- 2.45 mmol L^{-1} as described in previous studies.[18] The detection limit for chlorinated ethenes, ethene, and methane was ≤ 0.018 µmol L^{-1}. The concentrations of these compounds in the liquid were calculated using Henry's constants (K_H) for each compound:

Equation 1: $$[Compound]_{liq} = [Compound]_{gas}/K_H$$

Henry's dimensionless constants (mM_{gas}/mM_{liq}, T=30℃) were experimentally obtained for each compound.[22] The concentration reported were the nominal concentrations in the system (μmol L^{-1}).

H_2 was monitored by injecting a 200 μl gas sample from headspace into a GC (Shimadzu GC-2010; Columbia, MD) equipped with a thermal conductivity detector (TCD), a fused silica capillary column 30 m X 0.53 mm 30 μm df (Car boxen™ 1010 PLOT, Supelco). Argon was the carrier gas. The temperature of the injector and TCD were 150℃ and 180℃, respectively. The temperature of the oven was maintained at 80℃ for 3 min, followed by a temperature increase of 50℃ $min^{-1}$to 160℃, and then held for 1.5 min. H_2 detection limit for the instrument was 3.6 μM gas, as described in previous studies.[13] Gas pressure in bottles was measured using a frictionless syringe during every sampling event. Vacuum was detected in some conditions and the headspace pressure was then adjusted to 1 atm by adding UHP N_2.

Samples for chemical liquid analyses were prepared from 1.5 mL of liquid slurry, which was centrifuged at a speed of 10,000 x g for 4 min on an Eppendorf 5415 R centrifuge and filtered by a 0.2 μm syringe filter. Volatile fatty acids, alcohols and organic acids were analyzed using a High-Performance Liquid Chromatograph (HPLC, Schimadzu LC-20AT). The HPLC method run was set for 60 min with the detector at 40℃, and column at 65℃. The flow was 0.6 mL/min for first 29 minutes and then it was increased to 0.8 mL/min for the remainder of the method run. The detection limit for VFAs and alcohols was ≤0.1 mM and 0.5 mM, respectively.[13,23,24] The calibration for VFA's and alcohols was created using a 10 mM VFA standard, pure ethanol, and

butanol. The following concentration of VFA's in mM were used to create a standard curve: 1, 2, 6, 8 and 10. The following concentration of ethanol and butanol in mM were used to create a standard curve: 2.5, 5, 10 and 20. The pH was measured using a Thermo Scientific™ Orion™ 3-Star Benchtop pH Meter.

2.4 Microbial ecology analyses

Slurry samples (1 mL) were collected for DNA analyses and 2.5 mL of RNAprotect Cell Reagent (Qiagen, USA) were added to the slurry. 0.5 mL of the slurry and RNAprotect mixture were pelleted using an Eppendorf micro centrifuge 5415R (Hauppauge, NY) for 15 minutes at a speed of 13200 rpm and frozen at -20℃. DNA was extracted using the Qiagen Dneasy Blood & Tissue Kit (MO BIO Laboratories, Inc., (Carlsbad, CA, USA) by following the protocol recommended by the manufacturer for Gram-positive bacteria. DNA purity and concentrations for each sample were determined by measuring absorbance at wavelengths of 260 and 280 nm with a NanoDrop spectrophometer (NanoDrop Technology, Rockland, DE).[24]

To determine changes in the microbial community structures in the soil microcosms, microbial community amplicon sequencing was performed using the Illumina Miseq at the Center for Fundamental and Applied Microbiomics in the ASU KED Genomics Core, Arizona State University, Tempe. The universal bacterial primers were 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′)[25,] 18 for the V4 hyper-variable region of the 16S rRNA gene which captures Bacteria and Archaea.[26] Forward and reverse sequences (2 ×150 mode) were first paired (overlap ≥ 45 base-pairs) using PANDASeq.[27] Then, ASU

KED Genomics Core paired reads (average length 250 base-pairs) were processed using the bioinformatics software, Quantitative Insights into Microbial Ecology QIIME (version 2.0) pipeline.[26]

2.5 Electron balances

Electron balances were performed to understand the distribution of electrons provided from the substrates (acetate and ethanol) to end-products identified (fatty acids, alcohols, and H_2). Concentration of electron donors, acceptors, and end products in mM were converted to millielectron equivalents (e^-meq) using electron equivalents per mol values. The e^-meq used in the calculations were as follows: H_2, 2; DCE, 2; VC, 4; ethene, 6; acetate, 8; methane, 8; sulfate, 8; ethanol, 12; butyrate, 20; butanol, 26; and caproate, 32.[11, 23]

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Reductive dechlorination of TCE via MCE in soil microcosms

Results show that chain elongating microorganisms can produce H_2 for TCE-reductively dechlorinating microorganisms to convert TCE to innocuous ethene. TCE was added at three events as outlined in Table 3. The natural groundwater and synthetic groundwater conditions reduced TCE to ethene at concentrations of 0.67 and 0.63 mM, respectively (Figure 2). Upon the third addition of TCE to synthetic groundwater of 1.22 mM and 1.63 mM to natural groundwater, an additional 1.22 mM of TCE and 1.13 mM of TCE was completely converted to ethene within 115 days, respectively.

An important difference between the natural groundwater (Figure 2A) and synthetic groundwater (Figure 2B) conditions is the higher concentrations of electron acceptors in natural groundwater. Particularly, a higher abundance of microorganisms with a H_2 metabolism creates more competition for *D. mccartyi* to obtain H_2 (e.g., sulfate reduction, methanogenesis).[28,29] Competition for *D. mccartyi* was assessed by measuring methane and sulfate concentrations, both processes that require H_2 as electron acceptors. The synthetic groundwater w/out TCE condition was created to observe changes in MCE substrates and end products in the absence of TCE, as well as changes in the microbial community when reductive dechlorination is not occurring. Dechlorination was not observed in the condition w/out TCE (Figure 2C) or w/out bioaugmentation (Figure 2D).

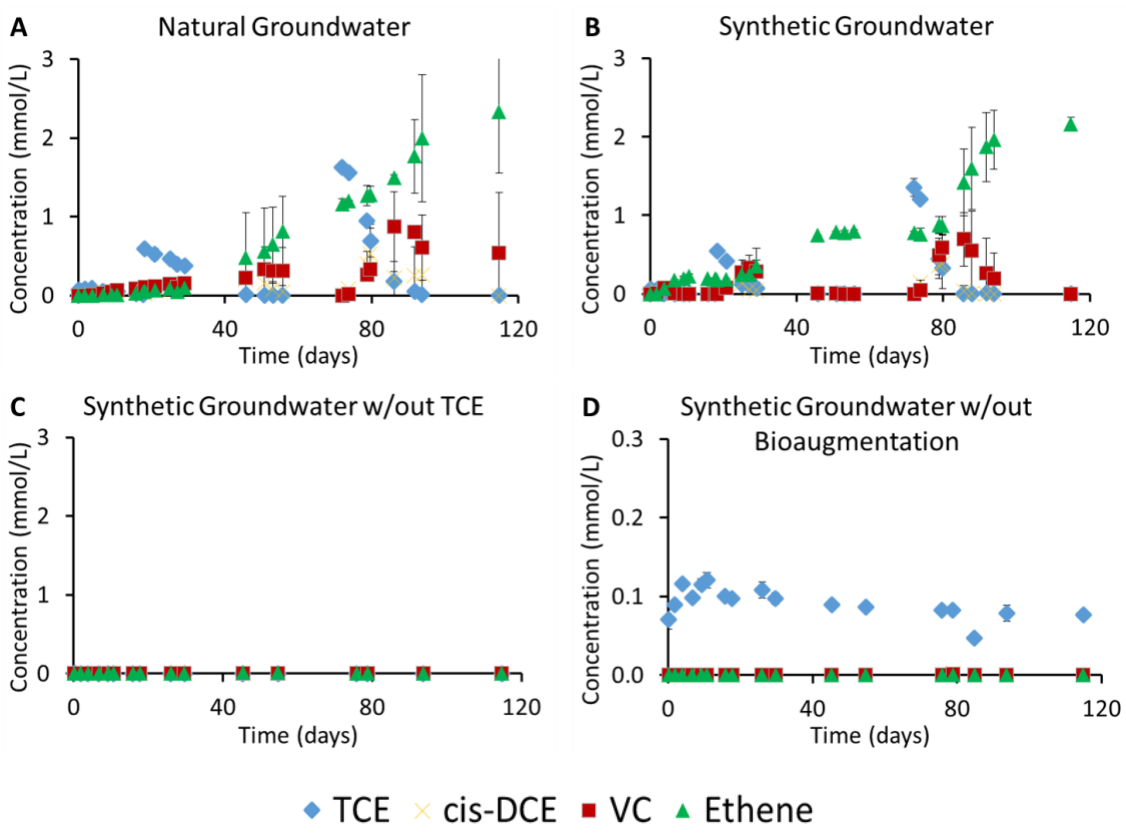

Figure 2: TCE reductive dechlorination in soil microcosms. The data are averages of triplicates and the error bars show standard deviation of the mean. The y-axis in panels A-C spans 0-3 mmol/L, while panel D spans 0-0.3 mmol/L

In reductive dechlorination via fermentation, molecular H_2 is crucial for complete reductive dechlorination. Many microbes have dechlorinating capabilities (e.g. *Geobacter Desulfuromonas,* and *Dehalobacter*), but only *D. mccartyi*, which solely uses molecular H_2 can dechlorinate past DCE.[8,30,31,32] H_2 concentrations were measured in all conditions and plotted in Figure 3. The conditions natural groundwater, synthetic groundwater and w/out TCE accumulated H_2 concentrations of 0.066 ± 0.010 mM, 0.523 ± 0.091 mM and 0.636 ± 0.071 mM, respectively. In general, H_2 accumulated more in the microcosms with a lower concentration of electron acceptors. H_2 went to other competing processes such as methanogenesis and sulfate reduction.

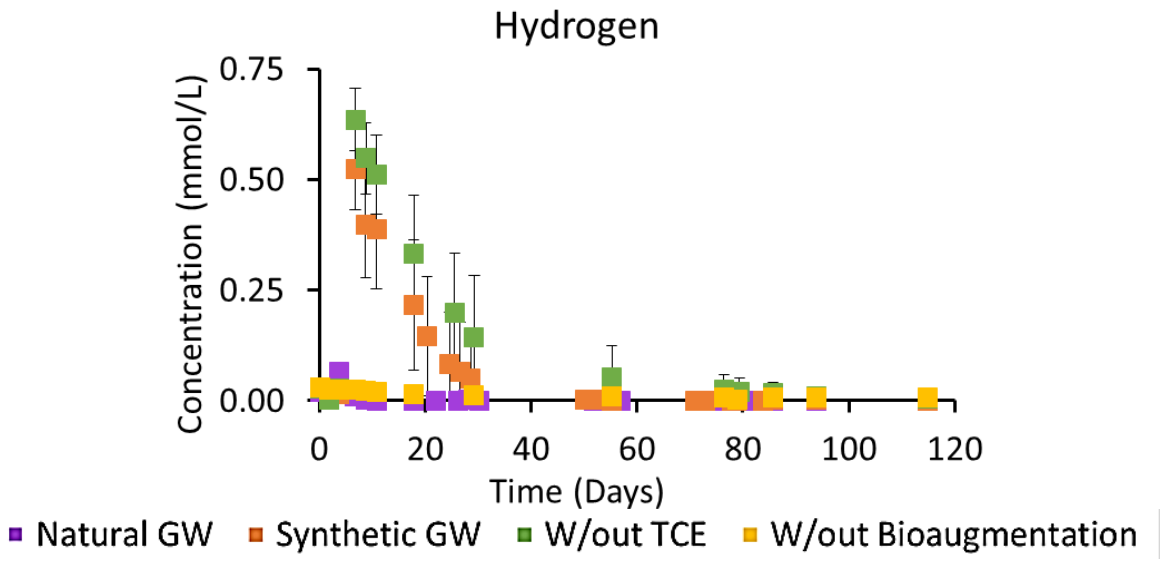

Figure 3: H_2 production and consumption in soil microcosms. The data are averages of triplicates and the error bars show standard deviation of the mean.

In the condition w/out bioaugmentation, the lack of detected H_2 production further confirmed the soil's poor microbial community with regards to chain elongation, as only 0.029 mM ± 0.002 mM of H_2 that was introduced in the anaerobic glovebox was measured at the beginning of the experiment and 0.008 ± 0.009 mM of H_2 were measured at day 115.

The soil microcosms received 50 ± 9 mM of ethanol and 50 ± 0.5 mM of acetate, known MCE substrates. Based on the stoichiometry of ethanol and acetate reaction from Table 2, it is expected that 52 mM of ethanol and 31 mM of acetate will produce 21 mM of H_2 and 42 mM of butyrate. Acetate was added in excess. This reaction is thermodynamically favorable with a Gibbs free energy of -177.85 kJ/mol (Table 2).[15] The microcosm conditions with natural groundwater, synthetic groundwater, and w/out TCE show butyrate and hexanoate production, as well as MCE substrate consumption (Figure 4). All ethanol was completely consumed within 9 days (Figure 4A-C), while butyrate

was produced following the stoichiometry shown in Table 2. The natural groundwater condition showed less butyrate production and less acetate consumption and more acetate production (Figure 4A). It is likely that some ethanol was fermented to acetate (Table 1). Acetate could have also been produced by acetogens using H_2 and CO_2. In the condition w/out bioaugmentation less than 1 mM of butyrate and butanol were produced throughout the experiment and MCE substrate were largely unconsumed (Figure 4D).

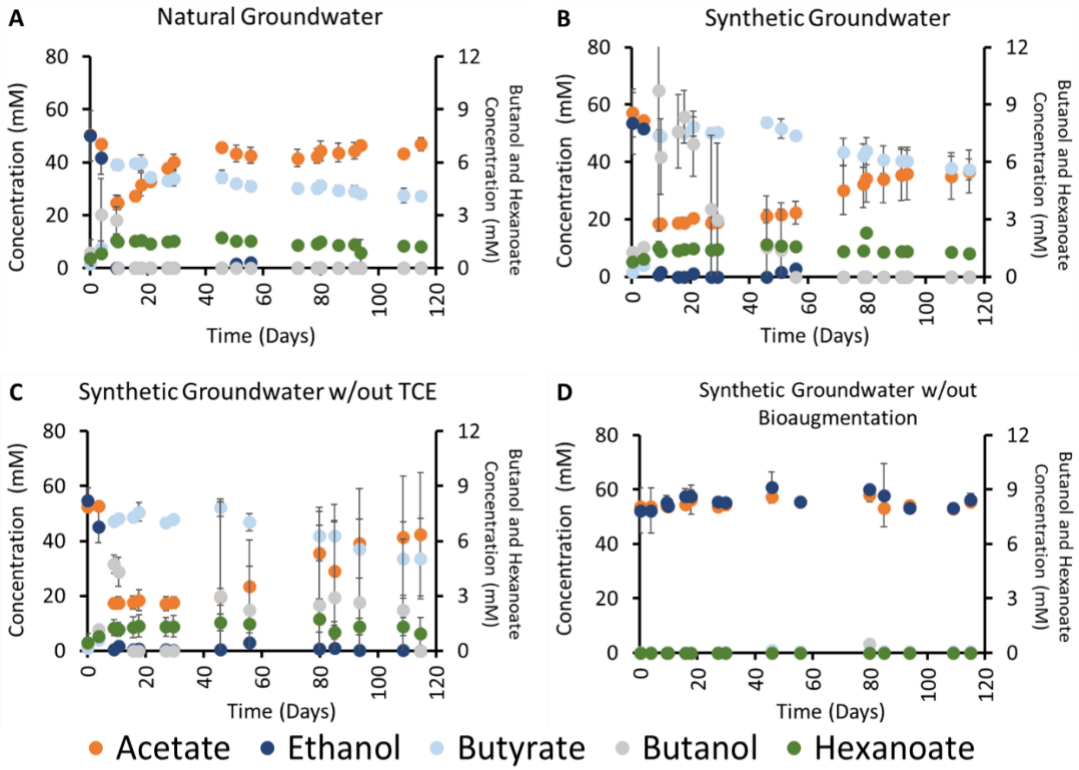

Figure 4: Fatty acid and alcohol concentrations in soil microcosms. The data are averages of triplicates.

The synthetic groundwater condition produced the highest concentrations of butanol compared to all other conditions (Figure 4), but by day 29 butanol was no longer detectable. All conditions also showed a steady increase in acetate after consumption of ethanol. Butanol could have been fermented explaining the decrease in butanol and

increase in acetate. Based on the stoichiometry mentioned before and on average ethanol concentrations added to soil microcosms, it is expected that 42 mM of butyrate would be produced if all ethanol is consumed. At day 9, natural groundwater, synthetic groundwater, and w/out TCE produced the following concentration of butyrate; 41 mM, 48 mM, and 46 mM, respectively. For day 9, these experiments although using a mixed consortia of chain elongators, closely follow the stoichiometry based on a chain elongating pure culture, *Clostridium kluyveri*, that utilizes acetate and ethanol.[15]

Overall, dechlorination and MCE data for soil microcosms showed that these processes occur simultaneously and that MCE promotes complete dechlorination.

3.2 Competing microbial processes in reductive dechlorination of TCE

In reductive dechlorination via fermentation whether at a contaminated site or in lab experiments, there are many competing processes that occur, such as methanogenesis and sulfate reduction. Methanogens and sulfate reducers will compete with dechlorinators for H_2.[28,29] For methanogenesis, hydrogenotrophic methanogens become large H_2 sinks for reductive dechlorination (Equation 2).

Equation 2: $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$.

In soil microcosms, methane concentrations were recorded for all conditions (Figure 5). Results revealed that in the absence of TCE, methane concentration were the highest (11.44 ± 9.86 mmol/L). The condition w/out bioaugmentation only produced 2.99 ± 1.42 μmol/L. In natural groundwater, methane production was 6.22 ± 0.16 μmol/L, while for synthetic groundwater was 2.6 ± 1 mmol/L within 115 days. More importantly, to support that when TCE, DCE and VC are absent in soil microcosms H_2 goes to production of

methane, the same trend was found in synthetic groundwater. When dechlorination was complete in synthetic groundwater at day 46, there is a steady increase of methane until TCE is re-added at day 72 were methane production is once again stalled.

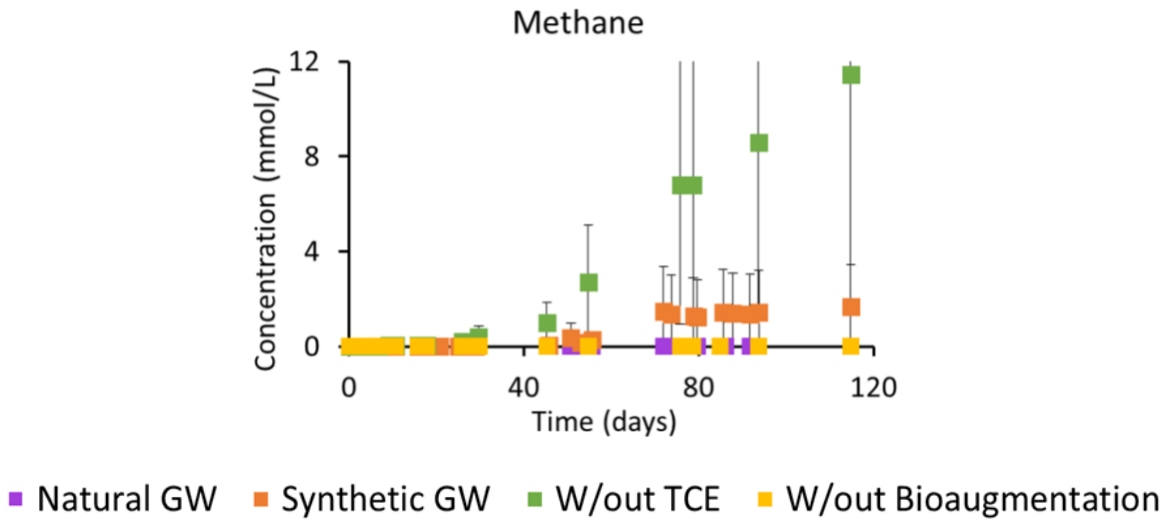

Figure 5: Methane concentrations in soil microcosms. The data are averages of triplicates and the error bars show standard deviation of the mean.

In addition to methanogenesis, sulfate reduction also competes with dechlorination for H_2.[28] Sulfate concentrations were measured all conditions (Figure 6).

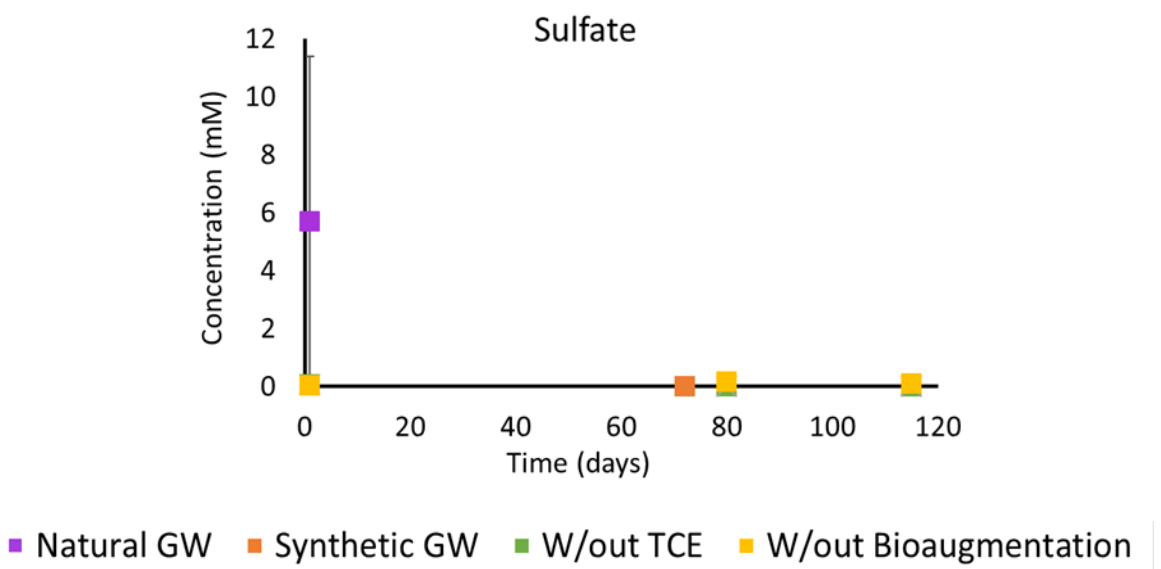

Figure 6: Sulfate concentrations for soil microcosms. The data are averages of triplicates and the error bars show standard deviation of the mean.

Natural groundwater had a concentration of 5.69 mM at day 1 and was undetected by day 115. Synthetic groundwater, w/out TCE and w/out bioaugmentation all contained less than 0.08 mM of sulfate at day 1. By day 115, sulfate was undetected in synthetic groundwater and w/out TCE, and was 0.1 mM in the condition w/out bioaugmentation. An electron balance at day 115 was created for soil microcosms (Figure 7).

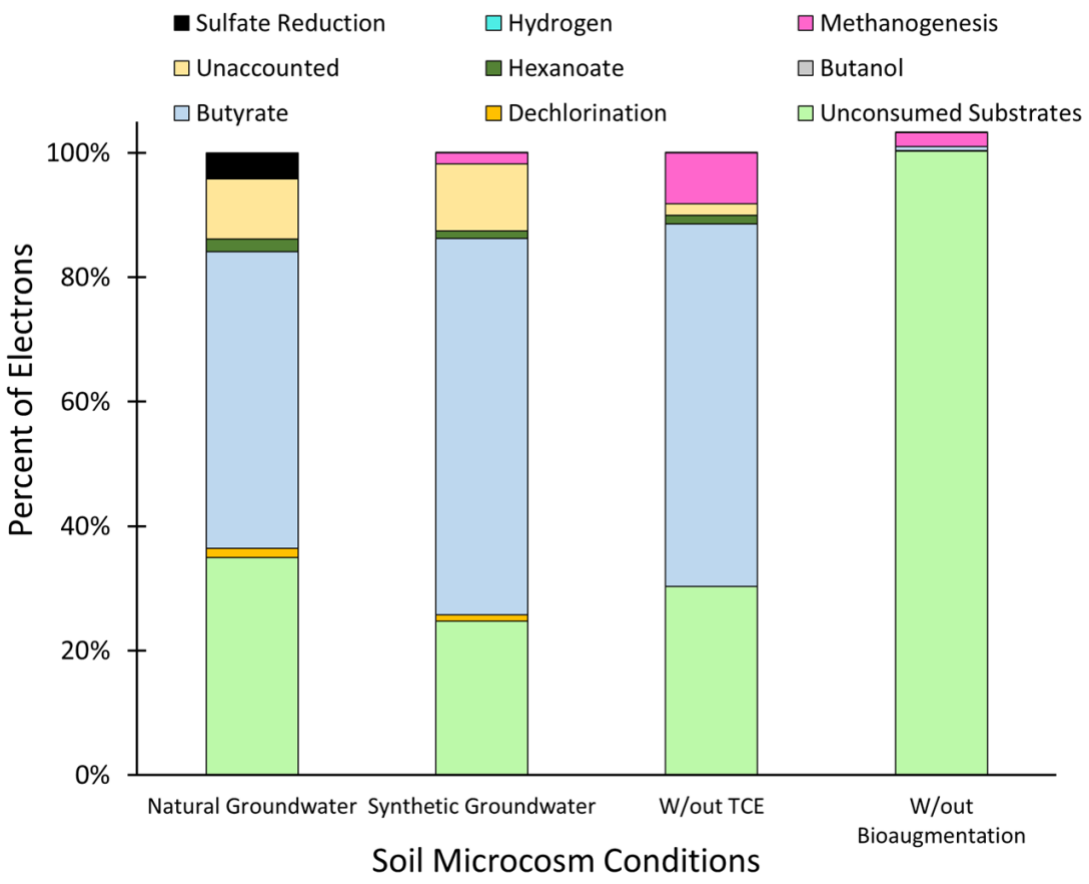

Figure 7: Soil microcosms substrate electron distribution to end products on day 115. The data are averages of triplicates.

Percent of substrates electrons to end products gives a fuller understanding on the dominating processes and differences in conditions. The distribution of electrons to end products reveals that most electrons went to the production of butyrate. In soil

microcosms, a reduction in electron acceptors increased butyrate production, but decreased butyrate production in the absence of TCE. In the absence of TCE, electrons went to the production of methane.

3.3 Reductive dechlorination via MCE in transfer microcosms

Microcosm transfers were established to explore dechlorination and/or MCE in more defined conditions (no soil) and using a more enriched and stable community. Ethanol only, as an MCE substrate, is known to produce larger concentration of hexanoate than ethanol and acetate combined (Figure 8A). This condition (Figure 8B) was created to explore if this would translate to increased dechlorination rates by providing more H_2 for *D. mccartyi* or have an adverse effect on reductive dechlorination through MCE products and processes.[16,17,33] Acetate only (Figure 8C) was setup as a dechlorination and MCE negative control

Over the course of 90 days, ethanol and acetate combined and ethanol only, completely dechlorinated the concentration of added TCE to mostly ethene (Figure 8). The condition with ethanol showed the fastest rates of dechlorination. Ethanol and acetate accumulated higher concentrations of DCE and VC before producing ethene while the acetate only condition stalled at DCE. The stall at DCE is likely due to the absence of H_2 in these microcosms, which is required by *D. mccartyi* for VC and ethene production.[34] Other microbes such as *Geobacter*, *Desulfuromonas,* and *Dehalobacter* can use acetate to produce DCE, but not *D. mccartyi*.

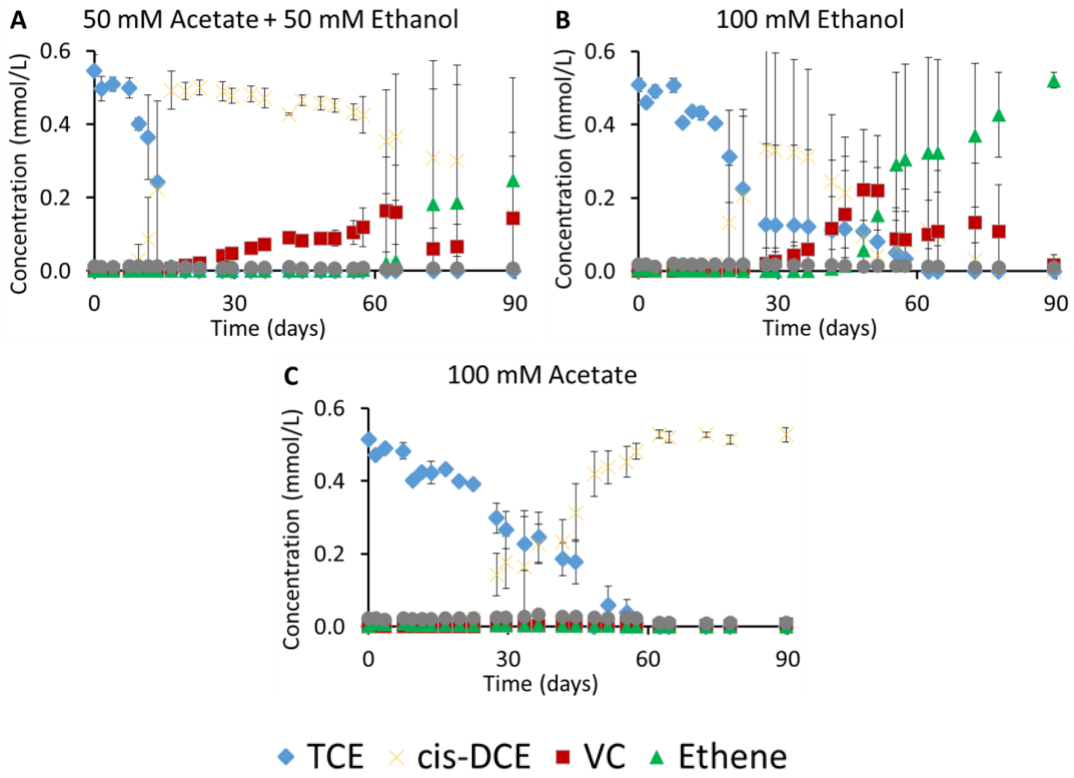

Figure 8: TCE reductive dechlorination in microcosm transfers. The data are averages of triplicates and the error bars show standard deviation of the mean.

In the transfer condition with acetate and ethanol, H_2 was accumulated slower than in soil microcosms. Moreover, production and consumption of H_2 were slower in transfer microcosms than in soil microcosms. Peak H_2 accumulation was reached between 30 and 60 days in transfer microcosms, compared to 10 days in soil microcosms. A slower production of H_2 can be due to a smaller concentration of chain elongators used in transfer microcosms compared to soil microcosms and slower consumption is due to less competition for H_2, once *D. mccartyi* were enriched. Ethanol was not fully consumed in the ethanol only condition, therefore H_2 increased (product of MCE) and decreased (electron donor for reductive dechlorination) through the end of the experiment

remaining at 0.295 ± 0.015 mM of H_2. The acetate only condition completely consumed all carryover H_2 (0.033 ± 0.005 mM) within 30 days (Figure 9).

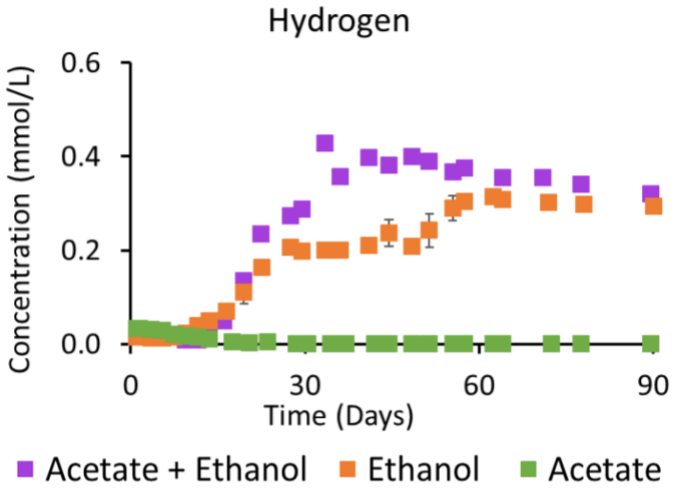

Figure 9: H_2 accumulation for transfer microcosms. The data are averages of triplicates and the error bars show standard deviation of the mean.

MCE products were detected in the acetate and ethanol and ethanol only condition, but not in the acetate only condition. Two other MCE reactions that can occur, but were not dominant in soil microcosms, but were observed in the ethanol only conditions are ethanol oxidation (Equation 4), and ethanol-butyrate to hexanoate (Equation 5).[13,15,16,17]

Equation 4: $C_2H_5OH + H_2O \rightarrow C_2H_3OO^- + H^+ + 2\ H_2$

Equation 5: $C_2H_5OH + C_4H_7OO^- \rightarrow 1\ H_2O + C_6H_{11}OO^-$

Chain elongation observed in the acetate and ethanol condition although stoichiometry reflected finding in soil microcosms after ethanol was completely consumed did not follow the same trend. Ethanol was not fully consumed before butyrate was produced (Figure 10A). Ethanol only produced the highest concentration of hexanoate (8 mM) as shown in Figure 10B. In the acetate only condition, no significant chain elongating end products were observed (Figure 10C). Acetate is only the backbone for chain elongation

and requires an electron donor substrate like ethanol to chain elongate. At day 90, the acetate and ethanol condition produced 39 mM of butyrate and 2 mM of butanol (Figure 10).

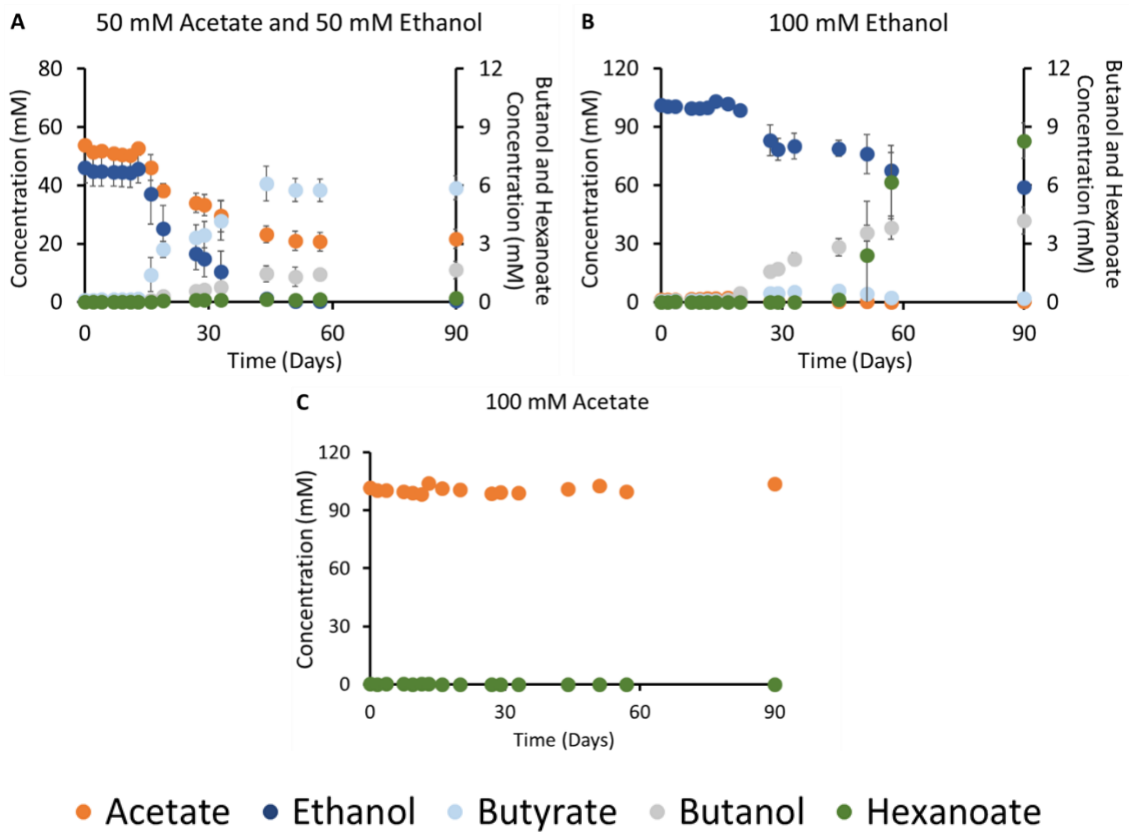

Figure 10: Fatty acid and alcohol concentrations for transfer microcosms. The data are averages of triplicates and the error bars show standard deviation of the mean.

An electron balance for the transfer microcosms was plotted at day 90 to show the substrate electron distribution to products for each condition (Figure 11).

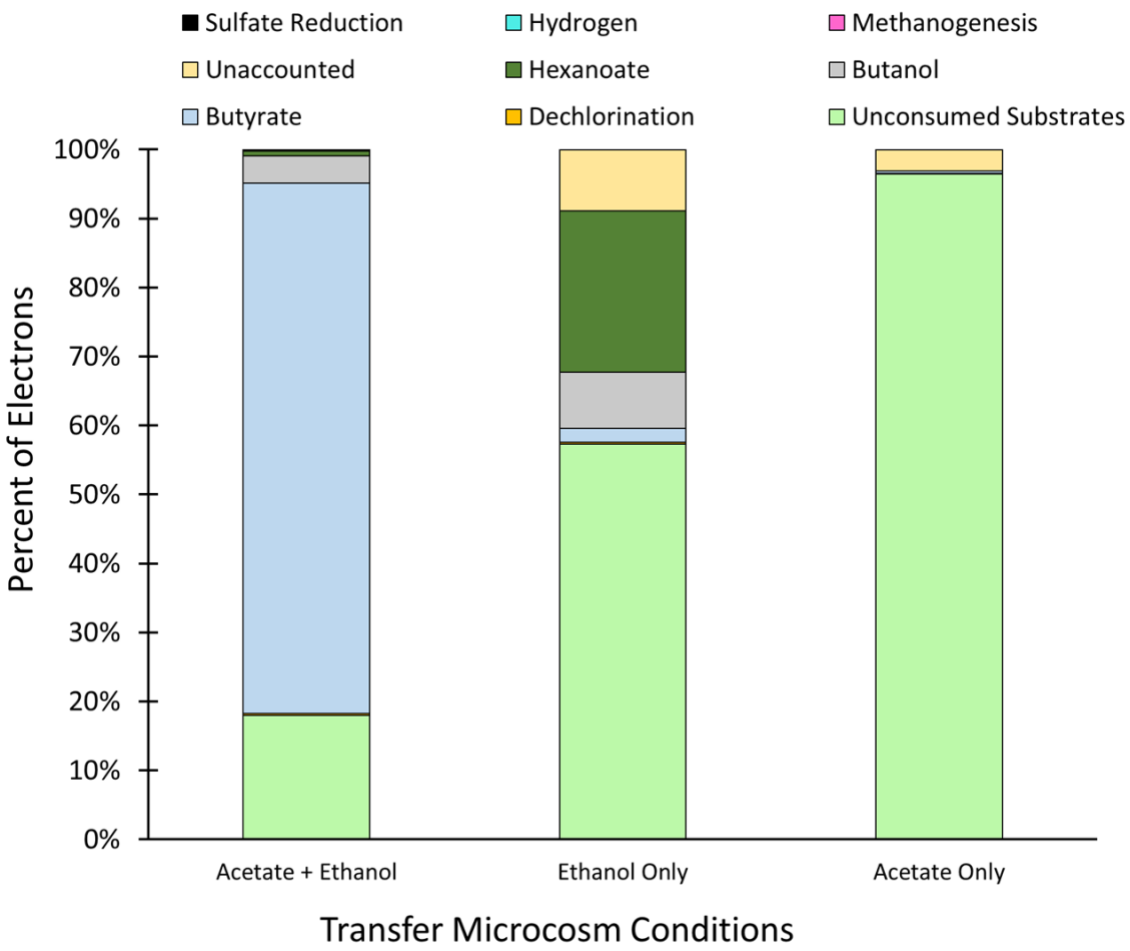

Figure 11: Transfer microcosms substrate electron distribution to end products on day 90. The data are averages of triplicates.

Percent of substrate electrons distributed to end products gives a fuller understanding of the dominating processes, differences in conditions, and changes from soil to transfer microcosms. The distribution of electrons to end products confirms that most electron went to production of butyrate in microcosms using acetate and ethanol combined as MCE substrates, in the ethanol only microcosms most of the consumed substrate went to hexanoate production, and in the acetate only condition a small portion of electrons went to unknown or unaccounted processes, while acetate went unconsumed. From soil microcosms to transfer, the ethanol and acetate combined condition showed more

consumption of substrates and more production of end products in a shorter time span. Likely ethanol and acetate consuming chain elongators were enriched from soil to transfer conditions.

3.4 Microbial community structure and relative abundance in soil microcosms

Soil microcosms successfully enriched for chain elongating and reductively dechlorinating microorganism via the addition of MCE substrates. A figure identifying dominant phylotypes at day 0, start of experiment for all soil microcosms; day 59 for natural groundwater and day 49 for synthetic, w/out TCE and w/out bioaugmentation, point where only VC and ethene was observed; and day 115, end of experiment, for all conditions was created. The addition of acetate and ethanol dramatically changed the microbial community structure. Initially microcosms were dominated by phylum proteobacteria, through addition of MCE substrates phylum *Firmicutes* for chain elongation and phylum *Chloroflexi* for reductive dechlorination were significantly enriched (Figure 12).

Looking deeper into the microbial community structure, within *Firmicutes* the following genera' *Clostridium* and *Desulfosporosinus* and family *Lanchospiraceae* were the most abundant (Figure 12). While, within *Chloroflexi* the genera *Dehalococcoides* was the most abundant.

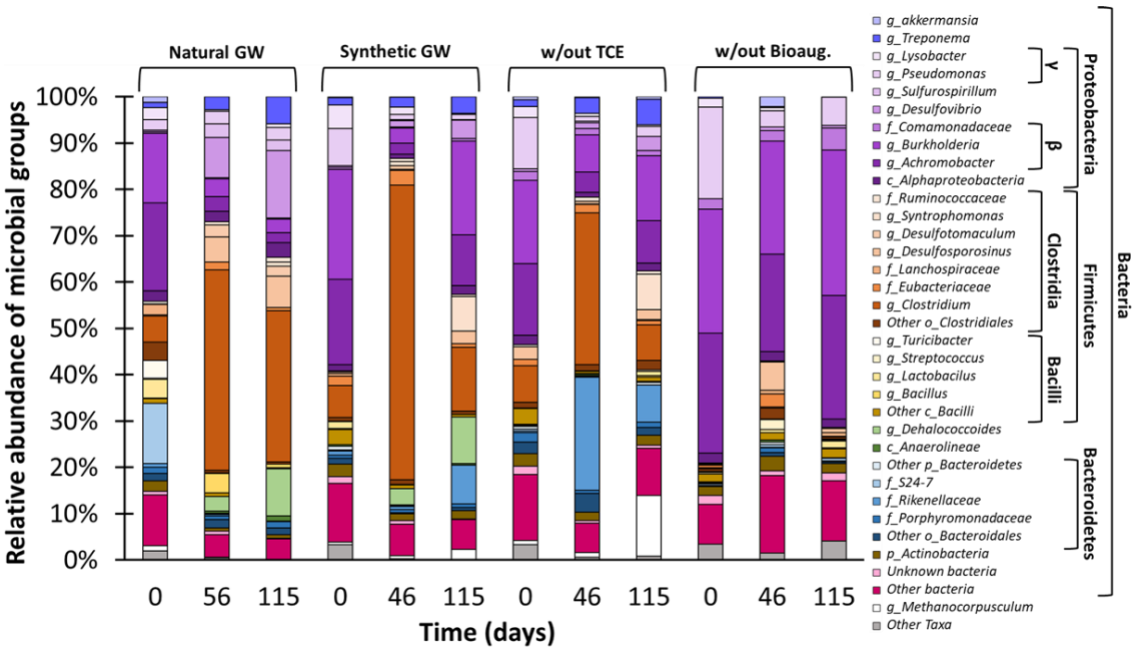

Figure 12: Relative abundance of taxa in soil microcosms on day 0, 46/56, and 115. The data are averages of triplicates.

Some known chain elongators belong to phylum *Firmicutes* and genus *Clostridium*. For instance, *Clostridium kluyveri*, isolated from canal mud, is the most studied anaerobic microorganism and uses acetate and ethanol for chain elongation.[15,17] Shown in Figure 13, *Clostridium kluyveri* was detected in all conditions except in the condition without bioaugmentation. In natural groundwater, synthetic groundwater, and without TCE species *kluyveri* went from 0.12% to 38.96%, 0.38 to 61.12%, and 0.00% to 31.22%, respectively (Figure 13). There are a few more chain elongating microbes that have been reported such as, *Eubacterium pyruvativorans* isolated from oxygen limited environments, *Megasphaera elsdenii* isolated from sheep rumen fluid, *Clostridium sp. BS-1* isolated from anaerobic digester sludge[15] and *Azospira oryzae* isolated from organic waste streams.[35]

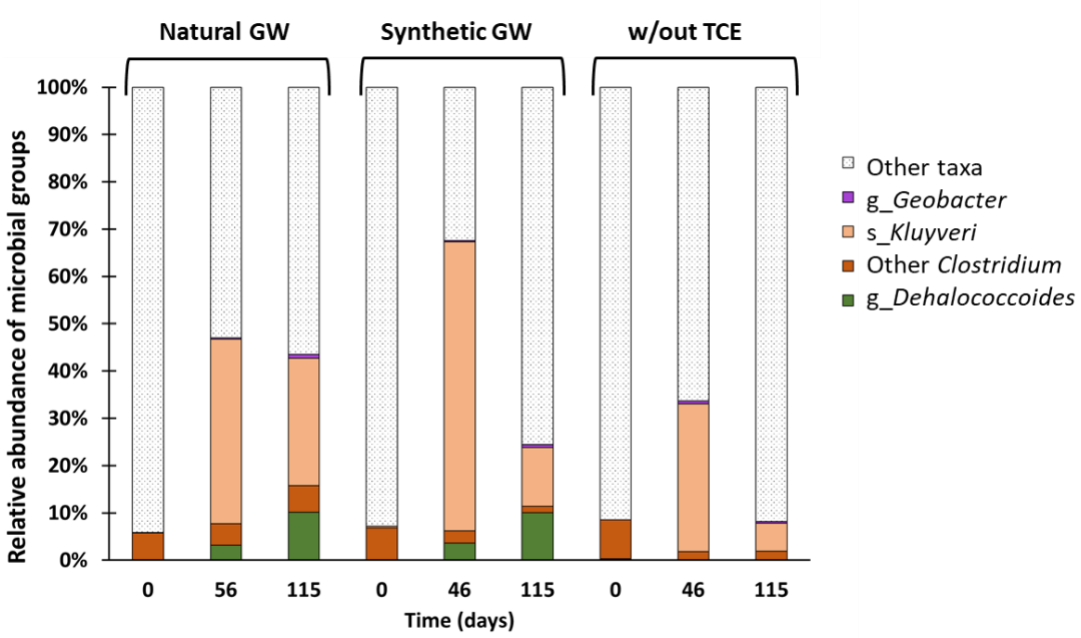

Figure 13: Relative abundance of known chain elongating and dechlorinating microorganisms in soil microcosms on day 0, 46/56, and 115. The data are averages of triplicates.

As mentioned previously, only *Dehalococcoides* can complete reductive dechlorination and they became the most abundant genera within the phylum *Chloroflexi*.[36] The inoculant used in these experiments contained *Dehalococcoides* stains 195, VS and GT, they were enriched through the addition of MCE substrates.[19,21,22] Other dechlorinators not in phylum *Chloroflexi* were from phylum *Proteobacteria*, and genera *Geobacter* accounted for 1% of the microbial community.[32]

CHAPTER 4

CONCLUSION AND OUTLOOK

Microbial chain elongation successfully drove complete reductive dechlorination of TCE in soil and transfer microcosms using 50 mM ethanol and 50 mM acetate, and 100 mM ethanol. In soil microcosms, MCE products were found in all conditions, but a significantly lower concentration was observed without bioaugmentation as well as a negligible percent of the microbial community belonging to phylum *Firmicutes*. Reductive dechlorination did not occur without bioaugmentation. Microbial community analysis revealed that the genus *Dehalococcoides* was not present in the soil. Methanogenesis, a main competing process typically observed in reductive dechlorination via fermentation, was inhibited and/or suppressed when chain elongation and reductive dechlorination occurred simultaneously.

In transfer microcosms, slower dechlorination rates and production of MCE end products were observed in the ethanol and acetate condition compared to soil microcosms, while methanogenesis was not observed and the MCE by-product, hydrogen, production increased. By using ethanol only as an MCE substrate, complete reductive dechlorination was faster than in the ethanol and acetate condition, as well as higher concentrations of butanol and hexanoate were produced. Future work will be dedicated to enriching transfer conditions and finding dominant microbial groups, as well as exploring other MCE substrates for the reduction of chlorinated solvents and other oxidized contaminants.

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