

ABSTRACT

HIORTDAHL, KIRSTEN MARIE. Enhanced Anaerobic Bioremediation of Tetrachloroethene Dense Non-Aqueous Phase Liquids in Column Studies. (Under the direction of Robert C. Borden).

Dense non-aqueous phase liquids (DNAPL) present many remediation challenges due to their recalcitrant nature, ability to sink into an aquifer, and slow dissolution rates. In situ bioremediation (ISB) is a common remedial strategy used to treat dissolved plumes. ISB treatment of plume source areas can potentially shorten treatment and monitoring time. However bioremediation of source areas has several challenges; high concentrations of chlorinated compounds have inhibitory effects on microorganisms, high acid production from dechlorination and fermentation lower pH, and slow dissolution of the DNAPL limits mass transfer. A minimal re-application technology that addresses these challenges is warranted given the persistence of many source zones.

Emulsified vegetable oil (EVO) has been shown to provide a long lasting carbon source and electron donor for bioremediation. In this research, EVO is combined with magnesium hydroxide (MH) and bioaugmented to treat a tetrachloroethene (PCE) DNAPL in a column experiment (EVO-MH). A conventional EVO column, with bioaugmentation, and a control column, without substrate or bioaugmentation, are run for comparison. MH is a good choice for pH regulation since it has a low aqueous solubility and slow dissolution rates are not governed by carbon dioxide dissolution. Laboratory columns containing neat PCE were treated with a commercially available EVO (EOS 598 B42, referred to as EOS) and EVO-MH (MH5, this study) in combination with a dechlorinating bioaugmentation culture (SDC-9) to enhance PCE biodegradation to ethene.

Short-term abiotic experiments were conducted to determine if EVO-MH can be transported through 1.5 m long laboratory columns packed with washed fine sand and if the EVO-MH will enhance PCE transport through the column. Experiments demonstrated that both EOS and MH5 emulsions could be transported through these columns. Injection of MH5 was effective in increasing the sediment alkalinity and pH throughout the columns. Injections of both EOS and MH5 were effective in redistributing a portion of the PCE DNAPL. This redistribution should bring the PCE into close contact with oil and MH throughout the column.

Long-term biotic experiments were conducted to determine if the EVO-MH will neutralize acid produced during anaerobic bioremediation, enhancing removal of a PCE DNAPL relative to EVO only. MH5 was effective in distributing MH throughout the column, maintaining near neutral pH until most of the MH had been depleted. Injection of MH5 and a bioaugmentation culture was effective in enhancing bioremediation of a pure PCE DNAPL. Over 99% of the ethenes released from the MH5 column during column running (Phase 2) were cDCE indicating extensive dechlorination of PCE resulting in more rapid removal of total ethenes than EOS addition. MH was depleted more rapidly than expected, rapid transport of MH through the column during the initial injection (Phase 1) and neutralization of VFAs and carbonic acid produced by substrate fermentation during column running (Phase 2) hastened its depletion. The extent of PCE dechlorination was likely greater than would be apparent based only on measurements of ethene concentrations in the column effluent as supported by evidence of dechlorination products accumulated in the column

(high ratio of cDCE to PCE in the sediment samples from the MH5 column) and measurements of dissolved Cl produced.

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Enhanced Anaerobic Bioremediation of Tetrachloroethene Dense Non-Aqueous Phase
Liquids in Column Studies

by
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DEDICATION

This thesis is dedicated to those who are no longer with us, my father for encouraging me to be ever curious, my Opa for my cherished memories and childhood inspirations, and my sensei for his support, wisdom, and unending compassion.

BIOGRAPHY

Kirsten (Kitty) Hiortdahl is from Baltimore, Maryland. She received her BS in Environmental Biology from Francis Marion University in December 2008 and worked from May 2006 through May 2009 at the USDA-ARS research facility in Florence, South Carolina. Through her work at the USDA on livestock waste/wastewater, she developed an appreciation for the chemical, biological, and physical principles at play in waste treatment and became interested in science as an applied field. This prompted her to pursue a career in engineering and in June 2009 she began exploring environmental engineering at NCSU. In January 2010 she became a graduate research assistant under the direction of Dr. Robert C. Borden.

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I am extremely grateful to Dr. Borden for being my adviser, for his time, patience, and guidance. I would like to acknowledge David Black, for his endless help, my committee members, Dr. Aziz and De los Reyes for having an open door to my questions, our research group members who have helped make this work possible, Michelle Roberts, Jaimie Potter, Andrew Hunter, Liu Han, Stewart Farling, and Sean Lai, and our current research group for support and friendship.

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Chapter 1: Background

1.1 Introduction

Groundwater contamination by chlorinated solvents remains a remediation challenge particularly due to their ability to form dense non-aqueous phase liquids (DNAPL). Tetrachloroethene (PCE) has a high density (1.62 g/mL) and low solubility (150 mg/L). When release into an aquifer PCE often sinks and diffuses into low hydraulic conductivity zones; dissolving slowly into groundwater (Stroo, 2010). PCE forms DNAPLs that are source zones for contamination and can persist for hundreds of years (Yang and McCarthy, 2000), and is a persistent organic pollutant in many aquifers (USGS, 2006). The US EPA classifies PCE along with its degradation byproducts as potential or known carcinogens and these compounds are strictly regulated in groundwater (US EPA, 2011).

Reductive dechlorination is a microbially mediated process where PCE is reduced to trichloroethene (TCE), dichloroethene (cDCE), vinyl chloride (VC) and ethene. The most common DCE isomer produced in this process is 1,2-*cis*-dichloethene or cDCE. There are a variety of anaerobic bacteria that can dechlorinate PCE and TCE to cDCE (He et al., 2007). However the *Dehalococcoides* genera (DHC) are the only identified organisms capable of dechlorinating cDCE completely to ethene, a non-toxic end product (Maymo-Gatell, 1997). Note, DHC has recently been renamed *Dehalococcoides mccartyi* (Löffler et al., 2012). Microbially mediated reductive dechlorination can be used to remediate contaminated aquifers by providing a fermentable organic substrate as an electron donor and carbon source

(termed biostimulation). In some cases, a microbial culture known to contain DHC is also added in a process called bioaugmentation.

The efficiency of ISB depends on the microbial population and environmental conditions. In field applications, remediation can stall at any step in the dechlorination process because of biological and environmental factors (ITRC 2008). In situ bioremediation (ISB) has been applied to treatment of DNAPLs in laboratory experiments with varying levels of success, ranging from minimal (Amos et al., 2008) to substantial degradation (Adamson et al., 2003).

1.2 ISB for DNAPL Source Areas

A DNAPL source zone is an area that is either fully or partially saturated with contaminants and can act as a reservoir which maintains a groundwater contaminant plume. Common objectives for DNAPL source zone remediation include: preventing further migration, reducing vapor potential, reducing plume size, depleting the source zone, and reducing the duration of treatment (ITRC 2008). Technologies used to treat DNAPL source zones include alcohol and surfactant flushing, thermal treatment, air sparging, in situ chemical oxidation (ISCO), and in situ bioremediation (ISB) (Christ and Abriola, 2006). Reductive dechlorination of DNAPL source areas is of interest due to the ability of microbial isolates and mixed consortia cultures to dechlorinate PCE and related contaminants to non-toxic end products. However, a variety of processes and factors can limit ISB effectiveness including number and composition of the microbial community, inhibition at low pH, slow mass transfer from the NAPL to the aqueous phase, and toxic inhibition

1.2.1 Microbial Ecology

Reductive dechlorination is a microbially mediated process where PCE is reduced to trichloroethene (TCE), dichloroethene (DCE), vinyl chloride (VC) and ethene. The most common DCE isomer produced in this process is 1,2-*cis*-dichloroethene or *c*DCE. Typically degradation can only take place anaerobically and under low redox conditions (ITRC 2008). Microbially mediated reductive dechlorination can be used to remediate contaminated aquifer by providing a fermentable organic substrate as an electron donor and carbon source (termed biostimulation). In some cases, a microbial culture known to contain DHC is also added in a process called bioaugmentation.

There are a variety of anaerobic bacteria that can dechlorinate PCE and TCE to *c*DCE including *Desulfuromonas*, *Sulfurospirillum multivorans*, *Dehalobacter*, and *Geobacter* (He et al., 2007, and Mohn and Tiedje, 1992). In addition, sulfate reducing, methanogenic, and acetogenic bacteria can degrade chlorinated alkyl halides due to the presence of corrinoids, hemes, and F 430 (Mohn and Tiedje, 1992), and reduced transition metals cofactors (Maymo-Gatell et al., 1999) through a cometabolic process that does not yield energy for growth (ITRC 2008).

The *Dehalococcoides* genera or DHC (Maymo-Gatell, 1997) within the phylum *Chloroflexi* (Spormann, 2011) are the only identified organisms capable of dechlorinating *c*DCE completely to ethene, a non-toxic end product. However, not all strains of these organisms

gain energy from the reduction of VC to ethene. Given the intricacies of horizontal gene transfer within microbial communities of *Chloroflexi*, functional/operational unit identification has become the most reliable method of dechlorinator population detection (Spormann, 2011). The known functional units are highlighted at each step in Figure 6.

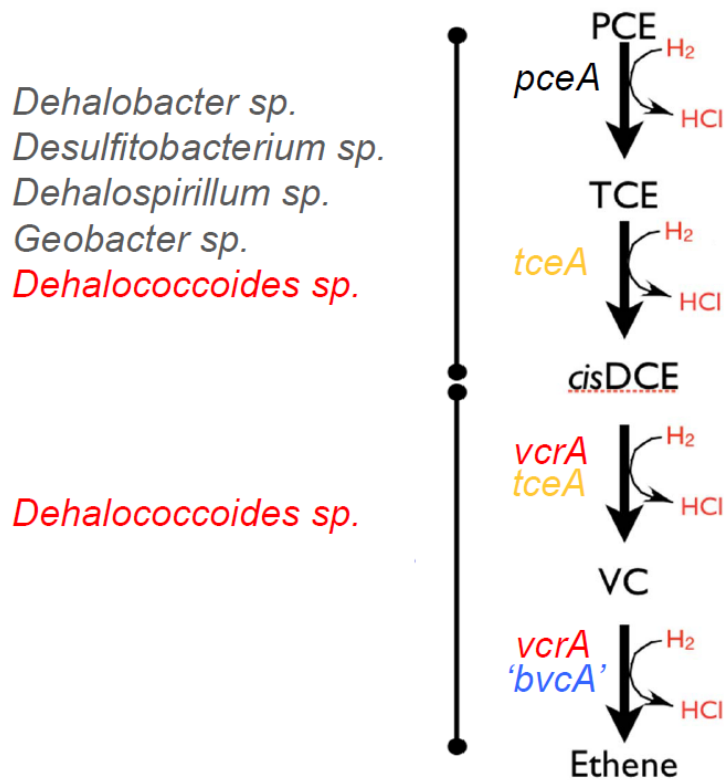


Figure 1-PCE degradation, the microorganism and functional genes associated with each step (Spormann, 2011).

1.2.1.1 Incomplete Dechlorination

While microorganisms can dechlorinate PCE all the way to non-toxic end product (ethene), dechlorination is incomplete at many source zone sites, resulting in cDCE accumulation (Adamson et al., 2004, McCarthy et al., 2007). Incomplete dechlorination has been attributed to insufficient residence time (Amos et al. 2008b), low pH (Adamson et al. 2004, Eddy 2008, Kouznetsova et al., 2010, McCarthy 2007, Robinson 2009), and inhibitory parent compound concentrations (Chu et al., 2004, Yu et al. 2005, Salbalowsky and Semprini 2010a and b). Partial degradation can potentially reduce source zone persistence by increasing mass flux (Adamson et al. 2004, Capiro et al., 2010) and allow treatment of more soluble, less inhibitory byproducts (Salbalowsky and Semprini 2010a and b) to occur downstream.

cDCE and VC are often the main degradation products produced in laboratory studies of DNAPL ISB (Adamson et al., 2003, McCarthy et al., 2007, Amos et al., 2008b). Adamson et al. (2003) utilized an experimental controlled release system (ECLS) to evaluate dechlorination of an inoculated PCE DNAPL. Overall the system predominantly produced cDCE for the first 225 days with VC production increasing over time to become the major product after 245 days demonstrating that re-inoculation was not necessary. cDCE was found in the source area however VC and ethene were not, suggesting that dechlorination past cDCE could be limited to down gradient of the DNAPL zone (Adamson et al., 2003).

1.2.2 pH

pH is believed to have a particularly strong impact on biological reductive dechlorination; cDCE to VC to ethene has been shown to be inhibited below pH ~ 6 (Eaddy, 2008), as these dechlorinators are highly sensitive to pH. High levels of fatty acids are produced from fermentation of substrates (Kouznetsova et al., 2010, Chu et al., 2004) and rapid dechlorination produces large amounts of hydrochloric acid (HCl) and carbonic acid (H₂CO₃). Acid production within source zone areas can be higher due to high dechlorination rates which can cause shock for pH sensitive dechlorinators. Multiple studies (Adamson et al., 2004, Eaddy, 2008, Kouznetsova et al., 2010, McCarthy et al., 2007, Robinson et al., 2009) have reported that low pH was a contributing factor to limited dechlorination. Amos et al., (2008b) with a mixed NAPL column experiment and Adamson et al., (2004) with a NAPL source zone tank experiment demonstrated that rapid biodegradation of a NAPL source zones produced large amounts of hydrochloric acid, and substantial amounts of sodium bicarbonate were added to both experiments to regulate pH.

1.2.2.1 Inhibition at Reduced pH

Eaddy, (2008) developed an enrichment culture from the site treated with Emulsified Oil Substrate (EOS[®], EOS Remediation, Raleigh, NC) that contained novel DHC strains and was able to degrade PCE to ethene. EOS[®] was found to be a superior to lactate as an electron donor, promoting faster dechlorination with less cDCE and VC accumulation. Figure 2 shows the effect of varying pH levels (5.5, 6.0, 6.5, 7.0, 8.5 and no pH buffer) on dechlorination of VC to ethene for Eaddy's enrichment culture maintained at pH 6.5-7.5 in

buffered media. The highest ethene production occurred in incubations buffered to pH 7. Once the pH drops to 6 or below, ethene production was minimal. Eaddy found that dechlorination of cDCE was completely inhibited at pH of 5.5 (data not shown). However, there was still significant methanogenesis at this low pH. At pH 8.5 the culture was strongly inhibited with minimal TCE production, but continued to produce methane. When the culture produced hydrochloric acid with no pH buffer, pH declined and ethene production was inhibited below 6.

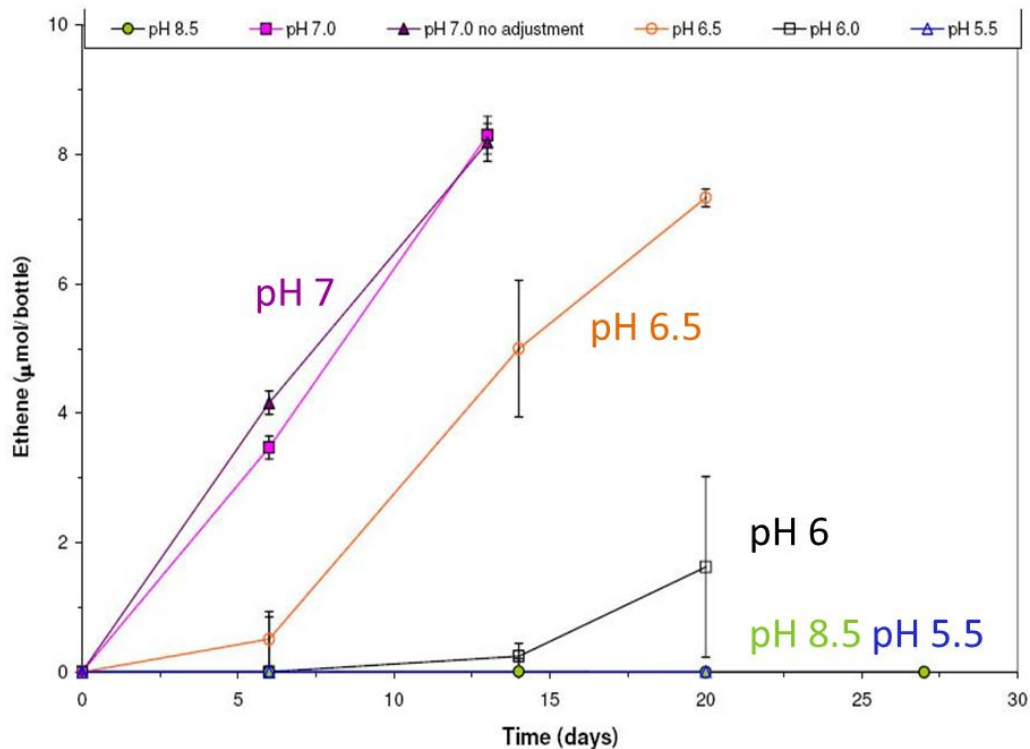
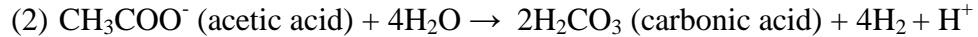
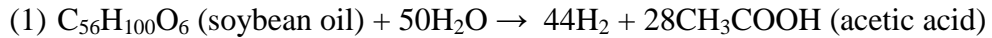


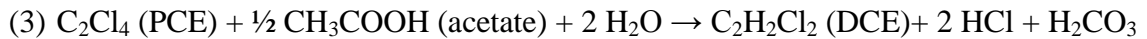
Figure 2- Various pH were maintained to observe dechlorination within a culture capable of full dechlorination to ethene. The accumulation of ethene as shown above was highest at a pH of 7.

1.2.2.2 Acidity Production during ISB

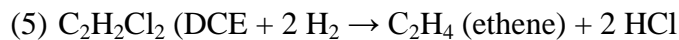
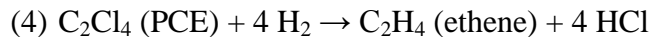
During ISB soybean oil is commonly added to the subsurface where it ferments to hydrogen (H₂) and acetic acid (1). Acetic acid can be utilized to facilitate PCE to cDCE (3), or further ferment to carbonic acid and hydrogen (2).



During reductive dechlorination of PCE, acetic acid can be consumed with concurrent production of HCl and carbonic acid to cDCE (3).



PCE can be reduced to cDCE with acetate or hydrogen utilizing the products from both reactions (1) and (2) as seen in reaction (4). However PCE can only be completely reduced to ethene with hydrogen from reaction (2) as seen in reaction (5). Both (4) and (5) utilizing hydrogen release HCl only.



Regardless of the specific pathway followed, reductive dechlorination has the potential to produce large amounts of acidity in the form of fatty acids from substrate fermentation, and carbonic acid, and HCl from dechlorination directly.

The total amount of acidity produced during reductive dechlorination can be substantial. For example, reduction of 8,400 μM TCE (aqueous equilibrium solubility) to ethene using hydrogen as an electron donor will release 26.4 mM of HCl, requiring 3200mg/L of

alkalinity to maintain a pH of 6.5 (McCarthy et al., 2007). One potential alternative for reducing the alkalinity requirement is to use sodium formate as an electron donor for reduction of PCE to cDCE (McCarthy et al., 2007), since this substrate self neutralizes, releasing bicarbonate. However, available information indicates that DHC cannot use formate for cDCE or VC to ethene so additional H₂ releasing donors would be required.

1.2.2.3 pH Buffering in Aquifers

Naturally occurring buffering processes in aquifers include carbonate dissolution, proton exchange with clays and various reduction reactions (nitrate, ferrous hydroxide and bicarbonate).

In the open systems that typically occur at the surface, CO₂ can degas essentially stripping carbonic acid out of the water. However below the water table, CO₂ may not be able to degas, reducing pH. At the surface, calcium carbonate (CaCO₃) is an effective buffer since continuous stripping of CO₂ results in reasonably rapid CaCO₃ dissolution and acidity consumption. However below the water table, CO₂ may accumulate, causing the groundwater to become supersaturated with CaCO₃, greatly reducing the effectiveness of CaCO₃ as a buffer (McCarthy et al., 2007).

Exchange of protons (H⁺) and aluminum ions (Al⁺³) onto clays and iron hydroxides can provide a strong pH buffer in the subsurface. This strong buffer can reduce the pH decline in many systems, but can also greatly increase the amount of base required to increase aquifer

pH (Borden, 2011). Kouznetsova et al. (2010) discounted the potential importance of cation exchange as a pH buffer since this process is most important at pH below 5. However, Adamson et al. (2004) reported pH values below 4.5, supporting the need to consider cation exchange in such applications.

During ISB, a variety of other electron acceptors are often consumed in thermodynamic order; oxygen (O_2), nitrate (NO_3^-), ferric iron ($Fe(OH)_3$), sulfate (SO_4^{2-}) and then CO_2 during methanogenesis (Christ and Abriola, 2006). All of these reactions can result in release or consumption of hydroxides which can alter pH (Borden, 2011). When chlorinated solvent concentrations are low such as dissolved phase NAPL plumes, hydroxide release during $Fe(OH)_3$ reduction may be sufficient to maintain neutral pH without buffer addition (Borden, 2011). However for higher chlorinated solvent concentrations such as source areas DNAPLs, the large amounts of hydrochloric and carbonic acid produced from dechlorination and fermentation may require buffer addition to maintain a neutral pH (Borden, 2011, Kouznetsova et al., 2010, Robinson et al., 2009, McCarthy et al., 2007).

Given the importance of pH and acidity production on ISB, a variety of different bases have been examined to control pH declines. Bases such as sodium hydroxide or sodium carbonate can increase aquifer pH excessively. Lime is slow to dissolve and can lead to calcium carbonate precipitation (McCarthy et al., 2007). For highly contaminated areas, the large amount of base required can greatly increase the dissolved solids concentration, and could potential result in sodium chloride precipitation (McCarthy et al., 2007).

Magnesium hydroxide (MH) can potentially be used as a base to neutralize acidity produced during ISB. MH has a relatively low pKa (approximately 10.5, Takeno, 2005) and dissolves slowly providing a long-lasting alkalinity source that does not result in the very high pH values associated with other more soluble bases (i.e. NaOH pKa = 13.8). MH can be procured with mean particle diameters of 1 micron, so it is possible to transport this material through porous media as a colloidal suspension. MH is nonmagnetic with a moderate density (2.2 g/cm³), so flocculation and settling tends not to be significant. The point of zero charge (PZC) is 11 giving MH a net positive charge under typical aquifer conditions (zeta potential = + 25 mV at pH10, Pokrovky and Schott, 2004). As a result, colloidal MH may be strongly retained by negatively charged aquifer sediments. Addition of MH to the subsurface could aid DNAPL source area treatment since it is less likely to be flushed from the system like other more soluble bases.

1.2.3 Bio-Enhanced Dissolution

One factor limiting treatment of chlorinated solvents is the slow rate of PCE dissolution into the aqueous phase. ISB can potentially enhance DNAPL dissolution and reduce source area longevity by reducing the parent compound concentration in the aqueous phase, increasing the net dissolution rate. Several studies have shown that biological activity can increase the mass flux of dissolved ethenes released from DNAPL zones, potentially reducing source zone persistence (Phillips et al., 2011, Christ and Abriola, 2006, Chu et al., 2004).

Amos et al 2008b summarizes results of prior studies on bioenhanced PCE dissolution research including microcosm studies with a surfactant mixed NAPL (Carr et al. 2000 and Cope and Hughes 2001), DNAPL residual column experiments (Yang and McCarthy 2002, and 2000), 2 and 3-D tanks containing DNAPL (Sleep et al. 2006 and Adamson et al. 2003) and a DNAPL flow cell (Glover et al. 2007). For most experiments cDCE was the dominant byproduct and some experiments showed significant VC or TCE production. Maximum bio-enhanced dissolution rates range from 1.5 with heterogeneous sand and DNAPL pools (Glover et al., 2007), to 14 times the abiotic dissolution rate with pre-mixed tridecane-PCE NAPL (Carr et al., 2000) .

Amos et al. 2008b examined bio-enhanced dissolution in column studies with a strict PCE to cDCE dechlorinator *Sulfurospirillum multivorans*. Two different NAPLs were examined: (a) pure phase PCE; and (b) an ex situ mixed PCE-hexadecane NAPL with an effective PCE solubility of 300 μM , or roughly one fourth that of pure PCE. In the column with a pure PCE DNAPL, there was no evidence of bioremediation or bio-enhanced dissolution. The PCE concentration reached the abiotic equilibrium value (1200 μM) within one pore volume and cDCE production was below 10 μM . The strong microbial inhibition observed in this column is consistent with prior studies showing inhibition at 540 μM PCE (Amos et al. 2007), 300-400 μM PCE (Yu and Semprini 2004), and 800 μM PCE (Duhamel et al 2002). In the mixed NAPL column, PCE was extensively dechlorinated, producing cDCE concentrations of 1300-1500 μM which is somewhat greater than the aqueous solubility of pure PCE. However, total ethenes declined with time, eventually stabilizing 600 μM or half

the solubility of neat PCE). The decline may have been due to depletion of PCE from the NAPL, diffusion limits, cDCE toxicity, or pH drop. In the mixed NAPL column, approximately 53% of the added PCE was flushed out of the column in the form of cDCE. In contrast only 11.6% was flushed out of the pure PCE column. Overall, Amos et al. (2008b) demonstrated that ISB could be used to enhance the dissolution of an ex situ mixed PCE NAPL. However bio-enhanced dissolution of pure phase PCE DNAPL was not feasible due to toxic inhibition by high PCE concentrations. This study demonstrated the advantage of forming a mixed NAPL.

1.2.3.1 Electron Donor Supply

Electron donor (ED) can be added directly with diatomic hydrogen (H_2). However in practice, fermentable organic substrates are usually added to the aquifer. Ubiquitous fermentative and syntrophic microorganisms can convert these complex substrates into acetate and hydrogen that are used by microbial dechlorinators. Virtually any fermentable substrate can be converted by ubiquitous subsurface microorganisms into hydrogen for *Dehalococcoides* (ITRC 2008) including acetate, lactate, methanol, ethanol, butyrate, toluene, propionate, benzoate, glucose, emulsified vegetable oil (EVO), and polylactate ester-hydrogen releasing compound (HCR) (Duhamel et al., 2002, Eaddy, 2008, ESTCP, 2008, Borden and Rodriguez, 2006, ITRC 2008). Acetate (PCE to cDCE only) and hydrogen can be used for dechlorination; but can also be consumed by competing microbial groups including iron and sulfate reducers and methanogens. Studies by Yang and McCarthy (2002) indicate that methanogenesis will be inhibited by high chlorinated solvent concentrations (>

500 μM). However, extensive methane production is often observed (IRTC, 2008, Adamson et al., 2003, and Eaddy, 2008).

Yang and McCarty (2002) showed that dechlorination could be increased and methanogenesis reduced by mixing PCE with olive oil. As the olive oil slowly biodegraded, it provided a long lasting electron donor for PCE biodegradation. Capiro et al. (2010) described the use of partitioning electron donors (PEDs) for DNAPL bioremediation where concentrated pulse of the PED (n-hexanol or n-butyl acetate) would be injected into the aquifer, partition into the NAPL, and then slowly dissolve back into the aqueous phase providing a steady supply of electron donor to support reductive dechlorination. Since the organic substrate and chlorinated solvent are released from the same location, competition for the electron donor by methanogens would be reduced. Also, the mixed NAPLs reduce the effective solubility of the chlorinated solvent, reducing toxicity (Capiro et al., 2010, and McCarthy et al., 2007).

1.2.4 Microbial Inhibition

While bioenhanced dissolution requires microorganism to be in close proximity with contaminants, microorganisms can also be inhibited by high concentrations of these contaminants. A variety of studies have demonstrated toxic inhibition of reductive dechlorination by high concentrations of PCE (Huang and Becker 2010, Amos et al. 2007, Yu and Semprini 2005, and Phillips et al. 2011) and daughter products (Salbalowsky and Semprini 2010a and b, Cupples et al. 2004, Yu and Semprini 2005). PCE concentrations that

inhibit dechlorination vary by dechlorinator from approximately 400 to 800 μM (Yu and Semprini 2004, Duhamel et al. 2002, and Amos et al. 2007). In addition, Salbalowsky and Semprini (2010a) reported that inhibition by high chlorinated ethene concentrations can be compounded by non-neutral pH conditions.

Modeling of chlorinated solvent inhibition has been a main focus of several research groups leading to a better understanding of the complex interactions of PCE and its daughter product production and biomass response. Yu et al. 2005 investigated cultures that were capable of complete dechlorination to ethene focusing on production of daughter products leading to microbial inhibition. Based on these inhibition studies, the model shown below in Figure 3 was proposed. Solid arrows represent inhibition directions and dashed arrows represent very weak inhibition (Yu et al., 2005).

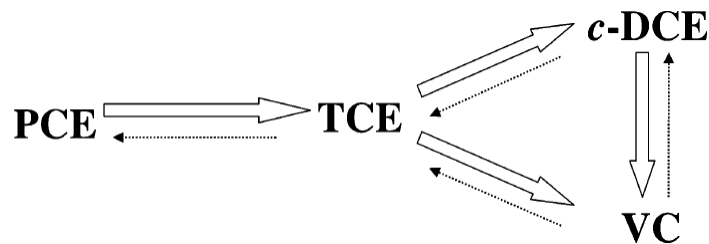


Figure 3- Inhibition model proposed by Yu et al. 2005.

Yu et al. 2005 found that the more highly chlorinated ethenes governed inhibition, except that cDCE was not inhibited by PCE which is consistent with microbial differentiation or stepwise dechlorination.

Given that the aqueous solubility of pure PCE (1200 μM) is 2-4 times levels reported to be inhibitory (300-540 μM , Yu et al., 2005 and Amos et al., 2007), and that the aqueous solubility of pure cDCE (280,000 μM) is also much higher than concentrations reported to be inhibitory (770-1100 μM , Chu et al., 2004; Salbalowsky and Semprini, 2010a); this suggests that microbial dechlorination may be strongly inhibited in areas with pure PCE DNAPLs.

1.2.4.1 Biomass

Higher tolerance to PCE concentrations has been shown in mixed cultures which can form biofilms, aggregates or attached growth; giving a biomass protection (Amos et al. 2007, Salbalowsky and Semprini 2010b). Microbial inhibition can be reduced by maintaining high biomass concentrations (Huang and Becker 2010), reducing PCE solubility using a mixed NAPL (Adamson et al. 2004, Amos et al. 2008b), and maintaining low PCE concentrations by rapid dechlorination (Amos et al. 2007 and 2008b, Duhamel et al 2002, Yu and Semprini 2005).

Huang and Becker (2010) studied the ability of PCE respiring dechlorinators (*Desulfuromonas michiganensis* and *Desulfitobacterium* strain PCE1) to function at high PCE concentrations including the aqueous equilibrium solubility of PCE, finding that high concentrations inactivated parts of the biomass permanently. High biomass concentrations were found to dechlorinate at higher PCE concentrations, with the amount of deactivated cell mass reduced. At high biomass concentrations (0.5 mg protein/L) and 800 μM PCE, the

system dechlorinated as predicted. However at 1200 μM (aqueous equilibrium solubility), the biomass was not able to dechlorinate (Huang and Becker, 2010).

Chu et al. (2004) utilized numerical modeling to explore the effects of ED supply, inhibition kinetics, and NAPL amounts and configuration on bioenhanced dissolution (Figure 4). The model was constructed using NAPL ganglion and accounting for dehalogenating and non dehalogenating biomass considering toxicity, affinity for ED, and pore space growth with respect to biomass, and electron donor availability. Two growth scenarios were explored where the electron donor was an up-gradient plug and where electron donor was an underlying plug with groundwater flowing perpendicular. Model simulations predicted that microbial biomass will be concentrated where high electron donor concentrations are high and at the PCE interface into the TCE zone.

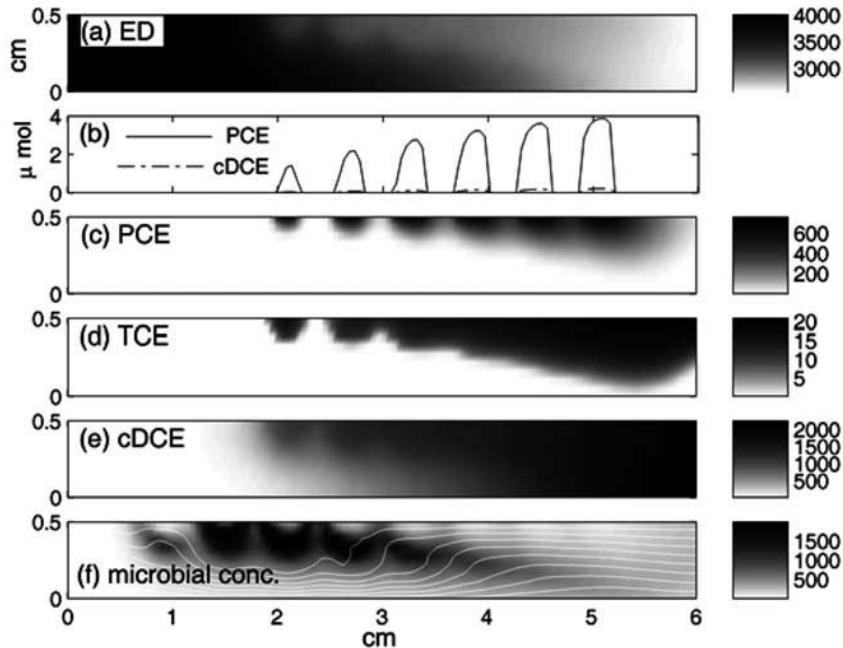


Figure 4- Numerical modeling of NAPL ganglia showing depletion of ED including inhibition effects and microbial biomass growth (Chu et al., 2004)

The model simulated bio-clogging after 55 days for a variety of conditions. However the compact biomass in the source zone did not greatly affect the dissolution rate once the dehalogenating microbes were established. Overall, ISB increased NAPL dissolution by a factor of 3 compared to abiotic conditions, reducing NAPL life by about two thirds the abiotic life.

Phillips et al. (2011) constructed a three layer diffusion cell to better understand the phenomenon controlling dissolution of a TCE DNAPL. The KB-1 bioaugmentation culture was used as inoculum and formate as an electron donor due to its buffer capacity (McCarthy et al., 2007). Dechlorination was inhibited in the sand from 0 to 1.5 cm above the TCE

DNAPL, presumably due to toxic inhibition. Dechlorination to cDCE occurred between 1.5 cm and 2.5 cm, with the largest activity occurring 2 cm from the TCE DNAPL. Beyond 2.5 cm, TCE was depleted and there was little additional degradation. The biotic concentration gradient and associated dissolution flux in the biotic system was approximately two times the abiotic values. Overall, ISB enhanced dissolution 2.4 times the abiotic system. The authors proposed that development of approaches that allow populations to grow closer to the DNAPL could accelerate dechlorination and that additional strategies are needed to deliver electron donor/carbon source and a pH buffer to target source zone (Phillips et al., 2011).

Chapter 2: The Need for a New Approach

Bioremediation of PCE DNAPL can be limited by low pH, inhibitory concentrations of PCE, and competition for available electron donor. In this study a combination of emulsified vegetable oil (EVO) with magnesium hydroxide (MH) was evaluated as a potential solution to address these challenges. This technology was developed to: 1) provide EVO as an electron donor and carbon source for the microorganisms, 2) reduced PCE inhibition to microorganisms through partitioning of PCE into the vegetable oil, and 3) provide MH to limit the pH decline resulting from hydrochloric acid (HCl), carbonic acid, and volatile fatty acid (VFA) production. To accelerate DNAPL dissolution and aquifer cleanup, the combined EVO-MH material must be effectively transported through the aquifer material to the DNAPL providing electron donor, carbon source, and pH buffer close to the site of active biodegradation.

Specific objectives of this research are:

- 1) To evaluate if the combination of EVO and MH can be successfully injected and distributed in low fines sand, compared to conventional EVO.
- 2) To evaluate if flushing the combined EVO-MH through a PCE DNAPL in moderate fines sand will transport a portion of the PCE further down gradient where it will be in close contact with the vegetable oil (carbon source and electron donor) and MH (neutralizing agent).
- 3) To evaluate if the addition of MH to the system will neutralize acids produced by substrate fermentation and reductive dechlorination, limiting pH declines.

- 4) To evaluate if the combined EVO-MH will enhance biodegradation of a pure PCE DNAPL, increasing bio-enhanced dissolution of the DNAPL, compared to conventional EVO.
- 5) To improve our understanding of processes limiting anaerobic bioremediation of PCE DNAPL.

2.1 Experimental Design

The experiment had two phases.

1. Short-term abiotic experiments to determine if EVO-MH can be transported through 1.5 m long laboratory columns packed with washed fine sand and if the EVO-MH will enhance PCE transport through the column (objectives 1 and 2).
2. Long-term biotic experiments to determine if the EVO-MH will neutralize acid produced during anaerobic bioremediation, enhancing removal of a PCE DNAPL relative to EVO only (objectives 3-5).

2.1.1 Experimental System

The experimental system consisted of 2.5 cm (1 inch) diameter x 1.5 m (5 ft) long clear PVC columns packed with sand. The columns were flushed with several pore volumes of water to saturate the sand. Then 1 mL of pure PCE was injected into the column at 15 cm (6 inches) from the inlet. The columns were then flushed with 0.4 pore volumes (PV) of dilute emulsion, 1.6 PV of water, and 0.4 PV of diluted bioaugmentation media. Two different emulsions were evaluated: (a) EOS 598 B42, a commercially available emulsified vegetable

oil (EVO) containing 60% vegetable oil, 4% soluble substrate, 2% yeast extract, 10% emulsifiers/additives, and 24% water (provided by EOS Remediation, LLC, Raleigh, NC); referred to as EOS and (b) a mixed EVO-MH developed for this work containing 50% vegetable oil, 4% soluble substrate, 7% emulsifiers/additives, 34% water, and 5% MH, referred to as MH5. This formulation (presented in the Appendix-3) appeared to be stable for over 1 year with no observed degradation or settling of the MH.

The amount of EOS and MH5 injected were selected to provide a large excess of biodegradable organic carbon to support reductive dechlorination and sufficient oil for effective transport through the columns. Previous work has shown that EVOs such as EOS are strongly retained by aquifer material and oil droplets will not be effectively transported until most of the attachment sites on the soil particles are filled with oil droplets. The maximum oil retention of sands is reported to vary between 0.0004 and 0.0095 g oil / g soil (ESTCP EVO Design Tool, 2008). In these experiments, the total amount of oil injected was 0.009 g oil/g for the EOS treatment and 0.007 g oil/g for the MH5 treatment to provide excess oil to saturate the sediment surfaces, allowing some oil to be discharged in the column effluent. The concentration of emulsion EOS vs MH5 injected was varied to provide the same amount of organic carbon to each column.

Preliminary work indicated that injection of large amounts of MH could increase the pH to between 9 and 10, inhibiting biological activity. As a result, 0.975 g of MH were injected into the MH5 columns providing 0.57 g MH to neutralize HCl released during dechlorination

of 1 ml (1.6 g) of pure PCE to cDCE, with 0.41 g of MH available to neutralize organic acids produced and/or HCl released during further dechlorination of cDCE to VC or ethene. This design was based on previous studies (Adamson et al., 2004, and McCarthy et al., 2007) showing that cDCE is the primary dechlorination product released during bioremediation of PCE DNAPLs.

2.1.1.1 Transport Experiments

Transport experiments were conducted in 1.5 m long columns to (a) determine if the new EVO-MH formulation (MH5) could be transported significant distances in sand with little silt/clay, and (b) that flushing EVO (EOS) or EVO-MH (MH5) through the DNAPL zone was effective in redistributing a portion of the DNAPL, improving contact between the PCE, oil and MH. The sand used to in these experiments was a washed masonry sand ($D_{50} = 434 \mu\text{m}$, 0.2 % finer than 200 sieve) selected to demonstrate transport in material with a relatively low oil retention. Prior to emulsion injection, 1.0 ml of neat PCE was injected into the column through a small hole at 15 cm above the column inlet.

The experimental procedure involved injecting 0.6 PV of diluted emulsion at 5 mL/min followed by 1.4 PV of maintenance media at 10 mL/min. After resting overnight, an additional 0.4 PV of groundwater (5 ml/min) and 1.4 PV of maintenance media (10 mL/min) were flushed through the column to displace EVO and MH from MH5 that had not been retained on sediment surfaces. The emulsion and flush water volumes and flow rates were

selected to match conditions expected to occur near an injection well during emulsion injection.

Transport of oil, MH and PCE through the column was monitored by measuring total organic carbon (TOC), volatile solids (VS), magnesium (Mg), pH, alkalinity and PCE in the column effluent over time. At the completion of column flushing, the columns were frozen, cut into 15 cm sections, homogenized, and analyzed for TOC, VS, pH, alkalinity, Mg, and PCE to determine the spatial distribution of these materials in the column.

2.1.1.2 Bioremediation Experiments

The biodegradation experiments were conducted in 1.5 m long columns packed with sand to determine if the EVO-MH (MH5) will neutralize acid produced during anaerobic bioremediation, enhancing removal of a PCE DNAPL relative to EVO (EOS) only. Columns were injected with EOS or MH5 following the same procedures used in the short-term transport experiments. However, the 0.4 PV of groundwater injected on the 2nd day was replaced with 0.4 PV of bioaugmentation culture (SDC-9 at an OD₅₅₀= 1) to enhance biological activity. Following injection of the emulsion and bioaugmentation culture, groundwater was pumped through the columns at 50 ml (0.2 PV) per week for approximately 60 weeks. A parallel control column without added EOS, MH5, or bioaugmentation culture was operated for 45 weeks to evaluate PCE flushing without substrate addition. The MH5 column was packed with a fine clayey sand ($D_{50} = 542 \mu\text{m}$, 12% finer than 200 sieve). The

EOS and control column were packed with sand from a different batch with a lower silt-clay content ($D_{50} = 585 \mu\text{m}$, 3.7 % finer than 200 sieve).

Transport and biodegradation of the oil, MH and PCE was evaluated by monitoring the column effluent for TOC, inorganic carbon (IC), VS, Mg, pH, alkalinity, PCE, TCE, DCE isomers, VC, ethene, ethane, methane, chloride (Cl), and volatile fatty acids (acetate, butyrate, propionate, and lactate). Most parameters were monitored weekly. However, Mg samples were composited and analyzed approximately once per month. At the completion of the biological monitoring period, the columns were frozen, cut into 15 cm sections, homogenized, and analyzed for TOC, VS, pH, alkalinity, Mg, PCE, TCE, and cDCE to determine the spatial distribution of these materials in the column.

Chapter 3: Methods and Materials

The experimental design consists of two phases, short-term abiotic transport experiments, and long-term bioremediation experiments. Short-term transport columns were tested for transport of oil, MH and PCE as monitored by total organic carbon (TOC), volatile solids (VS), magnesium (Mg), pH, alkalinity, and PCE in the column effluent. Long-term bioremediation columns were tested for transport, biodegradation of the oil and PCE, and MH consumption by monitoring the column effluent for TOC, inorganic carbon (IC), VS, Mg, pH, alkalinity, PCE, TCE, DCE isomers, VC, ethene, ethane, methane, chloroethane, chloromethane, chloride (Cl), and volatile fatty acids (acetate, butyrate, propionate, and lactate). At the completion of both experiments columns were frozen, cut into 15 cm sections, homogenized and analyzed for TOC, VS, pH, alkalinity, Mg, PCE, TCE, and cDCE to determine the spatial distribution of these materials in the column. Column sections are outlined in Figure 5; showing the site of PCE injection and sample collection.

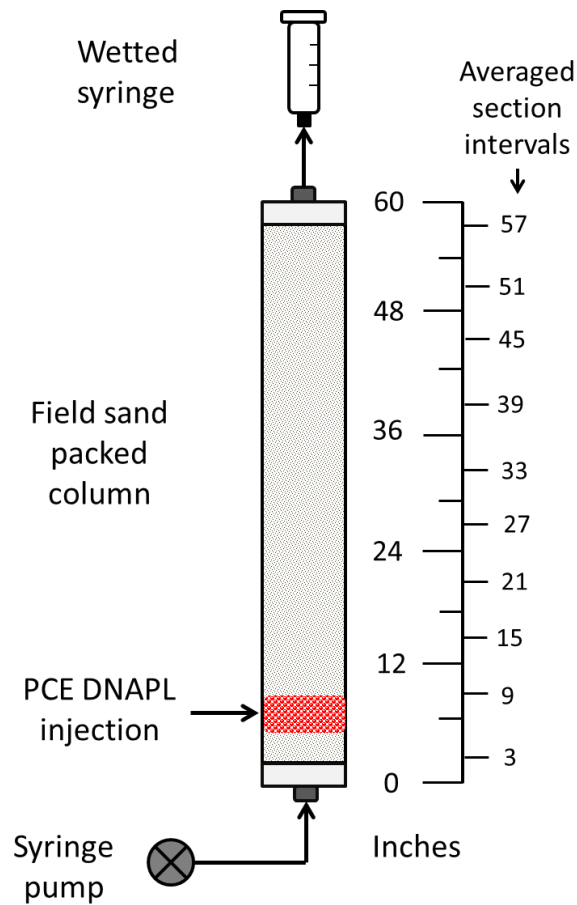


Figure 5-Schematic of column set up.

3.1 Column Sampling and Analysis

Column effluent was collected in a wetted glass syringe to maintain integrity of samples.

Samples for purge and trap (PCE, TCE, and DCE isomers) were prepared in 40 mL EPA VOA vials with Teflon septa and screw cap and diluted in DI water with no headspace.

Headspace analysis samples (VC, ethene, ethane, methane, chloroethane, and chloromethane) were prepared in 20 mL vials with Teflon septa, aluminum crimp cap and diluted with DI water. Samples for TOC, pH, alkalinity, anions (Cl, acetate, butyrate, propionate, and

lactate), and Mg were volatilized in a chemical hood prior to filtration (anions and Mg only), dilution, and analysis. Mg samples taken during Phase 1 of MH5 were digested and analyzed by North Carolina State University Environmental and Agricultural Testing Service (NCSU EATS) using inductively coupled plasma atomic emission spectroscopy (ICP-AES). Samples were stored at 4°C prior to analysis, except anion samples which were frozen.

At the completion of both experiments columns were frozen and cut into 15 cm sections. Frozen soil was homogenized and 5-15 g subsamples were weighted and added to 40 mL EPA VOA vials sealed with Teflon septa and screw cap. One mL of hexane was injected through the septa to trap any VOCs in the headspace; a total of 10 mL of hexane containing carbon tetrachloride as an internal standard and 10 mL of acetone were added. Samples were vigorously hand shaken and sonicated for 15 minutes, then vigorously shaken by hand again and allowed to settle. The hexane/acetone extractate was analyzed in triplicate for PCE, TCE, and cDCE. Duplicate soil samples of each 15 cm segment were dried and analyzed for VS, Mg, and TOC (solid and liquid). Dried soil was digested in 2 N HCl for TOC (liquid) and Mg analysis (ICP).

The alkalinity of the soil samples was determined by acidifying dried soil samples below pH 2 with 2N HCl, equilibrating the soils for typically one week or until stable pH was obtained, and then back titrating to pH 4.5 with 0.1 and/or 1N NaOH.

A variety of extraction methods were investigated to identify a method that was effective in extracting PCE and EVO from sediment. Preliminary work indicated that a combination of 1:1 v/v hexane and acetone (a miscible organic solvent) was most effective in liberating PCE-oil from soil in controlled tests using carbon tetrachloride as an internal standard. Ratios of PCE extractable from various concentrations of emulsions in soil with PCE were used to determine recoveries. Hexane extractable materials were measured gravimetrically to determine organic carbon residual imparted by emulsions. Controlled experiments demonstrated a 62% recovery for EOS and 91% for MH5 of partitioning hydrophobic substances (oil, glycerin, polysorbate, and glycerol monooleate; extractability of hydrophobic components were determined separately to define total recoveries). In controlled experiments PCE recoveries were 88% recovery for EOS and 99% for MH5; however column sediment samples recoveries were much lower than expected (<5%).

3.1.1 Analytical Chemistry

Headspace analysis of methane, ethene, ethane, chloroethene, VC, and chloroethane was performed with a Teledyne Tekmar 7000 headspace autosampler and Agilent Technologies 7890A GC with a flame ionization detector (FID) and J&W Scientific GS-Pro column. PCE, TCE and DCE isomers (1,1-dichloroethene, trans-1,2-dichloroethene, and cis-1,2-dichloroethene) were measured with a Teledyne Tekmar AQUAtek 70 vial autosampler, Teledyne Tekmar Stratum purge and trap system with concentrator, and a Shimadzu GC-2014 with FID (column: J&W Scientific DB-VRX). Anions (lactate, acetate, butyrate, propionate, chloride, nitrate, nitrite, bromide and sulfate) were analyzed by ion chromatography with a

Dionex ICS 2500 using a hydroxide mobile phase (column: Dionex IonPac AS11-HC). PCE/oil extractions for chlorinateds analysis were performed by direct injection with the Shimadzu GC with ECD (column: Agilent DB-VRX). Bulk gas analysis (carbon dioxide, oxygen, methane, and nitrogen) of SDC-9 culture was performed on an SRI 8610C GC with TCD. TOC and IC liquid samples were performed on a Shimadzu TOC-5000 with autosampler. The total carbon content of solid samples and ICP-AES for Mg analysis were performed at NCSU EATS (NC State University Department of Soil Science, 3319 Williams Hall, Campus Box 7619, Raleigh, NC 27695).

3.1.2 Culture maintenance

Bioaugmentation culture added to long-term biodegradation experiments was SDC-9 provided by Shaw Environmental Inc., containing a consortium of methanogenic, sulfate-reducing, and dehalogenating bacteria including strains of *Dehalococcoides* (DHC) and maintained as recommended by Vainberg et al. (2009). The bioaugmentation culture was sampled periodically for CVOCs, gas composition, anions, OD₅₅₀, and pH to monitor culture stability, dechlorination rates, and bacterial growth. The culture was maintained in serum bottles with rubber butyl stoppers and aluminum crimps initially and transferred to large laboratory grade glass carboys fitted with rubber butyl stoppers each having two ports fitted with air tight valves and sealed with silicon to maintain anaerobic conditions. The maintained culture was capable of complete dechlorination of PCE to ethene (data not shown).

3.1.3 Materials

PCE, TCE, and DCE isomers standards were purchased from Sigma-Aldrich, United States distributors. Gas standards (methane, ethene, ethane, chloroethene, VC, chloroethane, oxygen, and nitrogen) were purchased from Air Gas, Raleigh, NC. Emulsified oil substrate, EOS™, provided by EOS Remediation consisted of 60% vegetable/soybean oil, 4% soluble substrate, 2% yeast extract, 10% emulsifiers and additives, and 24% water by weight. MH amended emulsified oil substrate suspension (MH5, this study) consisted of 50% vegetable/soybean oil, 4% soluble substrate, 7% emulsifiers and additives, 5% magnesium hydroxide and 34% water by weight. Sand was obtained from Goodwin Sand and Gravel Inc., Raleigh NC. Groundwater (pH 8.2) was obtained from local well in Raleigh, NC.

Chapter 4: Results

4.1 Short-term Transport Experiments

Transport experiments were conducted to determine if the new EVO-MH formulation (MH5) could be effectively transported in washed masonry sand ($D_{50} = 435 \mu\text{m}$, 0.2 % finer than 200 sieve) and if flushing traditional EOS or MH5 through the DNAPL zone would redistribute a portion of the DNAPL.

4.1.1 PCE and MH Transport

Monitoring of the column effluent and analysis of the sediment in the columns demonstrated that both EOS and MH5 could be transported at least 1.5 m in the washed masonry sand columns. Amounts of PCE and Mg (correlating to MH) in the influent and effluent of each column are presented in Table 1. 32% of the Mg in the MH5 was discharged in the column effluent demonstrating MH could be effectively transported at least 1.5 m. Substantial amounts of PCE were discharged in the MH5 and EOS column effluents indicating that emulsion injection did transport a portion of the PCE through the column, improving contact with oil downstream of the original DNAPL location.

Table 1- Mg and PCE mass balance

Column	MH5		EOS	
	grams	%	Grams	%
Mg in Influent	0.410	100%	NA	NA
Mg in Effluent	0.133	32%	NA	NA
Mg Retained by Column	0.277	68%	NA	NA
PCE added to Column	1.6	100%	1.6	100%
PCE in Effluent	1.06	65%	1.04	64%
PCE Retained by Column	1.42	35%	0.56	36%

NA – Not Applicable

Figure 6 shows the pH of the EOS and MH5 column effluents. In the EOS column, the effluent pH dropped to near 4 when the emulsion was being flushed out of the column due to the lactic acid present in the concentrated EOS. However, when water was flushed through the column, the pH recovered to over 7. In the MH5 column, the pH increased to near 9 during emulsion breakthrough, then dropped to about 8 during water flushing.

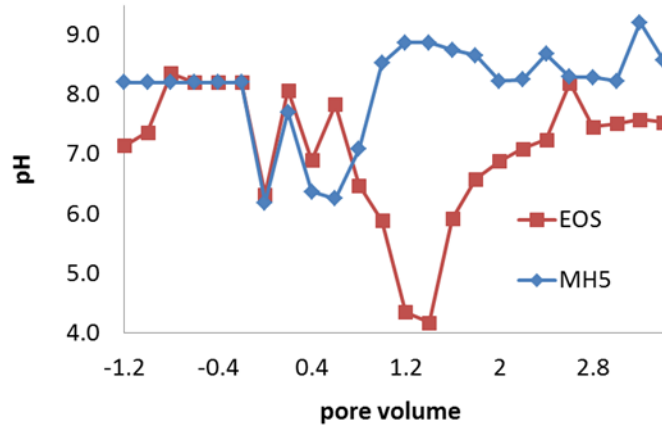


Figure 6-Transport experiment pH; columns MH5 and EOS in washed masonry sand.

Figure 7 shows the alkalinity and pH distribution in the column sediment after the end of the transport experiment. Throughout the entire column length, the sediment alkalinity of the MH5 column sediment was significantly higher than the EOS sediment, indicating the MH was distributed throughout the column.

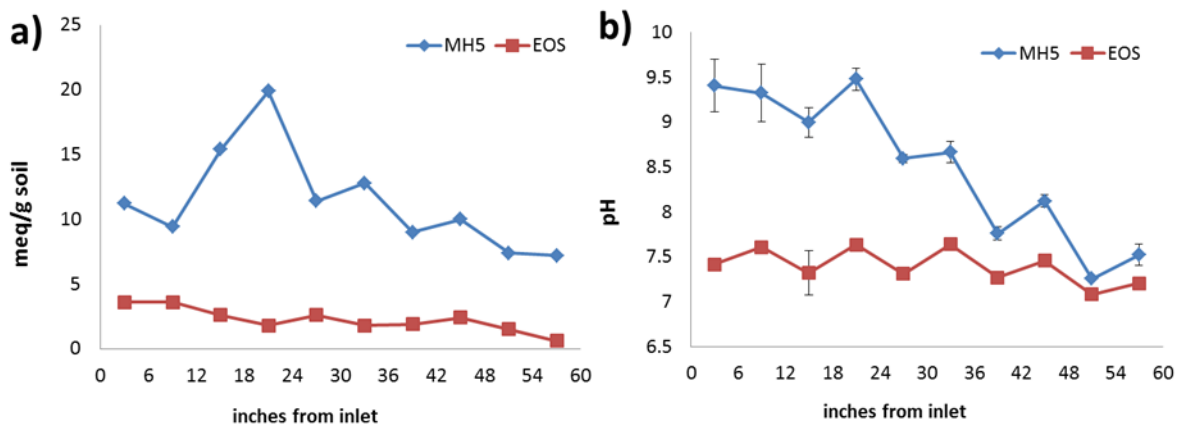


Figure 7-Transport Experiment: (a) Soil alkalinity; and (b) Soil pH, through the length of columns EOS and MH5.

4.1.1.1 PCE Transport

Figure 8 shows PCE concentrations in the effluent of the EOS and MH5 columns. PCE concentrations reached a maximum of near 50,000 μM or approximately 40 times PCE aqueous equilibrium solubility in water (1200 μM). The maximum PCE concentrations were observed at roughly 1 PV after emulsion injection when emulsion and sediments (brown/orange suspended solids) were visually observed in the effluent. Overall, 64% of the PCE DNAPL added to EOS column was discharged in the column effluent and 65% of the PCE was discharged in the MH5 column effluent.

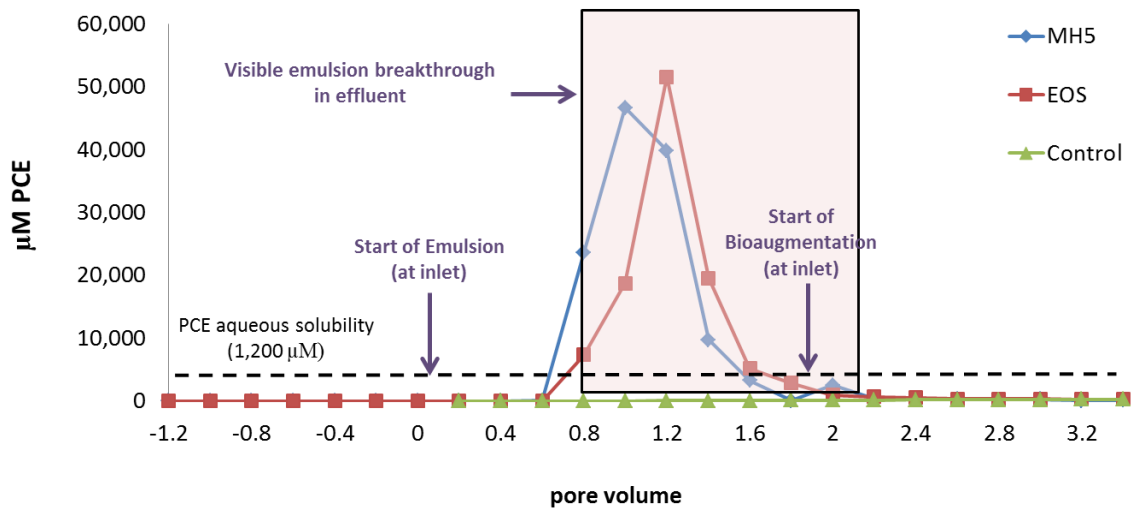


Figure 8-Transport of PCE corresponding with emulsion breakthrough in Transport Experiment columns EOS and MH5.

These transport experiments demonstrated that both EOS and MH5 emulsions could be transported through 1.5 m columns packed with masonry sand. Injection of MH5 was

effective in increasing the sediment alkalinity and pH throughout the columns. Injection of both EOS and MH5 was effective in redistributing a portion of the PCE DNAPL. This redistribution should bring the PCE into close contact with oil and MH throughout the column.

4.2 Long-term Bioremediation Experiments

Long-term biodegradation experiments were conducted to determine if the EVO-MH (MH5) would neutralize acid produced during anaerobic bioremediation, enhancing removal of a PCE DNAPL relative to EVO (EOS) only. The MH5 column was packed with a fine clayey sand ($D_{50} = 542 \mu\text{m}$, 12% finer than 200 sieve). The EOS and a parallel control column without added substrate were packed with sand from a different batch with lower silt-clay content ($D_{50} = 585 \mu\text{m}$, 3.7 % finer than 200 sieve). Sand used to pack MH5 was consumed and different sand was obtained to pack EOS and control. MH5 and EOS columns were operated for 60 weeks and the control column was operated for 45 weeks.

4.2.1 Phase 1 – Treatment of Columns with EVO/EVO-MH

4.2.1.1 PCE, TOC, and MH Transport

Table 2 presents a summary of materials added to and discharged from the bioremediation columns during the first phase of the experiment. The amount of MH discharged in MH5 column effluent was substantially more than in the previous transport experiment. The mechanism responsible for this is unknown. The amount of PCE in both column effluents was also lower, presumably due to the more limited oil transport.

Table 2-Phase 1 Mass Balance Summary

Column	MH5		EOS	
	grams	%	Grams	%
TOC in Influent	15	100%	15	100%
TOC in Effluent	4.5	30%	2.7	18%
TOC Retained by Column	10.5	70%	12.3	82%
Mg in Influent	0.410	100%	NA	NA
Mg in Effluent	0.224	55%	NA	NA
Mg Retained by Column	0.186	45%	NA	NA
PCE added to Column	1.6	100%	1.6	100%
PCE in Effluent	0.18	9%	0.28	17.5%
PCE Retained by Column	1.42	89%	1.32	82.5%

Figure 9 shows the pH of the EOS and MH5 column effluents. Overall results were similar to the previous transport column experiments.

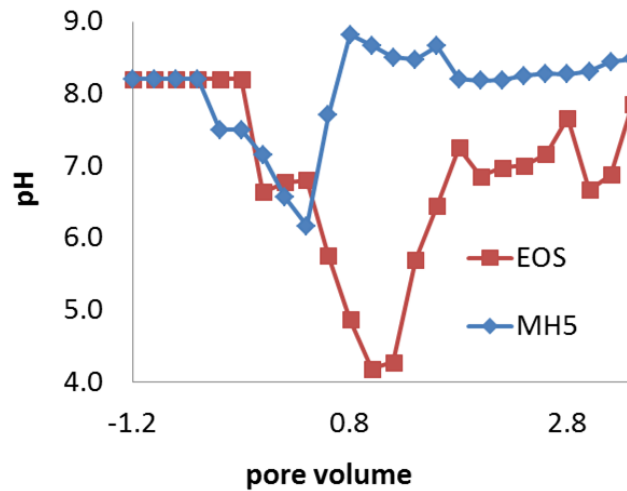


Figure 9-Bioremediation Experiment column effluent pH in EOS and MH5 during Phase 1.

As seen in Figure 10, the overall pattern of PCE transported was similar to the previous transport experiments (Figure 6). PCE concentrations reached a maximum when the emulsion front discharged in the column effluent. However, the maximum PCE concentrations in the effluent of MH5 were significantly lower, presumably due to the reduced oil transport and higher fines content. It is not known why more PCE was discharged from the EOS column since the oil discharged from this column was lower than for the MH5 column.

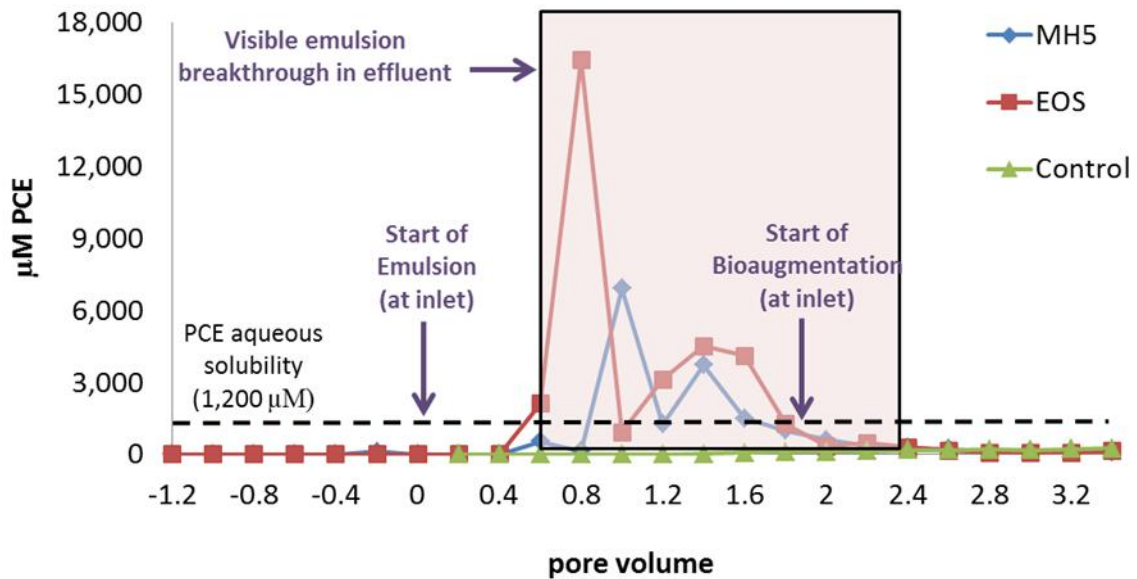


Figure 10-Transport of PCE corresponding with emulsion breakthrough in Bioremediation Experiment columns EOS and MH5 during Phase 1.

Both MH5 and EOS were effectively transported through the columns, enhancing PCE transport.

4.2.2 Phase 2 – Bioremediation Monitoring

4.2.2.1 EOS and Control Columns

Figures 11 shows ethene concentrations versus time in the EOS treated column (Figure 11a), ethene concentration vs time in the control column (Figure 11b) and pH vs time in both columns (Figure 11c). Shortly after startup and bioaugmentation, large amounts of cDCE were being produced from PCE dechlorination which resulted in a decline in pH to below 6. The low pH appears to have inhibited PCE dechlorination, slowing cDCE production and

allowing pH to recover somewhat. In the control column (which received no substrate or bioaugmentation addition), there was some limited dechlorination of PCE to cDCE reaching a maximum of over 250 μM cDCE produced. The smaller amount of cDCE produced is presumably due to the limited amount of bioavailable carbon in the sediment used to pack the column. There is evidence of a pH decline associated with cDCE production, starting around day 250. However, the observed pH decline was more limited, presumably due to the more limited cDCE production.

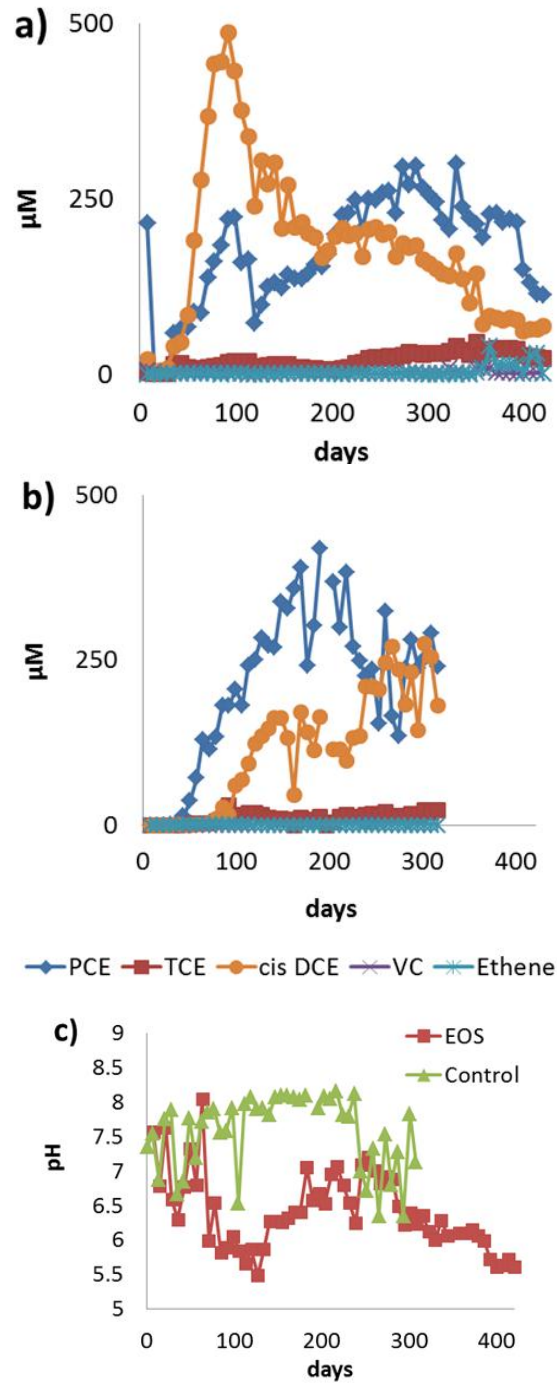


Figure 11-Bioremediation experiments: (a) EOS; and (b) Control, columns CVOC's; (c) pH of EOS and Control, during Phase 2

Figures 12 to 14 show the variation in TOC, methane, VFAs and non-VFA organic carbon with time in the EOS and Control columns. Total VFA organic carbon was calculated based on the measured concentrations of acetate, propionate, butyrate and lactate. Non-VFA organic carbon was calculated as the difference between the measured TOC and the Total VFA organic carbon. Large amounts of TOC were initially released from the EOS column, then TOC concentrations declined to around 100 mg/L after approximately 75 days. The high initial TOC concentrations were primarily non-VFA TOC, presumably glycerol or other higher molecular weight compounds. After 75 days, most of the TOC was associated with acetate, with smaller amounts of butyrate (<5 -17 mg/L). Large amounts of dissolved methane were produced in the EOS column with some gas bubbles released, contradicting previous reports that methanogenesis will be inhibited by high PCE levels (Yang and McCarthy, 2000). The control column produced little TOC, methane, or VFAs.

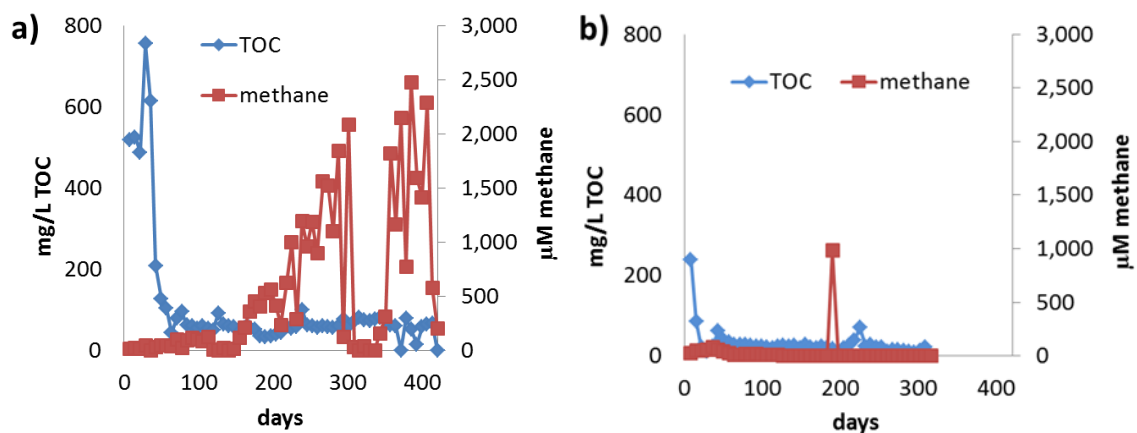


Figure 12-Bioremediation Experiments: (a) EOS; and (b) Control, TOC and methane during Phase 2.

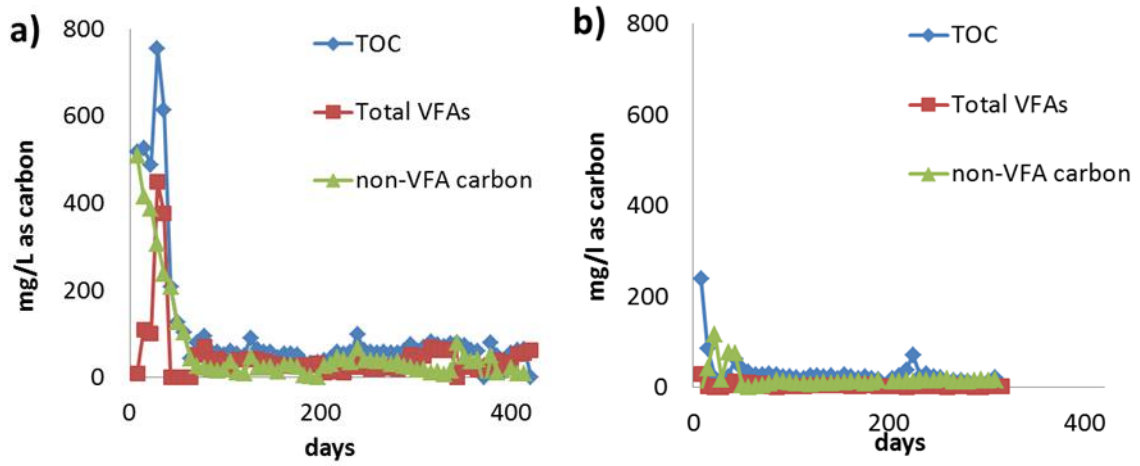


Figure 13- Bioremediation Experiments: (a) EOS; and (b) Control, TOC fractions during Phase 2.

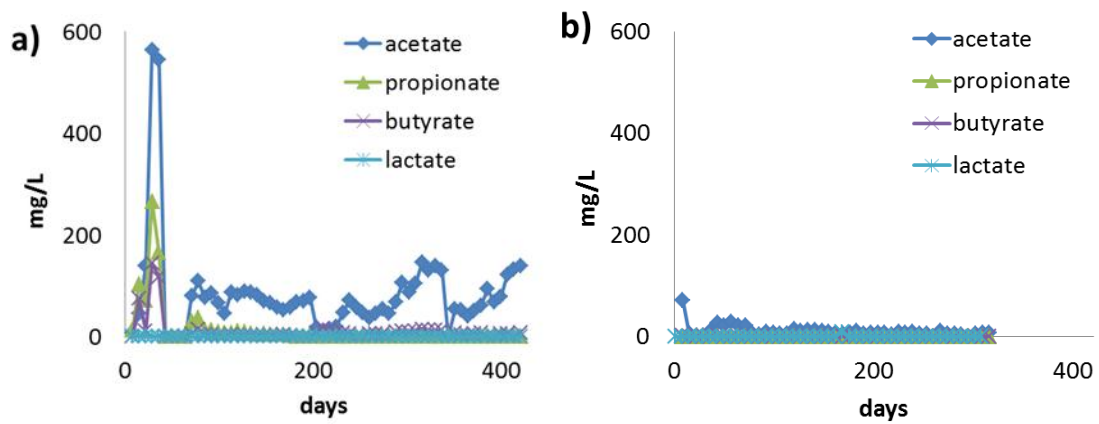


Figure 14-Bioremediation Experiments: (a) EOS; and (b) Control, VFAs during Phase 2.

4.2.2.2 MH5 column

Figure 15 shows ethene concentrations and pH versus time in the MH5 treated column.

Shortly after startup and bioaugmentation, large amounts of cDCE were being produced from PCE dechlorination. However in contrast to the EOS column, pH remained stable at 7 – 8 for about 275 days. By the end of the experiment, cDCE production increased to a maximum of 1,350 μM which is greater than the aqueous equilibrium solubility of PCE (1200 μM) indicating bioenhanced dissolution. pH declined to ~6 after 275 days, indicating MH dissolution was not keeping pace with the large amount of HCl produced by PCE dechlorination. By the end of the experiment, only 6% of the Mg injected into the column was still present. 55% of the injected Mg had been flushed through the column during the injection (Phase 1) and 40% had been discharged in the column effluent during active bioremediation (Phase 2).

VC and ethene concentrations remained low throughout the entire experiment, consistent with prior studies of DNAPL bioremediation (Adamson et al., 2004, McCarthy et al., 2007).

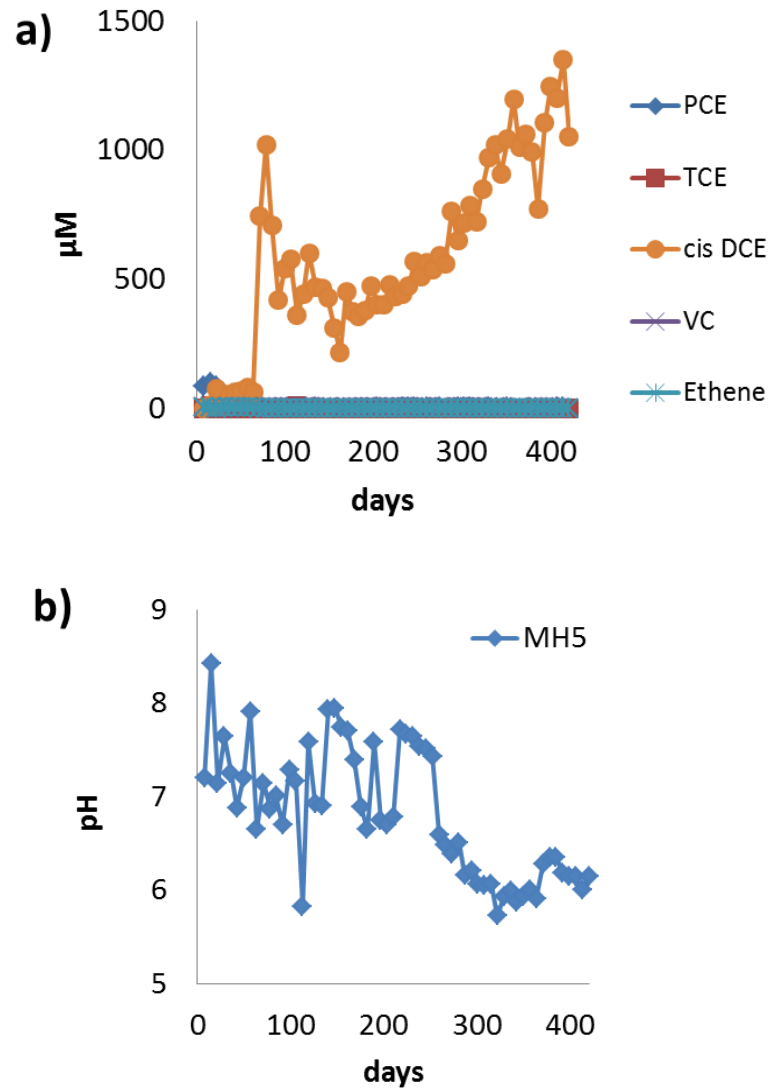


Figure 15-Bioremediation Experiments: (a) CVOC's and; (b) pH, in MH5 column over Phase 2.

Figure 16 show the variation in TOC, methane, VFAs and non-VFA organic carbon with time in the MH5 column. TOC and VFA behavior was similar to the EOS column with a large initial spike of non-VFA TOC, followed by a steady release of acetate with smaller

amounts of butyrate. However, methane production in the MH5 column was significantly lower than in the EOS column, presumably due to increased competition for H₂ and/or acetate by dechlorinators.

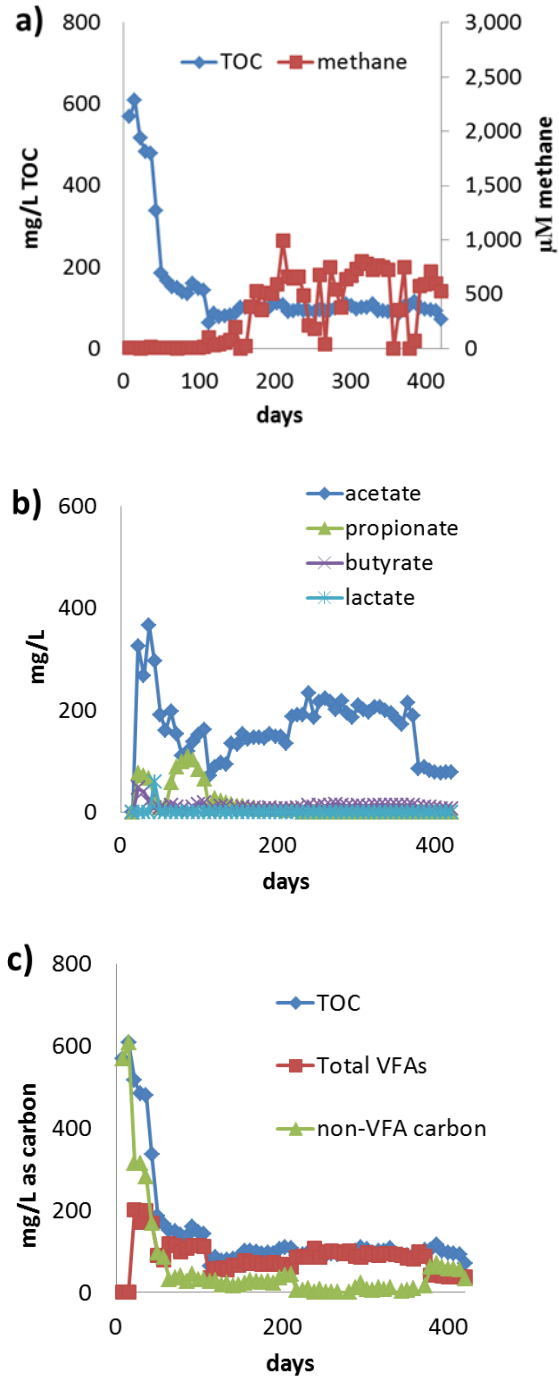


Figure 16-Bioremediation experiments, MH5: (a) TOC and methane; and (b) VFAs; (c)

TOC fractions, during Phase 2

4.2.3 Phase 3- Sediment Analyses

At the completion of the biological monitoring period (Phase 2), columns were frozen, cut into 15 cm sections, homogenized, and analyzed for TOC, VS, pH, alkalinity, Mg, PCE, TCE, and cDCE to determine the spatial distribution of these materials in the column. VS and alkalinity in soil are presented for both MH5 and EOS in Figure 18.

Soil samples were extracted using 1:1 v/v hexane and acetone to measure CVOCs. However, mass balance results were poor (less than 5% of the expected CVOCs were recovered).

Therefore the data are presented as a ratio of cDCE to PCE. Preliminary studies to develop the hexane/acetone extraction procedure showed that when the soil contained vegetable oil, CVOC recovery was often low. However, the ratio of different CVOCs recovered was consistent with the amount added to the soil.

As shown in Figure 17, the ratio of cDCE to PCE in the MH5 sediment varied over the length of the column. Near the column inlet where the DNAPL was injected, PCE was the primary CVOC. Farther from the inlet, the cDCE concentration is one to three orders of magnitude greater than the PCE concentration indicating very extensive dechlorination. In most of the EOS column, PCE concentrations were more variable and the ratio of cDCE to PCE was much lower, indicating limited dechlorination. However towards the end of the EOS column, cDCE increases relative to PCE indicating some dechlorination has occurred.

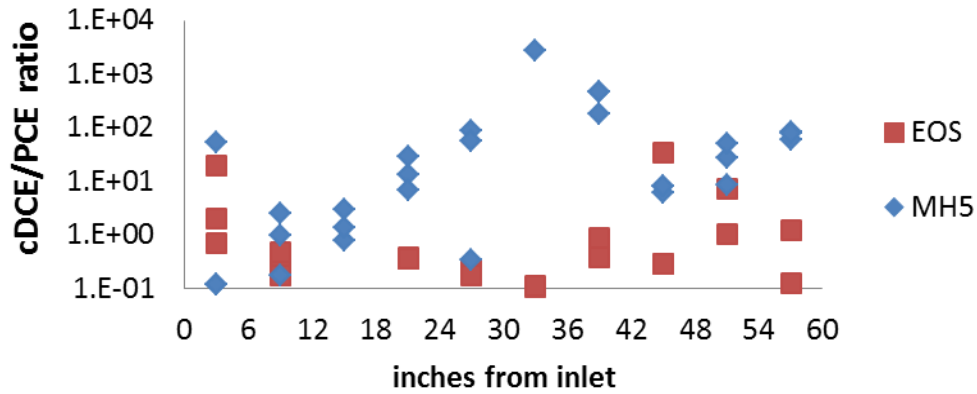


Figure 17- Bioremediation Experiments cDCE to PCE ratio in triplicate sediment sections of MH5 and EOS columns in Phase 3.

Figure 18 shows the variation in pH and alkalinity in the EOS and MH5 columns after the end of the bioremediation experiment. In the EOS column, alkalinity was near zero throughout the entire column. pH was near 7 in most of the column, but dropped to below 6 at the effluent end where the ratio of cDCE to PCE in the sediment increased. In the MH5 column, pH was near 6 throughout most of the column, while alkalinity was very low near the column inlet where the DNAPL was injected and increased over the length of the column. The higher alkalinity at the end of the column suggests that some buffering capacity was still present. However, this buffer was not sufficient to increase the pH above 6 for optimum biodegradation.

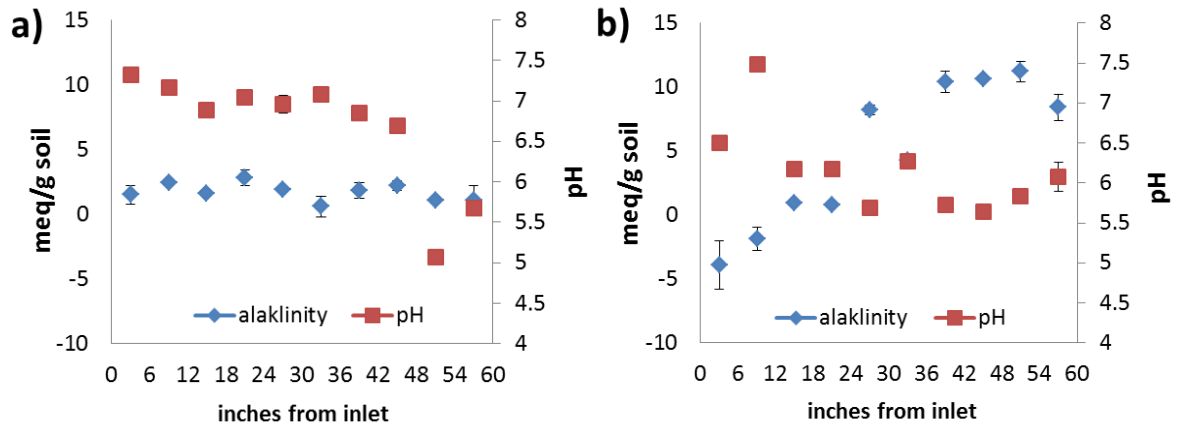


Figure 18-Bioremediation Experiments: (a) EOS; and (b) MH5, sediment sections pH and alkalinity in Phase 3.

Chapter 5: Discussion

Injection of MH5 and a bioaugmentation culture was effective in enhancing bioremediation of a pure PCE DNAPL bioremediation. Over 99% of the ethenes released from the MH5 column were cDCE indicating extensive dechlorination of PCE. At the end of the experiment, cDCE concentrations were 10 to 1000 times the PCE concentration in much of the column. MH5 injection was also effective in distributing MH throughout the column, maintaining near neutral pH until most of the MH had been depleted.

5.1 Bio-Enhanced Dissolution

One of the major reported benefits of DNAPL bioremediation is the potential for increasing dissolution of the DNAPL, reducing source zone longevity. However, injection of MH5 or EOS could potentially reduce PCE flushing, since the hydrophobic PCE is expected to partition into the oil, reducing the effective solubility of the PCE.

Figures 19a and 19b show the total ethene (TE) concentration (sum of PCE, TCE, DCE, VC and ethene) and the cumulative moles of TE released versus time in the effluent of the MH5, EOS and control columns. In the control column, TE began to increase at one month after the start of operation (~ 1 pore volume (PV) of fluid injected), reaching a steady-state concentration of 400-500 μM at 125-150 days, then remaining constant until termination of these columns after 308 days of operation. In both the MH5 and EOS columns, TE began to increase after 1 PV of fluid injected (30 days), reaching maximum concentrations of 700-1000 μM TE, associated with high cDCE concentrations. From 100 to 308 days, TE

concentrations in all three columns were very similar, even though conversion PCE to cDCE was much more extensive in the MH5 column. At 300 days, the TE concentration in the EOS column effluent began to decline, presumably due to reduced surface area of the PCE DNAPL. In contrast, cDCE production increased dramatically in MH5 after 300 days, increasing TE removal. The average TE concentration during the last 100 days of operation was 1060 μM or 2.9 times the average in the EOS effluent (364 μM).

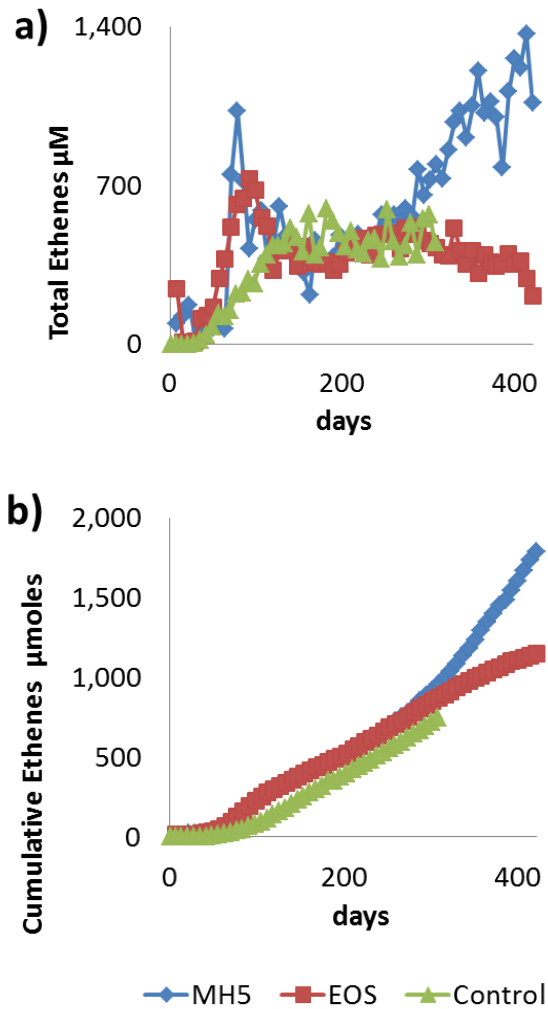


Figure 19-Total ethenes vs time during Bioremediation Experiments, Phase 2 in MH5, EOS and control columns: a) total ethene concentration; and b) cumulative mass of ethenes released.

Table 3 presents a mass balance analysis of ethenes released from the three columns during the bioremediation phase. PCE present in the column at the start of the bioremediation phase

(from Table 2) was slightly lower in the EOS and MH5 columns due to flushing of some material out of the column during the injection phase.

Table 3- Phase 2 Bioremediation mass balance presented ethenes released, aqueous Cl is compared to cDCE produced (main degradation product)

(units mM)	Control	EOS	MH5
PCE Present at start of Bioremediation Phase	9759	8052	8896
Recovered during Bioremediation phase			
PCE in effluent	466	531	20
TCE in effluent	24	57	2
DCE in effluent	251	543	1757
VC in effluent	1	5	4
Ethene in effluent	1	8	2
Total ethenes recovered	743	1,144	1,785
Effluent TE as % of starting PCE	7.6%	14.2%	20%

Note: Control column operated for 308 days compared to 421 days of operation for EOS and MH5 columns.

Active biodegradation in the MH5 column resulted in more rapid TE removal (20%) compared to 14.2% TE removal in the EOS column. There is no evidence that addition of emulsified oil present in MH5 or EOS slowed TE removal from the columns. When the MH5 and EOS columns were terminated after 421 days operation, the MH5 column had

released 1.54 times as much TE as the EOS column. It appears that the MH5 treatment would have increased flushing compared to the control column. However, this cannot be confirmed due to the shorter operating period for the control column.

5.2 Chlorine Number (Cl#) and pH

One approach for evaluating the extent of dechlorination is to calculate the chlorine number (Cl#) of a water sample where Cl# is calculated as:

$$Cl \# = \frac{4 * [PCE] + 3 * [TCE] + 2 * [cDCE] + 1 * [VC]}{[total \ ethenes]}$$

and ethene concentrations are in μM . PCE has a Cl# of 4, cDCE has a Cl# of 2, while a 50:50 mixture of PCE and cDCE would have a Cl# of 3.

Figure 20 shows the relationship between effluent pH and Cl# in the control, EOS, and MH5 columns. In the control column, the Cl# rapidly increases to near 4 as PCE breaks through into the column effluent, then slowly declines towards 3 as naturally occurring organic carbon in the sand is used to reduce a portion of the PCE. For the first 200 days, pH remains near 8 in the control column indicating the natural buffering capacity is sufficient to neutralize the small amount of HCl produced. However, when the Cl# reaches 3, the pH drops to 7 indicating the natural buffering capacity has been reached.

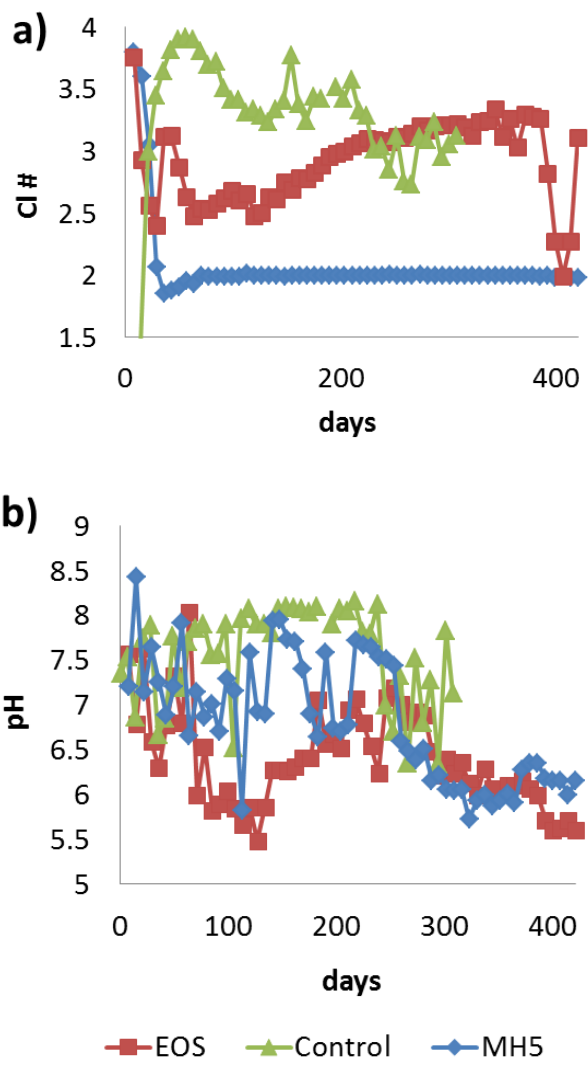


Figure 20- Bioremediation Experiment: (a) pH; and (b) Cl#, in EOS, Control, and MH5 columns during Phase 2.

In the EOS column, cDCE production is much more rapid causing a rapid drop in Cl# followed by a drop in pH to 6 and below. At approximately 125 days, the Cl# in the EOS column effluent begins to increase as PCE conversion to cDCE slows, presumably due to toxic inhibition by both low pH and high PCE. From 150 to 250 days, the Cl# continues to rise,

even though the pH has risen above 6, due to the reduced cDCE production. During this period, PCE concentrations vary from 220 to 300 M, which is approaching the 400 to 800 μ M PCE range reported to inhibit dechlorination in buffered media (Yu and Semprini 2004, Duhamel et al. 2002, and Amos et al. 2007). Around day 350, there was a sharp drop in Cl# associated with a drop in PCE concentration and increase in VC and ethene, which caused the pH to drop below 6. The cause of this rapid increase in PCE degradation is not known, but could be related to the gradual decline in PCE concentrations and associated toxicity.

In the MH5 column, cDCE production began immediately causing the Cl# to drop to 2 as PCE was converted to cDCE. PCE was typically below detection in the MH5 column after day 75. During the initial increase in cDCE the pH of the MH5 column effluent was mostly between 7 and 8, presumably due to the buffering capacity provided by MH. cDCE production increased throughout the remainder of the experiment, with a moderate pH. However around day 275, the pH dropped to ~6. This drop in pH did not inhibit further increase in cDCE production, as seen from the steady Cl# that remains 2 for the entire experiment. Eaddy (2008) noted that in batch studies, a pH of 6 (without excess PCE) was not inhibitory to cDCE production. However at pH 6, dechlorination stalled at VC. In MH5 column, 99% of the aqueous PCE released was converted to cDCE, unlike the EOS column where the low pH and moderate aqueous PCE concentrations appeared to inhibited dechlorination until PCE levels declined.

5.3 Cl and Mg ratio in MH5

Addition of MH in the MH5 emulsion was effective in maintaining a near neutral pH for the first 250 days of operation. However towards the end of the experiment, the pH dropped to near 6 indicating the added MH was not sufficient to neutralize the acid produced. When planning the experiment, sufficient MH was injected to neutralize all the HCl produced from dechlorination of the PCE DNAPL to cDCE with a small amount of MH available to neutralize VFAs and carbonic acid produced by substrate fermentation. However, 55% of the Mg added to the column was discharged in the effluent immediately after injection, so the total amount of MH available for buffering at the start of Phase 2 was significantly lower than planned.

Figure 21 shows the variation in the cDCE and the Cl/Mg ratio in the MH5 column effluent overtime. If MH ($\text{Mg}(\text{OH})_2$) is only used to neutralize HCl produced from dechlorination of PCE to cDCE, then 2 moles of Cl should be released for every mole of Mg. However if MH is also used to neutralize VFAs and carbonic acid, then Cl/Mg ratio will be less than 2.

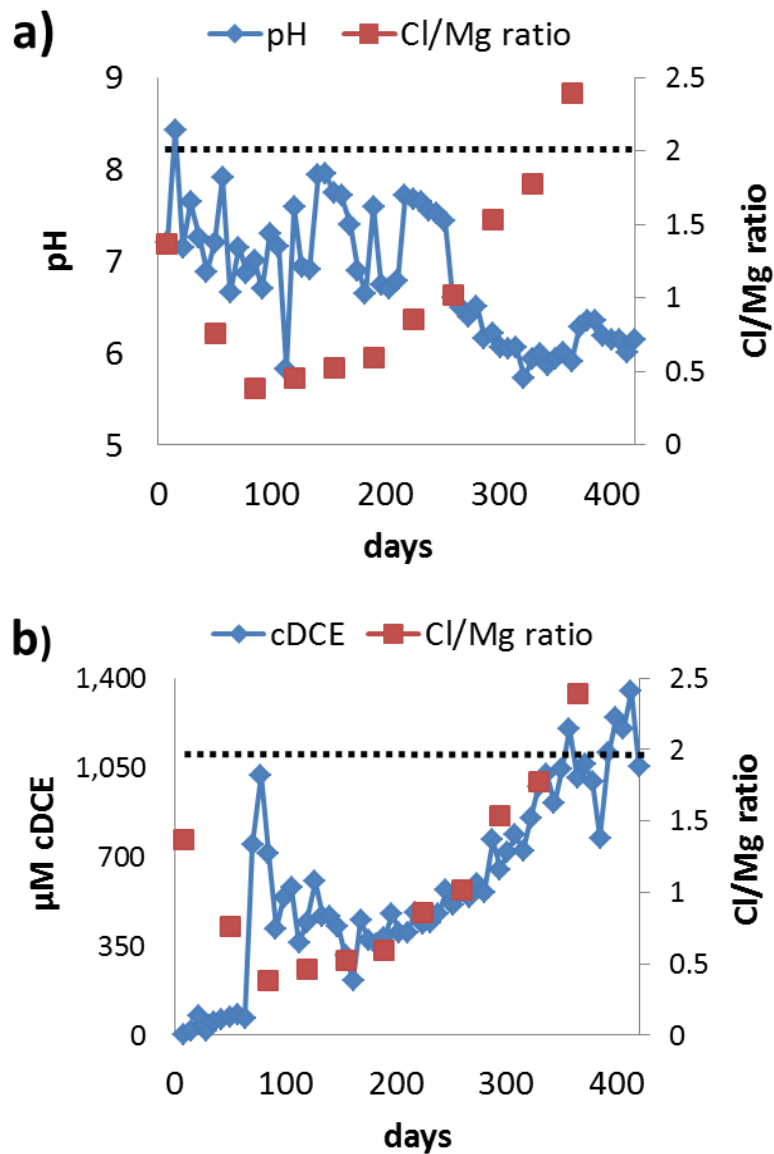


Figure 21-cDCE, pH and Cl/Mg ratio vs time in MH5 column during phase 2.

For most of the experiment, the Cl/Mg ratio is below 2 indicating sufficient MH is available to neutralize all HCl produced with an excess to neutralize other acids and maintain a neutral pH. However, when cDCE production begins to increase at 250 days, the Cl/Mg ratio begins to increase, followed by a sharp drop in pH. By the end of the experiment, the Cl/Mg ratio

exceeded 2 indicating there is not sufficient MH to neutralize the HCl produced and none available to neutralize VFAs and carbonic acid.

5.4 Measured versus Theoretical Chloride Production

Analysis of the sediment samples indicated that a substantial portion of the chlorinated ethenes remaining in the MH5 column at the completion of the experiment had been reduced to DCE. However, it is not possible to estimate the total PCE degraded due to the poor extraction efficiency of the sediment analyses. To provide some measure of the amount of PCE potentially degraded, the measured chloride concentration in the effluent of the MH5 and EOS columns was compared to a theoretical estimate of chloride produced where

$$\text{Calculated Cl} = (4\text{-Cl\#}) * \text{TE}$$

The value (4-Cl#) is the moles of Cl released per mole of total ethene (TE) released.

Figure 22 presents a comparison of measured and calculated Cl concentrations versus time in the MH5 and EOS column effluents. Between 30 and 75 days, there is a large spike in measured Cl associated with chloride present in the bioaugmentation culture. However, once this Cl flushes out, the measured and calculated chloride concentrations match reasonably well until the onset of rapid dechlorination in the MH5 column (Around 250 days) and EOS column around 350 days. After the onset of rapid dechlorination, measured Cl in the effluent exceeds calculated Cl, indicating and more DCE + VC + ethene is being produced than is measured in the column effluent.

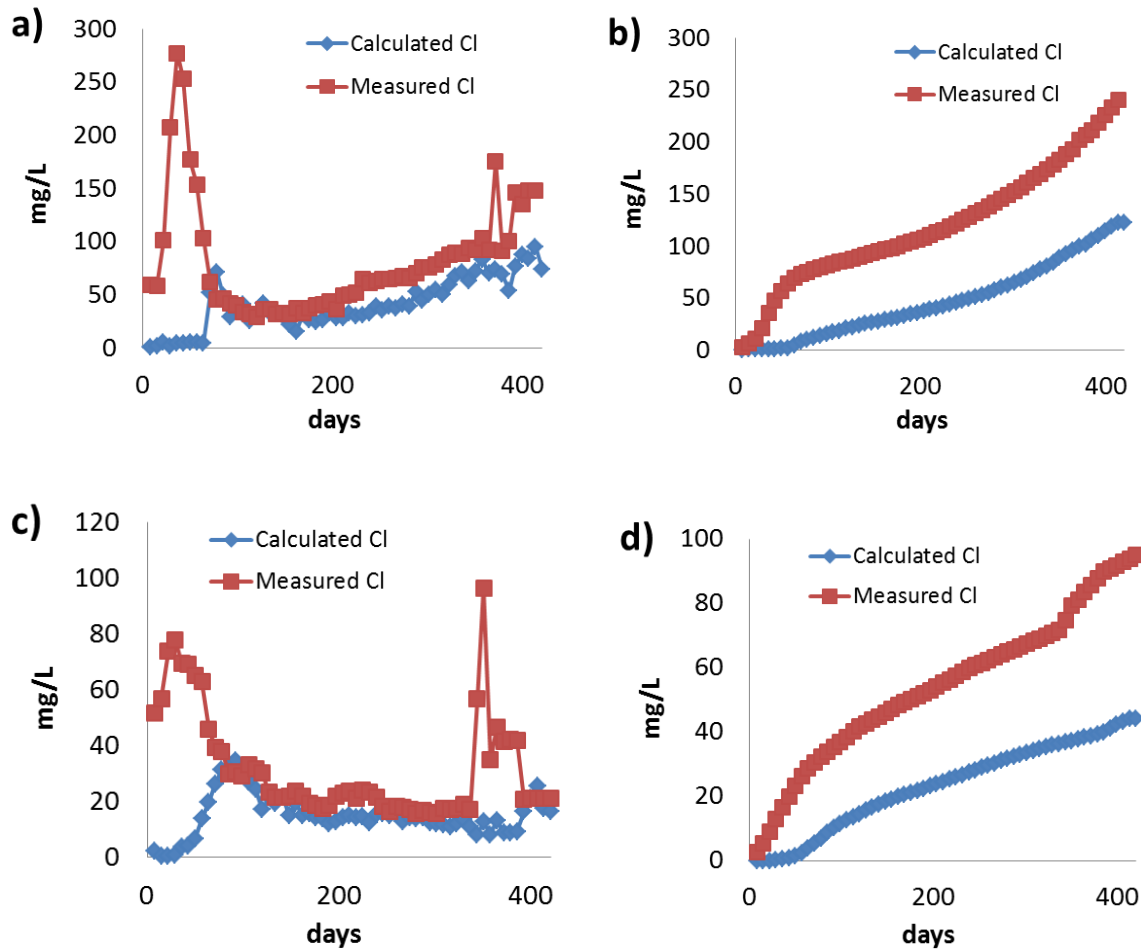


Figure 22-Comparison of measured and calculated Cl concentrations in column

effluents during the bioremediation experiment (Phase 2) (a) Cl concentration in MH5 column; (b) cumulative Cl released from MH5 column, (c) Cl concentration in EOS column, and (d) cumulative Cl released from EOS column.

Chapter 6: Conclusions

6.1 Transport Column Results

The short term transport experiments demonstrated that both EOS and MH5 emulsions could be transported through 1.5 m columns packed with masonry sand. Injection of MH5 was effective in increasing the sediment alkalinity and pH throughout the columns. Injection of both EOS and MH5 was effective in redistributing a portion of the PCE DNAPL. This redistribution should bring the PCE into close contact with oil and MH throughout the column.

6.2 Bioremediation Column Results

- MH5 injection was effective in distributing MH throughout the column, maintaining near neutral pH until most of the MH had been depleted.
- Injection of MH5 and a bioaugmentation culture was effective in enhancing bioremediation of a pure PCE DNAPL. Over 99% of the ethenes released from the MH5 column during Phase 2 were cDCE indicating extensive dechlorination of PCE.
- Addition of MH5 resulted in more rapid removal of total ethenes than EOS addition. There is no evidence that addition of emulsified oil present the MH5 or EOS slowed TE removal from the columns. It appears that the MH5 treatment would have increased flushing compared to the control column. However, this cannot be confirmed due to the shorter operating period for the control column.
- MH was depleted in the MH5 column more rapidly than expected, allowing the pH to drop to ~6. The more rapid depletion of the MH is believed to be due to rapid transport

of MH through the column during the initial injection and neutralization of VFAs and carbonic acid produced by substrate fermentation.

- The actual extent of PCE dechlorination in the columns was likely greater than would be apparent based only on measurements of ethene concentrations in the column effluent. The high ratio of cDCE to PCE in the sediment samples from the MH5 column indicates that dechlorination products accumulated in the column. This is supported by measurements of dissolved Cl produced.

6.3 Future Work

Further work should examine the mechanisms of enhanced solubility. Both EOS and MH5 increased PCE in the column effluent to several times the aqueous equilibrium solubility. However, the factors controlling this increase in flushing are unknown.

Future work should focus on understanding the mechanisms and factors that allowed MH5 to produce significantly more cDCE as compared to EOS.

Additional work is needed to develop a reliable method for extraction and detection of chlorinateds from soil treated with emulsified oils and to determine the amount of MH to add to systems based on transport, retention, acid production, and closed system kinetics.

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APPENDICES

1. Bioaugmentation Media Preparation

Media stock solutions: Make using DI water and 1L volumetric flask.

A. Buffer Solution (100x) Dissolve in 1 L DI H₂O:

KH ₂ PO ₄	27.0 g
K ₂ HPO ₄	35.0 g

B. Mineral Salts Solution (100x) Dissolve in 1 L DI H₂O:

NH ₄ Cl	53.0 g
CaCl ₂ -2H ₂ O	7.5 g
MgCl ₂ -6H ₂ O	10.0 g
FeCl ₂ -4H ₂ O	2.0 g

C. Trace Metals (1000x) Dissolve in 1 L DI H₂O:

MnCl ₂ -4H ₂ O	5.00 g
H ₃ BO ₃	0.05 g
ZnCl ₂	0.05 g
CuCl ₂	0.03 g
NaMoO ₄ -2H ₂ O	0.01 g
CoCl ₂ -6H ₂ O	0.50 g
NiCl ₂ -6H ₂ O	0.05 g
Na ₂ SeO ₃	0.05 g

Add ~1 mL of 38% HCl to keep metals in suspension

D. Resazurin Solution (1000x) Dissolve 0.01 g Resazurin in 100 mL DI H₂O.

1. Start the gassing station and autoclave all glass wear and DI water at 250 for 15 minutes if it is not directly boiled under nitrogen.
2. Mix solutions of the following with deionized water in a round bottom flask:
 - a. Buffer solution- 1 mL per liter final culture,
 - b. Mineral salt solution- 1mL per liter final culture,
 - c. Trace metals: 0.1mL per liter final media, and
 - d. Renazurin: 0.1mL per L of media
3. Using a snug fitting black rubber stoppers for round bottom flasks, put the gassing needle on the side of the lip of the container and fit the stopper in. Place round bottom flasks in the heaters and turn heat to 10. Boil while sparging with nitrogen and for 15 + minutes once the gassing station is ready. The media will take some time to reach a boil but do not leave the general area while boiling.
4. Cool the media to 28-30°C (room temp) with continuous sparging of nitrogen. Turn the heater off and let the flaks sit for a few minutes. Using an orange hot mit take the

flask out of the heater and place it on a cork bottom. When the glass has cooled to where it can be handled, place in an ice bath and making sure the stopper is snug.

5. Transfer to anaerobic hood and add:
 - a. 1.2 g of NaHCO₃ per liter of media
 - b. 1g of yeast extract
6. Mix thoroughly, and allow media to turn clear before using (reduce fully)

2. Bioaugmentation Culture Maintenance

Sampling schedule:

An SDC-9 culture containing a consortium of methanogenic and sulfate-reducing bacteria including strains of DHC was obtained from Shaw Environmental Inc. and maintained as recommended in Vainberg et al., 2009. Every three days the culture was sampled for chlorinates, gas composition, VFA, ions, and pH to monitor culture stability. OD₅₅₀ was sampled periodically to assess growth and biomass density. PCE is spiked and a pre and post sampling of chlorinateds is performed, followed by a lactate spike with sampling post spike after three days PCE addition, and a monitoring day three days after lactate addition. See “Sampling Procedure” for details of sample collection.

Culture transfer:

When the culture reaches an OD₅₅₀ of 1.3 or higher the culture should be transferred.

Following the “Media Preparation” protocol after step 4 follow below:

1. Transfer media, culture, and supplies into anaerobic hood
2. Add 1.2 g of NaHCO₃ and 1g of yeast extract per liter of media, mixing thoroughly allowing the colour to turn clear (yellowish colour from yeast extract)
3. Anaerobically inoculate the media with 1/5 of the final volume of culture of SDC-9 with an OD₅₅₀~1.3-1.5 or 1X stock culture to get the final OD of media at least 0.3. Concentrated consortium of SDC-9 (10X) also can be used for inoculation accordingly. Typically the amount of media to culture ratio is 200-250 per liter of final solution.
4. Mix thoroughly and note if colour changes, if the media turns blue or pink, allow it to turn clear in anaerobic hood
5. Re-seal carboys with fresh silicon and parafilm wrapping checking that all valves are closed and snug/ re-crimp serum bottles with new stoppers, and transfer culture back to incubator/shaker
6. Perform monitoring sampling

Additional steps for new culture (From Shaw Environmental), to follow step 4 from “Culture Transfer”, and conclude with step 5 from “Culture Transfer”:

1. Add 10 mg/l of PCE anaerobically to the bottle through stopper with a needle. Stock solution 10000 mg/l of PCE in DMF or Methanol can be used for injection.

2. Add Lactic Acid Sodium Salt (SIGMA) (60% w/w) at concentration 12mM to media.

Sampling procedure:

The culture was maintained in serum bottles with rubber butyl stoppers and aluminum crimps initially and transferred to large laboratory grade glass carboys. Carboys were fitted with rubber butyl stoppers with two ports fitted with air tight valves connected to pipetting needles which penetrated the stoppers. The stoppers were sealed with silicon to maintain anaerobic conditions; one port for air sampling/over pressure release, another with tubing and diffuser reaching the liquid for liquid sampling.

1. Remove serum bottles/carboys from incubator-shaker (maintained at 121rpm, 29°C)
2. With the 5 mL glass gas tight syringe remove 4.0 mL of fluid from the serum bottles using a needle to puncture the butyl rubber stopper. For carboys connect lure fitting on 5 mL glass gas tight syringe and open the valve with tubing and diffuser to sample 4.0mL of fluid. Close the valve and unscrew the syringe.
3. Purge and trap samples (store sample at 4°C):
 - a. Add 0.5 mL to a 40mL EPA VOA vial
 - b. Fill the vials to just a convex of DI water forms on top and carefully screw the cap on with the Teflon of the septa down.
 - c. Flip the vial over and check for bubbles
 - d. Analysis measuring 1,1-dichloroethene, trans-1,2-dichloroethene, cis-1,2-dichloroethene, trichloroethene, and tetrachloroethene was performed with a Teledyne Tekmar AQUAtek 70 vial autosampler, Teledyne Tekmar Stratum purge and trap system with concentrator, and a Shimadzu GC-2014 with FID (column: J&W Scientific DB-VRX).
4. Headspace samples (store sample at 4°C):
 - a. Add 2.5 mL to a 20mL vials fitted for a metal crimp cap and septa
 - b. Quickly cap and crimp with the Teflon of the septa down
 - c. Analysis of methane, ethene, ethane, chloroethene, vinyl chloride, and chloroethane was performed with a Teledyne Tekmar 7000 headspace autosampler and Agilent Technologies 7890A GC with FID (column: J&W Scientific GS-Pro).
5. IC (store sample at 0°C):
 - a. Using the syringe with the 1 mL of remaining fluid and place a 0.45 micron glass fiber filter the lure fitting of the syringe (multiple filters maybe needed depending on biomass density)
 - b. Dispense filtrate into IC vials
 - c. Conductivity detector measuring lactate, acetate, butyrate, propionate, chloride, nitrate, nitrite, bromide and sulfate was performed with a Dionex ICS 2500 using a hydroxide mobile phase (column: Dionex IonPac AS11-HC).
6. pH :
 - a. Use a few drops on a pH test strip to check if pH is neutral

- b. If pH is below neutral range adjust with NaOH 0.1N as needed by addition via ports, measure pH by withdrawing liquid and using pH meter for accurate pH adjustment

Additional sampling for monitoring sample days:

7. Bulk gas (performed on SRI GC with TCD):
 - a. Follow instructions for turning the instrument on and measuring standards. If standards do not run within parameters bake the column as noted in GC instructions
 - b. Run a test blank to purge line
 - c. Remove 2.5 mL of gas from culture vessel using a needle, three way plastic valve and a 3L plastic syringe. Purge 2.5 mL to flush syringe by turning the three way valve out. Take another 2.5 mL and turn 3 way valve off, take gas that is in syringe and gently, slowly hand inject into the lure lock port on the GC. Press the 'run' (white button). Three seconds later the SRI will make a noise.
 - d. Label the print out, and make sure that all gases were measured and recorded
 - e. Record gas volume sampled
 - f. Analysis was performed on an SRI 8610C GC with TCD.
8. Overpressure:
 - a. In a chemical hood using a 50mL wetted syringe with a wetted plunger (3-4 drops of DI water), attach lure to gas sampling port
 - b. Open the valve and allow gas to push the syringe up (close valve if more pressure than can be measured by a 50mL syringe, record, purge, and re-sample)
 - c. Record total mL of overpressure

Periodic sampling:

9. OD550 (performed in Unicam UV-1 spectrophotometer)
 - a. Using a fixed setting at 550nm zero the instrument
 - b. Fill quartz cuvet with approximately 1mL of sample, and wipe with kim-wipe the clear sides, placing the clear side in light path inside the instrument
 - c. Close the lid and measure

Spiking:

1. Tare a glass syringe with an unsheathed needle on a 100g scale, and fill with desired amount of spiking fluid (sodium lactate or PCE stock standard) as designated by:
 - a. Lactate spike 12mM:
 $1.344\text{g}/1000\text{mL} = x \text{ grams}/\#\text{mL}$ in serum bottle
 - b. PCE stock Std 10mg/L:
 $0.5\text{mL stock}/500\text{mL} = x \text{ grams}/\#\text{mL}$ in serum bottle
2. When spiking screw the syringe into the valve on the carboy reactors that does not have tubing connected to it.

3. Pull reactor liquid out of the valve with tubing that reaches the liquid and flush the valve that was injected in 2-3 times or until sufficiently flushed.
4. Make sure to maintain the anaerobic integrity of the culture and that all valves are closed to the atmosphere when not in use.

When done sampling:

1. Place samples in designated locations
2. Put the culture back on the incubator/shaker
3. Enter all information into spreadsheet
4. Clean all glassware and disinfect counter (EtOH or sponge and soapy water)

3. MH5 Preparation

Protocol written according to preparation of a 500g batch, changes may need to be made for scaling up.

Required Materials:

1. Chemicals (See Table 6)
2. Lab equipment
 - a. Laboratory vortex blender
 - b. 2 blender containers with lids
 - c. Transfer pipettes
 - d. Weight boats/tins
 - e. Spatulas
 - f. Scale (5kg capacity)
 - g. Hot mitts
 - h. PPE -gloves, eye protection, lab coat and lab apron (optional)
1. Pre-weight all solid chemicals; methyl paraben, propyl paraben, and magnesium hydroxide, in pre-labeled weight boats/tins. Set aside.
2. Tare a pyrex beaker and add water, adding 20-30 mL extra than specified, microwave for 2 minutes, or until just boiling. Use hot mitts to transport and pour.
3. Mix 2 → Tare a separate blender container and add the hot water, glycerol, GMO, and polysorbate 20 by weight. Use the transfer pipettes to reach the specified amount. Add the pre-weighted methyl paraben, and propyl paraben. In vortex blender, mix on high for one minute. Set aside.
4. Tare a pyrex beaker and add about 50-70 grams extra partially hydrogenated vegetable oil then specified and microwave for 3-4 minutes until liquid. Use hot mitts to transport and pour.
5. Mix 1 → Place a blender container on the scale and tare. Add appropriate amount liquefied partially hydrogenated vegetable oil by weight to blender container, aim to reach exactly the specified amount (manipulated the weight with the transfer

- pipettes). Place the blender container on the blender on a low setting, very slowly add in the magnesium hydroxide and increase speed as needed. Add magnesium hydroxide in 5-6 parts waiting 1-3 seconds between additions. If needed use a scraper occasionally to scrape any clumped magnesium hydroxide off the sides and back into the mix. Blend this well and set aside.
- Remix mix 2, it should still be hot. Very slowly add the appropriate amount of the mix 1 to the blender with mix 2. Add in 5-6 batches and allow mixing thoroughly giving 3-5 seconds between additions. If needed scrape the sides of the blender top. To ensure complete transfer, pour the final mixture into the mix1 blender and blend to incorporate any of mix 1 that is stuck in the blender. Confirm visual mixing; make sure none of mix 1 is stuck to the sides or at the top. Use scraper if needed. Mix on high for 5 minutes.
 - Allow mixture to cool on low blender setting with the lid off. Once at room temperature (30-45 minutes) store in a labeled container.

Table 4-Percentage of components in MH5

Components:	%
Mix 1	
Partially hydrogenated vegetable oil	50
Mg(OH) ₂	5
Mix 2	
DI water	33.8
glycerol	4
GMO	2.8
polysorbate	4.2
methyl paraben	0.1
propyl paraben	0.1
Total:	100

4. Column Treatment

Used for Short-term Transport Experiments and Long-term Bioremediation Experiments (Phase1). For sampling of headspace, purge and trap, ion chromatography, and total organic carbon see “Sampling Procedure”. Dilutions may vary.

Column preparation

Stage 1: Rapid flush

- a. Rapidly flush column with 1.0 liters of groundwater (peristaltic pump)
- b. Initial parameters cumulative sampling of: volatile solids, headspace, purge and trap, ion chromatography, pH, and total organic carbon- Label { F 1-3, triplicate cumulative samples }

Stage 2: DNAP Injection

- a. Drill small hole using a 1/8th inch drill bit in side of column 6 inch (15 cm) from inlet
- b. Using syringe, inject 1.0 mL of red dyed (Oil-O red) neat PCE into soil, draw slight amounts of water from the inlet to aid PCE in entering column using a syringe connected to the inlet, epoxy, and let dry over applying multiple layers of epoxy as needed
- c. Pump water at 10 mL/d through column for 2 days (syringe pump)
- d. Composite sample from serum bottle: volatile solids, alkalinity, headspace, purge and trap, ion chromatography, and total organic carbon- Label { i }

Note: Maintenance media made as described in “Media Protocol”. Add 5mL SDC-9 culture to consume oxygen (0.5%) if needed. Treatment “4” made as described in “MH5 Preparation”, treatment “3”, EOS, and GW obtained as described in “Materials and Methods: Materials”.

Column Treatment

Stage 3: Treatment addition

- a. Using a syringe pump and gas tight 50mL glass syringe, flush 100 mL of diluted emulsion, MH5- treatment 4, EOS- treatment 3, or GW-treatment 1 (20 mL treatment + 80 mL media, prepared in 2-50mL syringes), collect 40 out of 50 mL per syringe, 10mL to cumulative sampling and 40 to incremental). Syringes prepared in anaerobic hood.
- b. Incremental sampling: prepare 3-2mL GC vial to fill without headspace and crimp (backup sample), sample for headspace, purge and trap, and the remainder of the liquid is placed in a 20mL glass scintillation vial to be purged of volatiles and used for pH. Labeled { T }.
- c. Cumulative samples: Collection of remaining 10mL for every 50mL in stoppered vented serum bottle { T-Ma-C } comprises cumulative volatile solids, total organic carbon, and ion chromatography samples.

Stage 4: Media (media phase A)

- a. 400 mL anaerobic media stored in IV bag (preparation in anaerobic hood, monitor colour of media to check if bag has been compromised) and pumped with peristaltic pump- collect 40 out of 50 mL per syringe, 10mL to cumulative, 40 to incremental as described in “Stage 3:Treatment addition” steps “b.” and “c.”. Record rate of pumping, and monitor for clogging.
- b. Incremental sampling: Label: { Ma }
- c. Cumulative samples: same serum bottle as “Stage 3”

- d. Let sit overnight

Stage 5: Bio-augmentation culture

- a. **Bioremediation Experiments:** 100mL bioaug culture (prepared in anaerobic hood in 2-50mL gas tight syringes) pumped using syringe pump.
- b. **Transport Experiments:** 100mL GW pumped using syringe pump.
 - a. Incremental sampling: Label: {B}
 - b. Cumulative samples: Label: {B-Mb-C}

Stage 6: Media (media phase B)

- e. 400 mL anaerobic media stored in IV bag (preparation in anaerobic hood, monitor colour of media to check if bag has been compromised) and pumped with peristaltic pump- collect 40 out of 50 mL per syringe, 10mL to cumulative, 40 to incremental as described in “Stage 3:Treatment addition” steps “b.” and “c.”. Record rate of pumping, and monitor for clogging.
 - a. Incremental sampling: Label: {Mb}
 - b. Cumulative samples: same serum bottle as “Stage 5”

Note: Store all samples at 4°C until analysis, for long term storage of total organic carbon, and ion chromatography samples store at 0°C (2 weeks post sampling).

5. Bioremediation Experiments Phase 2

Weekly column sampling: for more detail see “Sampling Procedure”. Dilutions may vary. See Figures 83-85 for further detail.

- 1) Gather sampling vials needed, and syringes as needed and label all sample vials with week number “N-week#” and column ID on 2 mL GC vials, 20 mL scintillation vial, 40mL EPA VOA vials with caps and Teflon septa, and 20mL headspace vial with metal crimp cap and Teflon septa
- 2) Check that there is no air in the line from the bottom of the column to the valve that will connect to the syringe, if so, bleed the line until bubbles have been removed
- 3) Fill a 50 mL gas tight syringe with ground water, remove all air bubbles, and place syringe in syringe pump.
- 4) Open the bottom most valve on the end of the tubing coming out of the bottom of the column and allow the liquid to flow out until there is a small enough gap that in the connection of the valve to the syringe no air is introduced into the system
- 5) Open all valves connection the bottom to tubing, top to tubing, and tubing into wetted syringe for collection at the top of the column.
- 6) Turn on syringe pump to maximum setting (X100) and note the time and measure the flow rate.



Figure 23- Image of weekly sampling, pumping GW

- 7) Make sure the syringe gets pushed and the black piece that rests on the cogs does not pop off the track
- 8) Look for sample to collect in the wetted syringe and confirm that sample is flowing and in the right direction



Figure 24-Image of weekly sampling, sample collection in wetted syringe



Figure 25-Image of weekly sampling, opening valves at inlet of column to start pumping of GW

- 9) Record the amount of liquid pumped and the amount in the effluent, any bubbles, and the time to finish pumping
- 10) Dispense 20 mL of the sample to the serum bottle (by opening the valve to the serum bottle tube) record as 20mL to "C"
- 11) Under the snorkel on the red tray, dispense the remaining sample into 3 pre labeled-2mL GC vial, set wetted syringe down carefully on the tray, and crimp the vial
- 12) Dispense about half of the contents of the wetted syringe into the scintillation vial and quickly remove liquid from the bottom with a clean gas tight syringe/s in order to make dilutions for purge and trap and headspace samples as needed. Record these dilutions. Crimping and seal vials.
- 13) Dispense the rest of the sample from the wetted syringe into the scintillation vial and cap
- 14) Place wetted syringe back, and close all valves so that no air can enter the column
- 15) Place samples in appropriate locations for storage prior to analysis

6. Column Sectioning

Used for Short-term Transport Experiments and Long-term Bioremediation Experiments (Phase 3). This protocol is written for a freezer that cannot accommodate a 5 foot column with end-caps, if a larger freezer is available, the column can be drained as written to avoid bursting and frozen whole, then sectioned once fully frozen.

- 1) Label the column using a sharpie with foot and 6 inch intervals; clearly in large writing identify sections by feet from inlet (1a, 1b, 2a, etc.).
- 2) Wearing gloves drain the top 1 foot of the column and transfer to chemical hood. With one person holding the column at a slight angle, using pipe cutters, cut the top foot of the column. Immediately wrap exposed ends with parafilm and packing tape and place securely in a freezer at 0°C.
- 3) After 2 days (allow time for material to fully freeze) transfer frozen column (lower 4 feet) to chemical hood and section using pipe cutters, cut the column at foot intervals, immediately wrap exposed ends with parafilm and packaging tape.
- 4) Re-freeze sections taking one section at a time out of the freezer for coring.
- 5) To core sections pre-weighted pre-labeled mason jars (10 jars- 1a, 1b, 2a, etc.) and 40mL EPA VOA vials with caps and Teflon septa pre-weighted pre-labeled (4 vials per 10 subsections).
- 6) Core foot sections in 2-6 inch intervals using the incremental marks on the column, homogenize quickly in mason jar keeping the jar covered with one gloved hand and homogenizing with a metal spatula with the other.
- 7) Immediately dispense homogenized sample into 40mL EPA VOA vials and weigh (4-per each 6 inch section). The 40mL EPA VOA vials will be used for hexane/acetone extractions, see “Hexane/Acetone Extractions” for instructions.
- 8) Sample 30grams of the material in a crucible and allow soil to volatilize in chemical hood until appears “dry”
 - a) Use dried material for volatile solids, total organic carbon-solid sampler module, and pH/alkalinity. Total organic carbon and magnesium as detected by inductively coupled plasma mass spectrometry were measured from the liquid fraction of the soil post hydrochloric acid digestion (2N).
- 9) Store mason jars at 4°C

7. Hexane/Acetone Extractions

Through various trials it was determined that PCE partitioned strongly into the hydrophobic emulsions and made for inaccurate extractions. Ratios of PCE extractable from various concentrations of emulsions in soil with PCE were determined to estimate recovery expected. “Standards and Controls” describes the making and testing (purge and trap) of these ratios. “All Samples” describes the treatment of samples including the standards and controls and the samples from the sectioned column, see “Column Sectioning”. Hexane extractable materials were measured as described in step 11 gravimetrically to determine if the “substrate/oil” fraction of the materials could be recovered.

Standards and Controls:

- 1) Weigh 10g of masonry sand + 2mL DI into 12-43.86 mL PT vials (in triplicate)
 - a. E- EOS-PCE spike
 - b. M- MH5-PCE spike

- 2) Dilute EOS and MH5 1:10 – 1mL spike
 - a. .01g oil: .001g PCE (1:10)
 - b. .01g oil = for 200mL volumetric flask
 - c. Spike 1mL in duplicate into crucibles-105 and 550
 - i. EOS=3.33g
 - ii. MH5 = 4g
- 3) Dilute EOS and MH5 1:5 – 0.5mL spike
 - a. .005g oil: .001g PCE (1:10)
 - b. Spike 1mL in duplicate into crucibles-105 and 550
- 4) Spike into 10g soil emulsion dilutions (1:10 and 1:5) and 100uL of PCE stock 10,000mg/L
 - a. Take 12 purge and trap dilutions
- 5) Let sit for 1 day
- 6) Make carbon tetrachloride (CT) to hexane stock-5ug/g stock (day of addition):
 - a. 10,000 mg/L CT stock in n-hexanes= 2g CT into 200mL vol n-hexanes= 2000mg/0.2L =10,000 mg/L stock
 - b. 10mL of n-hexanes used for extraction, spike n-hexane stock
 - c. PCE control is 20ug/g =10uL/vial of 10,000mg/L stock ; 5ug/g = 2.5uL/vial of 10,000mg/L stock, into 1000mL =250uL of 10,000mg/L stock = 0.25mL of 10,000mg/L stock into 1000mL volumetric flask of n-hexane = solvent stock
 - d. Pour into numerous small mason jars and seal immediately
 - e. Pipette with 5mL pipette and plastic tip

All Samples:

- 7) Add 20 mL of 1:1 v/v n-hexanes with CT and acetone to 10 grams of duplicate samples
 - a. Use syringe with needle to inject 1mL of CT hexane
 - b. 9 mL carbon tet n-hexanes (pipette)
 - c. 10mL acetone (pipette)
- 8) Sonicate for 15-20 minutes
- 9) Let sit overnight
- 10) Quickly extract 2mL into a headspace free 2mL GC vial for direct injection into FID-PCE and CT detection
 - a. Tetrachloroethene, 1,2-cis-dichloroethene, and trichloroethene were analyzed for chlorinateds performed by direct injection with the Shimazu GC with ECD (column: Agilent DB-VRX).
- 11) Hexane extractable materials (in chemical hood, transfer a 100g scale into chemical hood):
 - a. Weight pre-fired (550 for 1 hour and stored in desiccator) crucibles with lids (need lids for MH samples due to light ash formation during volatile solids testing)

- b. Extract top layer with 50mL glass gastight syringe and 1inch 22gauge pipetting needle into crucibles
- c. Weight crucibles once extricate is added
- d. Wipe pipetting needle off with kimwipe
- e. Rinse: Withdraw 5-10 mL of acetone (separate clean stock) into syringe, pull plunger and shake syringe
- f. Dispense into crucible
- g. Place crucible on tray to allow organic solvents to evaporate
- h. Rinse syringe with acetone two more times
- i. Repeate a-i until all samples are dispensed
- j. Stay in vicinity of samples until samples appear to have volatilized off and no longer smell of solvents (carefully wafting) due to the explosive nature of both hexane and acetone, place proper signage on chemical hood
- k. Allow samples to sit overnight to minimize flammability risk
- l. Place samples in oven at 105C for 24 hours
- m. Weigh crucibles (Standard Methods total solids procedure- store 105C crucibles in desiccator until no residual heat is displaced ~30-45mins)
- n. Fire crucibles at 550C in furnace for 1 hour, then cooling furnace to 105 post volatilizing
- o. Weigh crucibles (Standard Methods volatile solids procedure - store 105C crucibles in desiccator until no residual heat is displaced ~30-45mins)
- p. Analyze data subtracting control volatile solids from samples