

**Biotic and Abiotic Dehalogenation of Halogenated Methanes:
Trichlorofluoromethane, Dichlorodifluoromethane, and
Tetrachloromethane.**

**A Dissertation Presented for the
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Abstract

Trichlorofluoromethane (CFC-11), dichlorofluoromethane (CFC-12), and tetrachloromethane (CT) are fully halogenated methanes that were produced as refrigerants in the early part of the 1900s and later used in many industrial processes. They are ozone-depleting agents and common groundwater contaminants. They are volatile chemicals that are moderately soluble in water. Due to their volatility when released to the environment, they are predominantly found in the atmosphere, though they also dissolve into the groundwater. In anaerobic environments, they can undergo dehalogenation reactions with several redox-active compounds. This dissertation presents results from two treatability studies from sites contaminated with CFC-11, CFC-12, and CT. Additionally, the effect of pH on the dehalogenation of CFC-11, CFC-12, and CT is examined, and a sulfidogenic enrichment culture grown in the presence of CT is characterized. The first treatability study indicates that the addition of reactive iron species (*i.e.*, zero-valent iron or ferrous sulfide) combined with the bioaugmentation culture KB-1 Plus and lactate can facilitate the transformation of CT into non-halogenated end products. The most effective remediation strategy for CT observed during the treatability study for the second contaminated site was the addition of zero-valent iron; this facilitated the transformation of CT to chloroform (CF). CF is a non-desirable end product, and additional remediation efforts are recommended for the second contaminated site. A shift in the type of transformation products formed during the reduction of CFC-11, CFC-12, or CT by super-nucleophilic cobalamin was observed as pH increased. Mackinawite and vivianite were identified as the two precipitate phases formed in the presence of the sulfidogenic enrichment culture. Vivianite formation likely occurs via precipitation with the phosphate present in the medium, and that mackinawite forms via precipitation with hydrogen sulfide produced by the sulfidogenic bacteria present in the enrichment. Additionally, a greater decline in CT was observed in microcosms that contained active enrichment culture than in heat-killed controls, suggesting that the consortium aids in the degradation of CT, probably via mackinawite formation, as it is a reactive iron species.

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INTRODUCTION

This dissertation is focused on the degradation of the halogenated methanes trichlorofluoromethane (CFC-11), dichlorodifluoromethane (CFC-12), and tetrachloromethane (CT). Chapter 1 presents a literature review, which encompasses the production, occurrence, and use of CFC-11, CFC-12, and CT as well as toxicological profiles, physical and chemical properties, and a discussion of the processes that contribute to the dehalogenation of CFC-11, CFC-12, and CT. Chapter 2 describes a high-resolution site characterization and treatability study for a site contaminated by CT, chloroform (CF), tetrachloroethene (PCE), 1,1-dichloroethene, CFC-11, and CFC-12, and aims to identify contaminant distribution in the vadose and saturated zone at the site as well as identify remediation strategies that may be effective. Chapter 3 details a treatability study performed for a site contaminated with CT, CF, and PCE and aims to determine effective remediation strategies for the site. Chapter 4 uses 16S rRNA amplicon sequencing to identify the community structure of an enrichment culture grown in the presence of CT and utilizes X-ray diffraction to characterize the precipitates formed in the enrichment cultures. Additionally, changes in CT concentration over time are monitored by gas chromatography (GC) with a flame ionization detector (FID). Chapter 5 focuses on the dehalogenation of CFC-11 and CFC-12 by super-nucleophilic vitamin B12. Additionally, a summary of the findings from each chapter can be found in the conclusions section.

CHAPTER I

TRANSFORMATION OF TRICHLOROFLUOROMETHANE, DICHLORODIFLUOROMETHANE, AND TETRACHLOROMETHANE BY BIOTIC AND ABIOTIC PROCESSES: A REVIEW

Abstract

Trichlorofluoromethane (CFC-11), dichlorodifluoromethane (CFC-12), and tetrachloromethane (CT) are ozone-depleting agents, and groundwater contaminants found the world over. The first few sections of this review discuss the global production occurrence and use, toxicology, physical properties, and fate in environmental systems of CFC-11, CFC-12, and CT. Then there is a detailed discussion of the possible degradation pathways for CFC-11, CFC-12, and CT. Degradation of CFC-11, CFC-12, and CT is favorable in anaerobic environments and can occur via hydrogenolytic, radical-radical coupling, combinatory, hydrolysis, or surface-associated carbene reduction pathways. The latter sections of this review discuss the redox-active compounds that facilitate the degradation of CFC-11, CFC-12, and CT and explores possible avenues of future research.

Production, use, and occurrence in the environment.

Trichlorofluoromethane (CFC-11) and dichlorodifluoromethane (CFC-12) are anthropogenic chlorofluorocarbons (CFCs) that were developed in the 1930s for use in refrigeration.¹ Their use was later expanded to foam blowing agents, aerosol propellants, insulation, solvents and as building blocks in the production of fluorinated polymers.² By 1995, estimated global accumulative production for CFC-11 and CFC-12 were 8.6 million and 11.3 million metric tons, respectively.³ The alternative fluorocarbons environmental acceptability study (AFEAS) estimated that 87.2% of the CFC-11 and 95% of the CFC-12 produced had been released into the environment as of 1995. Unfortunately, CFC-11, CFC-12, and carbon tetrachloride (CT) are ozone depleting agents and greenhouse gasses.^{4,5} Once these chemicals reach the upper atmosphere they undergo photochemical decomposition and produce chlorine radicals.⁵ These chlorine radicals then drive the ozone destruction cycle.⁶ Due to their ozone destruction potential the production of CFC-11, CFC-12, and CT has been phased out under the Montreal protocol.⁷

CT is a toxic chlorinated solvent, predicted carcinogen, and an ozone-depleting agent.⁸ It was widely used during the twentieth century as a dry-cleaning agent, industrial degreaser, grain fumigant, fire extinguishing agent, and in the synthesis of other chemicals most prominently for the production of CFCs.⁹ Production of CT peaked in 1974 with production volumes reaching 1200 million pounds per year.¹⁰ Production volumes declined during the latter part of the twentieth century due to regulations. Under the Montreal Protocol, production of CT as an emissive chemical has been completely banned since 2010.¹¹ CT still produced as an intermediate for the synthesis of chloromethanes and tetrachloroethene, and production volumes as of 2018 were estimated to be between 33 – 55 million pounds per year. CT is released into the environment via evaporation, dumping and spills.⁸ It can be found in sediment, groundwater and the atmosphere. Global atmospheric emissions for 2014 were estimated to be around 77 million pounds due to emissions from current production, legacy sites, and inadvertent formation and loss during the production of chlorine (Cl₂) gas.¹¹ As of 2019, there were 301 active Superfund sites containing CT in the United States and many others abroad.¹² CT is ranked 6th on the EPA's national priorities list for hazardous substances and has an MCL of 0.005 mg/L in drinking water.

This review summarizes the abiotic and biotic mechanisms of CFC-11, CFC-12 and CT degradation, and identifies research needs to advance the remediation of CFC-11, CFC-12, and CT.

Toxicology.

The NIH cancer institute performed a bioassay for carcinogenicity for CFC-11, but was unable to determine if CFC-11 is carcinogenic to rats because too many rats died in the early stages of the study.¹³ However, a yearlong study of Swiss mice found that exposure to CFC-11 was not correlated with a significant increase in tumor formation.¹⁴ Aviado and colleagues reported that inhalation of CFC-11 had toxic effects on the respiratory and circulatory systems of mice, rats, dogs, and monkeys.¹⁵ Increased cardiac sensitivity to adrenaline and noradrenaline is the primary health risk from exposure to CFC-11 and other halocarbons.¹⁶ Increased cardiac sensitivity to adrenaline and noradrenaline can cause arrhythmia and death.

CFC-12 is considered non-carcinogenic, though there has been little research on the topic.¹⁷ In one study, CFC-12 exposure was found not to effect tumorigenesis in Sprague-Dawley rats or Swiss mice.¹⁸ Short term effects on the central nervous system were observed in guinea pigs exposed at high concentrations for an hour or more.¹⁹ Similarly to CFC-11 the primary health risk from exposure to CFC-12 is increased risk of cardiac arrhythmia due to cardiac sensitivities to adrenaline and noradrenaline, though higher concentrations of CFC-12 appear to be required to produce cardiac sensitivities and cardiac arrhythmias.¹⁷

The primary health concern from exposure to CT is hepatotoxicity, while hepatic cancer is thought to occur secondarily.^{20–26} Chronic exposure to CT leads to severe cirrhosis, liver tumors, and chronic respiratory disease.^{27–29} The hepatotoxicity and carcinogenicity of CT is thought to occur via interactions between cellular macromolecules and the chlorine and trichloromethyl radicals that form during the degradation of CT.^{30–35} The effects of CT exposure on human and animal health were thoroughly reviewed by the International Program on Chemical Safety (IPCS).²⁶

Physical and chemical properties.

CFC-11, CFC-12, and CT are volatile chemicals that are moderately soluble in water, and have densities greater than water **Table 1**, Appendix A. When these chemicals reach saturation in water they form dense non aqueous phase liquids. The octanol–water partitioning coefficients of CFC-11, CFC-12, and CT suggest that adsorption onto organic particles is feasible **Table 1**, Appendix A.^{36,37}

Fate and longevity in environmental systems.

Due to their volatility, CFC-11, CFC-12, and CT are predominantly found in the atmosphere.¹ Atmospheric concentrations are globally consistent, though elevated concentrations have been measured over large industrial cities like New York, Heidelberg, and Taipei.^{38–42} Atmospheric concentrations as of 2020 for CFC-11, CFC-12, and CT are roughly 224, 499, and 77 parts per trillion (ppt) respectively.⁴³ Researchers predict that atmospheric concentrations of CFC-11, CFC-12, and CT will slowly decline for the next hundred years or so. This prediction is based on the calculated lifespans of these chemicals in the atmosphere and the expectation of decreased emissions due to the limited amount of production currently occurring.^{4,5,44}

Once CFC-11, CFC-12, and CT reach the stratosphere they undergo photolytic dissociation.⁷ The process of photolytic dissociation produces a two odd electron species (free radicals).⁵ Photolytic dissociation of CFC-11 produces CFCl_2 and a chlorine radical (Cl), CFC-12 produces CF_2Cl and

Cl, and CT produces CCl_3 and Cl. The carbon containing free radicals can generate additional one atom chlorine radicals in future reactions. CFC-11 has the potential to generate three chlorine radicals, while CFC-12 and CT have the potential to generate two and four chlorine radicals respectively. Chlorine radicals destroy ozone (O_3) in a chain reaction where Cl reacts with O_3 to produce ClO and O_2 . The radical is regenerated when ClO reacts with O to generate Cl and O_2 . Free radical reactions continue until they react with another free radical (*i.e.*, OH, Cl, or NO).

An alternative environmental fate for CFC-11, CFC-12, and CT is retention in the subsurface, where they can be stored in the unsaturated or saturated zones. In the unsaturated zone CFC-11, CFC-12 and CT concentrations are driven by barometric pumping and diffusion.¹ In porous unsaturated soils barometric pumping of gasses only effects the top few meters of the unsaturated zone.^{45,46} Barometric pumping as a mechanism of subsurface gas transport is much more effective in fractured rock, where it can transport gasses hundreds of meters over a few months.^{47,48} Diffusion is the dominant mechanism of gas transport in unsaturated porous sediments.⁴⁵ Research suggests that CFC-11 and CFC-12 concentrations decline as depth increases.⁴⁹ Field studies indicate that CFC-11 and CFC-12 concentrations are between 7 – 10 % of atmospheric concentrations at depths of 43.9 m and 57.5 m in porous unsaturated sediments.^{49,50}

Sorption and desorption of CFC-11, CFC-12, and CT may play a role in the transportation and transformation of these chemicals in the subsurface.⁵¹ Sorption of CFC-11 and CFC-12 to dried limestone, Ottawa sands, Yolo sandy loams, and dry soils has been reported.^{51–53} Russel and colleagues reported that CFC-11 and CFC-12 desorbed when soils were wetted, but desorption from the other matrices was not explored.⁵⁴ Adsorption of CT to dry soils increases as the surface area of the soil increases or the percent of organic carbon in the soil increases.^{55,56} Adsorption of CT dry soil is a physical process that can be completely reversed.^{56,57} More information regarding the sorption and desorption rates of CFC-11, CFC-12, and CT on a variety of matrices could provide a better understanding of the transport and transformation of these chemicals in the subsurface.

Introduction of CFC-11, CFC-12, and CT into the saturated zone can occur via gasses dissolving into the groundwater from the atmosphere, industrial solvent spills, and contaminants leaching from landfills.^{58–63} CFC-11 and CFC-12 are used as groundwater tracers of young groundwater (*i.e.*, ~100 years old).⁵⁸ They are considered good groundwater tracers, because they were released and monitored globally, their rates of production and solubility are known, and they are considered stable in the groundwater.^{1,58,64,65} However, CFC-11 and CFC-12 cannot be used as groundwater tracers if their concentration in the groundwater exceed the values possible by equilibrium with modern air (*i.e.*, if the groundwater was contaminated by a solvent spill or landfill leachate).^{1,59–61,63,66–68} Transformation of CFC-11, CFC-12, or CT has not been observed in aerobic groundwater.^{1,7,68,69} However, transformation of CFC-11, CFC-12 and CT has been observed under reducing conditions.

Degradation pathways.

Transformation of CFC-11, CFC-12, or CT can occur via several pathways. Each transformation pathway consists of sequential chemical reactions that produce a variety of transformation

products.^{69,70} Some transformation products are more favorable than others depending on their toxicity, carcinogenicity, or recalcitrance. Redox conditions and the presence of reactants determine which transformation path occurs. Transformation of CFC-11, CFC-12, and CT is favorable in reduced conditions. The transformation of CT has been explored more thoroughly than the transformation of CFC-11 or CFC-12, because CT causes more severe health hazards and is a regulated chemical in the subsurface under the clean water act.^{37,63,68,71} Reported reaction pathways for CT include hydrogenolysis, radical-radical coupling, free radical addition to alkenes, combination, carbene hydrolysis, and surface associated carbene reduction reactions **Figure 1**, Appendix A.^{69,70,72–76} Figure 1 depicts transformation pathways for CT. Transformation products for CFC-11 and CFC-12 have been reported via hydrogenolysis, and carbene hydrolysis, since the formation of radicals from CFC-11 and CFC-12 is possible coupling and free radical addition reaction pathways are also included for CFC-11 and CFC-12 in **Figure 2**, Appendix A through **Figure 4**, Appendix A.^{77,78}

Hydrogenolysis.

Hydrogenolysis is a reductive chemical reaction where a single bond between a carbon and a halogen (ie., Cl or F) is cleaved (Equation 1).^{70,79} The halogen leaves, and is replaced by two electrons and a hydrogen. Hydrogenolysis can occur sequentially, in this process halogens are consecutively replaced by hydrogens and two electrons.^{70,80}

Radical-radical coupling.

Radical-radical coupling reactions occur when two radicals form a covalent bond (Equation 2). For example, when two trichloromethyl radicals couple to form hexachloroethane **Figure 1**, Appendix A.⁷² This can lead to the formation of tetrachloroethene when two of the chlorines leave the hexachloroethane compound.

Free radical addition to alkenes.

Free radical additions to alkenes occur when a radical attacks a carbon-carbon double bond (Equation 3). This forms a carbon radical that will subsequently attack other compounds, and perpetuate a chain of radical reactions (Equation 3). The hepatotoxic nature of CT is related to the formation of the trichloromethyl radical and its subsequent tissue injury (*i.e.*, lipid peroxidation).^{30,81}

Combination reactions.

A combination reaction occurs when two compounds react to form one compound (Equation 4).⁸² An example of a combination reaction is when a trichloromethyl radical and oxygen gas combine to form a new compound **Figure 1**, Appendix A. This is the first step in the transformation path way that leads to the formation of phosgene. There is uncertainty regarding what the subsequent steps in the formation of phosgene are.⁸³

Carbene hydrolysis.

Hydrolysis reactions occur when water breaks a chemical bond (Equation 5).^{70,76} A carbene hydrolysis reaction can occur between a dichlorocarbene and a water molecule react to form carbon monoxide **Figure 1**, Appendix A.

Surface associated carbene reduction.

Surface associated carbene reduction occurs when a carbene is associated with a reactive surface; it was proposed by McCormick and Adriens to explain the formation of methane without observing the transformation products associated with hydrogenolysis.⁷⁶ During surface associated carbene reduction a reactive surface donates electrons and hydrogen while stabilizing carbene and radical intermediates **Figure 1**, Appendix A.

Transformation of CFC-11, CFC-12, and CT.

Biologically mediated transformation of CFC-11, CFC-12, and CT occurs via co-metabolic processes that do not support organism growth. No evidence of an organism using CFC-11, CFC-12, or CT as terminal electron acceptors exists. Biotic transformation of CFC-11, CFC-12, and CT occur via redox-active compounds with low molecular weights.^{68,84} Transformation of CFC-11, CFC-12 and CT has been reported under various reducing conditions, including sulfate reducing, iron reducing, methanogenic, acetogenic, denitrifying, fermentative and halorespiring conditions.^{77,78,85–92} This review focuses on the redox-active compounds that have been shown to facilitate the transformation of CFC-11, CFC-12 or CT. Some of the redox active compounds are biologically produced (*i.e.*, cobamides) while others are abiotic (*i.e.*, zero valent iron), but the line between abiotic and biotic reactions is fuzzy, as many of the abiotic redox active compounds can be reduced by microbial processes, and the biologically produced redox active compounds rely on metal atoms. Many of the reactions that dehalogenate CFC-11, CFC-12, and CT fall into the category of biologically mediated abiotic reduction.

Organometallic cofactors.

Three organometallic cofactors that are able to dehalogenate CFC-11, CFC-12, or CT are cobamides, cytochromes (which contain heme groups), and coenzyme F430.^{93–98} All three of these cofactors contain highly conjugated porphyrin ring systems that stabilize a metal atom in the center of the ring.^{97,99,100} The metal components in these cofactors are cobalt in cobalamins, iron in cytochromes, and nickel in coenzyme F430. When the centrally coordinated metal atoms in these cofactors are reduced they can act as electron donors to halogenated compounds.⁹⁴ Krone et al., reported the formation of carbon monoxide as the predominant transformation product of CFC-11 and CFC-12 dehalogenation by cobalamin with cobalt in the (+1) state (super-nucleophilic B₁₂).⁹³ Some carbon monoxide (~6% of observed transformation products) was formed when CT was dehalogenated by super-nucleophilic B₁₂, but chloroform (CF) was the predominant transformation product (54% of observed transformation products). Krone and colleague suggest that the formation of carbon monoxide occurs via a carbene intermediate that undergoes hydrolysis (**Figure 1-Figure 4**, Appendix A), and that more carbon monoxide forms in reactions between super-nucleophilic B₁₂ and CFC-11 or CFC-12 because trihaloradicals that contain fluorine are less stable than radicals that contain only chlorine (trichloromethyl radical), and that fluorine containing radicals are more likely to decompose into carbene intermediates (**Figure 2 -Figure 4**, Appendix A). Cytochrome P450 reduced CFC-11 to carbon monoxide, and CT to CF.^{96,97} The formation of carbon monoxide likely occurs via a carbene intermediate. Unfortunately, carbon monoxide inhibits the dehalogenation activity of cytochrome P450.^{95–97} The carbon monoxide induced inhibition of cytochrome P450 makes the dehalogenation of CFC-11 by cytochrome P450 an inefficient degradation mechanism. Additional experiments have been shown that cytochrome-c and cytochrome-b produced by *Shewanella putrefaciens* 200 can transform CT to CF.^{101,102} Transformation of CT to CF is thought to occur via a trichloromethyl

radical intermediate **Figure 1**, Appendix A. Experiments with showed that F-430 could dechlorinate CFC-11 and CT.⁹⁸ Unfortunately, no defluorination activity was observed. An additional limitation of F-430 is that it only produced methanogenic bacteria, and thus is encountered less frequently in contaminated environments than cobamides and cytochromes which are produced by several microbial groups.^{87,96,97,101–104}

Extracellular electron shuttles.

Extracellular electron shuttles are molecules that can be reversibly oxidised or reduced and commonly function outside of the cell. The regenerative nature of electron shuttles allows a single molecule to facilitate numerous redox reactions.¹⁰⁵ Electron shuttles donate electrons to halogenated pollutants like CFC-11, CFC-12, and CT, and facilitate redox reactions by lowering the activation energy.^{106–110} The redox potential of an electron shuttle is important, because the effectiveness of an electron shuttle is dependent on its ability to be reduced and its subsequent capacity to reduce the target contaminant.^{94,105} Some conditions that can effect the redox potential of an electron shuttle are pH and whether the electron shuttle is carrying one or two electrons.¹⁰⁵

Organic extracellular electron shuttles are molecules that can be reversibly oxidised or reduced, and are primarily carbon based. Humic substances and quinones are organic extracellular electron shuttles, that have been studied in depth.⁹⁴ Humic substances contain aromatic compounds that have redox activity, and quinones are a common constituent of humic substances.¹¹¹ Quinones contain cyclic diketones that is fully conjugated. Humic substances and quinones can be reduced biotically by iron reducing, sulfate reducing, halorespiring, fermentive, and methanogenic microbes, or abiotically by reductants found in the subsurface (*i.e.*, sulfide, zero valent iron (ZVI), Ti(III)citrate, and cysteine.).^{94,106,109,112–117} Standard redox potential and chemical structure influence the effectiveness of humic substances and quinones as electron shuttles.¹⁰⁵ In general, quinones and humic acids with lower standard redox potentials facilitate faster reduction of target contaminants.^{94,110,114,118} However, under some conditions chemical structure seems to have a greater effect on the rate of reduction than standard redox potential.^{94,108} For example, the rate of reduction of CT by sulfide was faster in the presence of juglone, naphthoquinone, and benzoquinone than in the presence of anthraquinone-2,6-disulfonate even though anthraquinone-2,6-disulfonate has a lower standard redox potential.¹⁰⁸ The authors attributed the increased reaction rates in the presence of juglone, naphthoquinone, and benzoquinone to chemical structure.^{94,108} They observed the formation of mercaptoquinones when juglone, naphthoquinone, and benzoquinone were reduced, while reduction of anthraquinone-2,6-disulfonate formed semiquinone radicals.

The most commonly encountered and best studied inorganic electron shuttles are reactive iron species.⁷⁰ Reactive iron species contain solid ferrous iron, which can donate electrons to chlorinated solvents.^{70,94} He and colleagues reviewed the literature on the degradation of chlorinated solvents by reactive iron minerals, and observed that the hierarchy of reactivity for reactive iron species towards chlorinated solvents is: disordered ferrous sulfide > ordered ferrous sulfide > zero valent iron (ZVI) > sorbed ferrous iron > green rust = magnetite.⁷⁰ Dehalogenation of CT by Mackinawite, FeS, FeS₂, Pyrite, Pyrrhotite, Magnetite, Green rust, and surface associated ferrous iron has been reported **Table 2**, Appendix A. Additionally dehalogenation of CFC-11 has been reported by ZVI.¹¹⁹ Dehalogenation of CFC-12 has been

reported by palladium catalysts, and by Molybdenum and Tungsten containing carbides, though these reactions are not environmentally relevant, as they occurred at temperatures greater than 140°C.^{120,121} Information regarding dehalogenation by inorganic electron shuttles, the transformation products formed, and the predicted dehalogenation pathways is available in **Table 2**, Appendix A. As mentioned above, the relationship between abiotic and biotic reaction is synergistic, examples of synergistic effects include ferrous sulfide production being supported by the byproducts of sulfate reducing bacteria, or the formation and regeneration of reactive iron minerals by dissimilatory iron reducing bacteria.^{70,74,94,122} Thus, understanding CFC-11, CFC-12, and CT degradation by reactive mineral phases requires the integrated study of biotic and abiotic processes.

Siderophores.

Pyridine-2,6-bis(thiocarboxylate) (PDTC) is a siderophore that dechlorinates CT.^{123,124} The primary transformation product formed when CT is dechlorinated by PDTC is carbon dioxide.^{125,126} Lewis and colleagues suggest that carbon dioxide formation occurs via interactions with a copper:PDTC complex that forms a thioester intermediate.¹²³ Unlike the extracellular electron shuttles previously discussed, the reaction between CT and PDTC is not catalytic.^{68,123} When PDTC reacts with CT dipicolinic acid is produced. PDTC is produced by *Pseudomonas stutzeri* strain KC.^{123,124} The dechlorination of CT occurs most rapidly when *Pseudomonas stutzeri* strain KC is grown under nitrate reducing conditions.¹²⁷

Conclusions and Future Work.

Despite the numerous mechanisms of CFC degradation known, these contaminants still trouble remediation efforts, this is likely due to the inhibitory nature of these chemicals on bacterial growth.^{30,31,34,95} There is one report in the literature of an organism regenerating a reactive iron barrier in the presence of CT.¹²⁸ Future research should look to identify other organisms that can promote the formation of reactive iron minerals in the presence of halogenated methanes. Alternatively research efforts could go into developing a bioremediation tool that utilizes B₁₂, as the literature suggests that the predominant transformation product formed via B₁₂ reduction is CO. However, a deeper understanding of how environmental factors alter the composition of transformation product formation is needed. It is especially important to understand which transformation products are produced, as they can be more hazardous than the starting chemical (*i.e.*, dichlorofluoromethane from CFC-11 or CFC-12).

Appendix A

Table 1. Physical and chemical properties of CFC-11, CFC-12, and CT.

Chemical	Molecular Weight (g/mol)	Density (g/mL)	Aqueous Solubility (mg/L) at 25°C	Henry's law constant k°_H (mol/kg*bar)	Octanol:water partitioning coefficient (log K_{ow})	Boiling point (°C)
CFC-11	37.368	1.49	1,100	0.0012	2.44	23.7
CFC-12	120.914	1.35	280	0.0025	2.05	-29.8
CT	153.823	1.59	793	0.033	2.83	76.7

Table 2. Transformation products and reaction pathways of dehalogenation of CFC-11, CFC-12, and CT by inorganic electron shuttles.

Chemical	Inorganic electron shuttle	Transformation products	Transformation pathway	Reference
CFC-11	ZVI	not reported	Unknown	119
CFC-12	Pd/Al ₂ O ₃ at temperatures greater than 140°C	CH ₂ F ₂	Hydrogenolysis	120
CFC-12	W ₂ C/y-Al ₂ O ₃ and Mo ₂ C/y-Al ₂ O ₃ carbides	CH ₂ F ₂	Hydrogenolysis	121
CT	Mackinawite	CHCl ₃	Hydrogenolysis	129
CT	FeS	CHCl ₃ , CH ₄ , C ₂ H ₄ , C ₂ H ₆	Hydrogenolysis, and radical-radical coupling	130
CT	FeS	not reported	Unknown	131
CT	FeS	CHCl ₃	Hydrogenolysis	73
CT	FeS	CHCl ₃	Hydrogenolysis	132
CT	FeS	CHCl ₃	Hydrogenolysis	74
CT	Pyrite	CHCl ₃	Hydrogenolysis	133
CT	Pyrite	CHCl ₃	Hydrogenolysis	134
CT	Pyrrhotite	CHCl ₃	Hydrogenolysis	134
CT	Magnetite	not reported	Unknown	135
CT	Magnetite	CHCl ₃ , CH ₄ , CO	Hydrogenolysis, carbene hydrolysis, surface associated carbene reduction	122
CT	Magnetite	CHCl ₃ and CO ₂	Hydrogenolysis	136
CT	Magnetite	CHCl ₃	Hydrogenolysis	129

Table 2. Continued.

Chemical	Inorganic electron shuttle	Transformation products	Transformation pathway	Reference
CT	Mackinawite	CHCl ₃	Hydrogenolysis	129
CT	Surface associated Fe ²⁺ Goethite	CHCl ₃	Hydrogenolysis	129
CT	lepidocrocite	CHCl ₃	Hydrogenolysis	129
CT	Siderite	CHCl ₃	Hydrogenolysis	129
CT	Hematite	CHCl ₃	Hydrogenolysis	129
CT	Green rust	CHCl ₃	Hydrogenolysis	137
CT	Green rust	CF, HCOOH, CO	Hydrogenolysis, carbene hydrolysis	138
CT	Surface associated Fe ²⁺ Goethite	not reported	Unknown	139
CT	Surface associated Fe ²⁺ Goethite	CHC ₃ , CO, formate	Hydrogenolysis, carbene hydrolysis	140
CT	Surface associated Cu ⁰ on green rust	CHCl ₃ , methane	Hydrogenolysis, and possibly surface associated carbene reduction	141

(Some of the information presented in this table is also presented in reference 70).

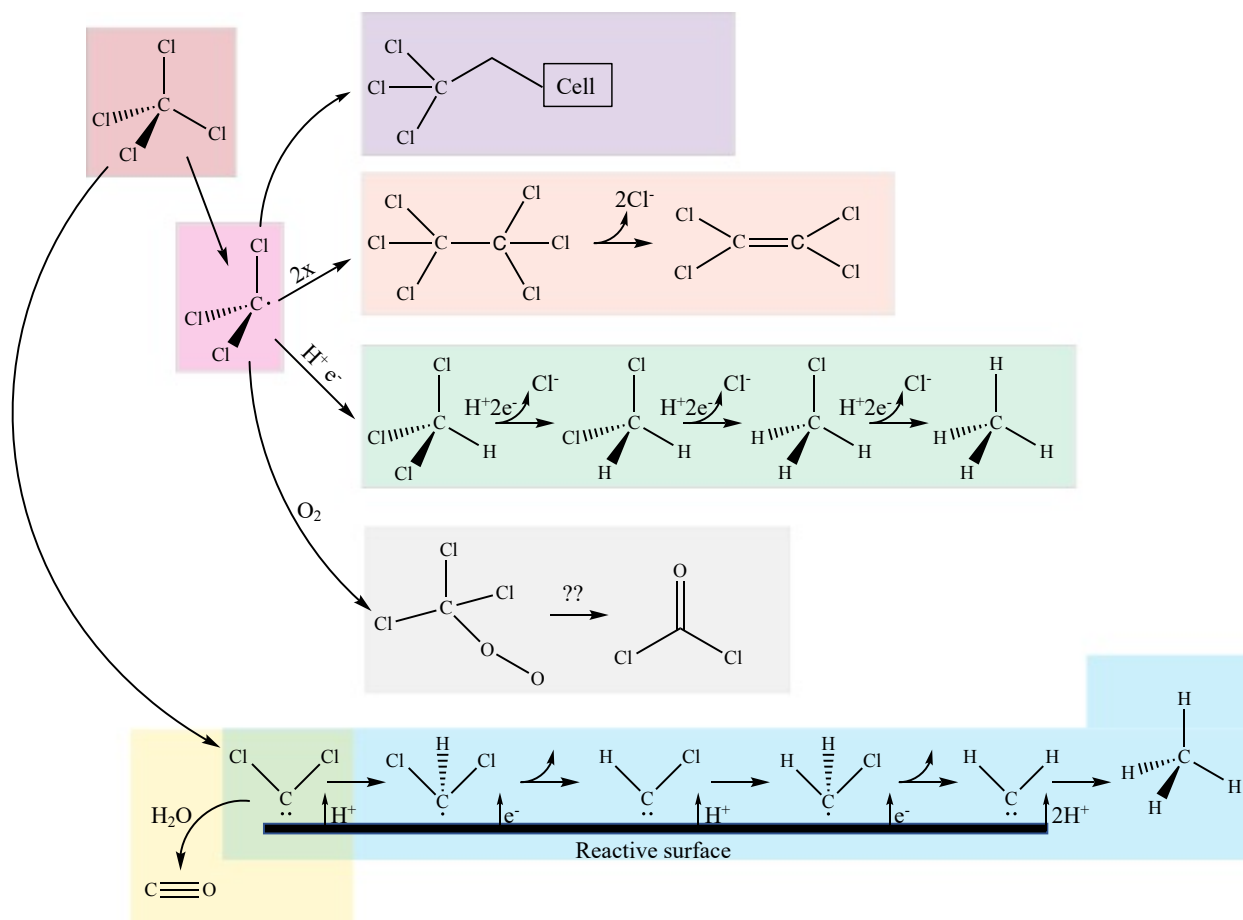


Figure 1. Tetrachloromethane degradation pathways

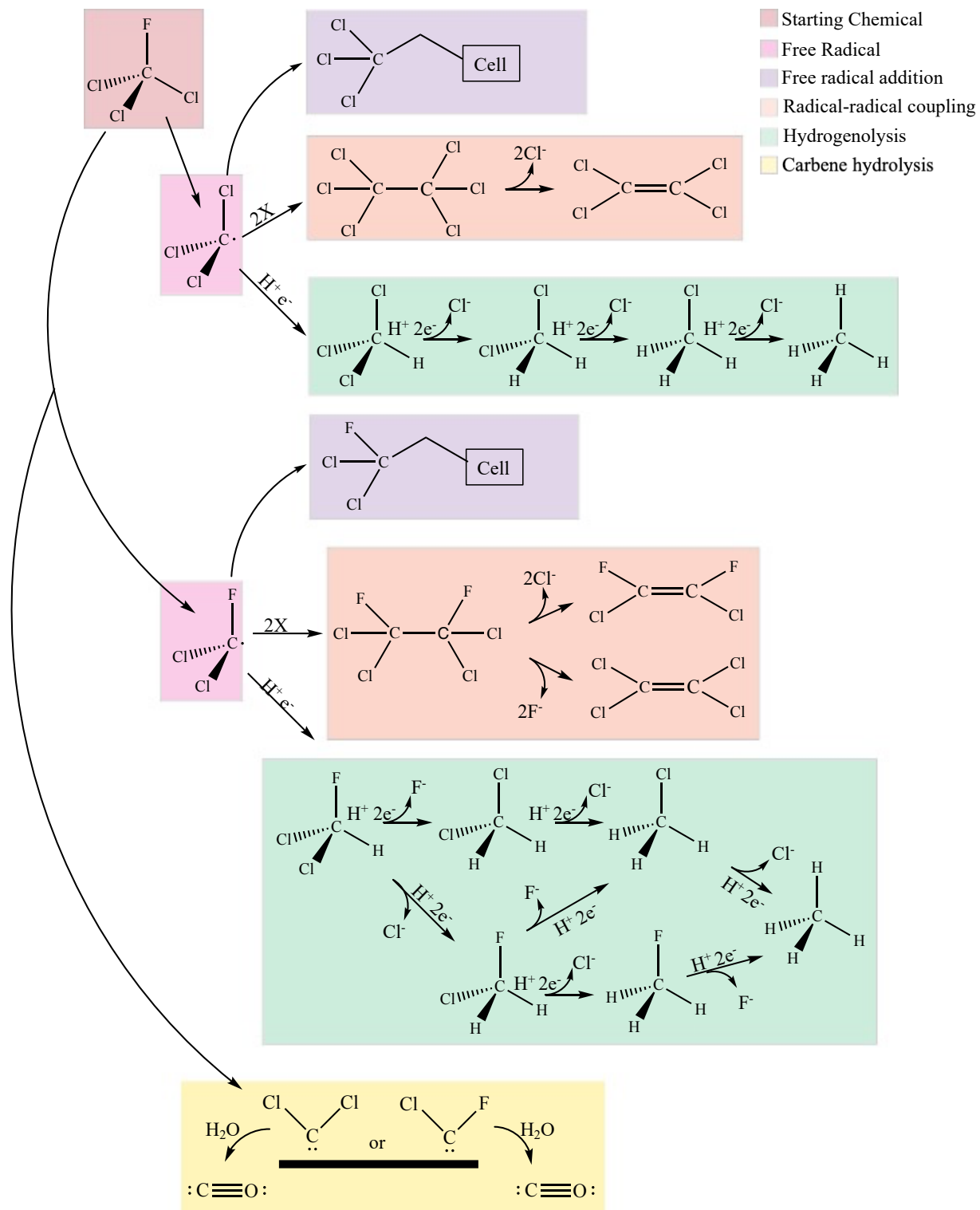


Figure 2. Trichlorofluoromethane degradation pathways

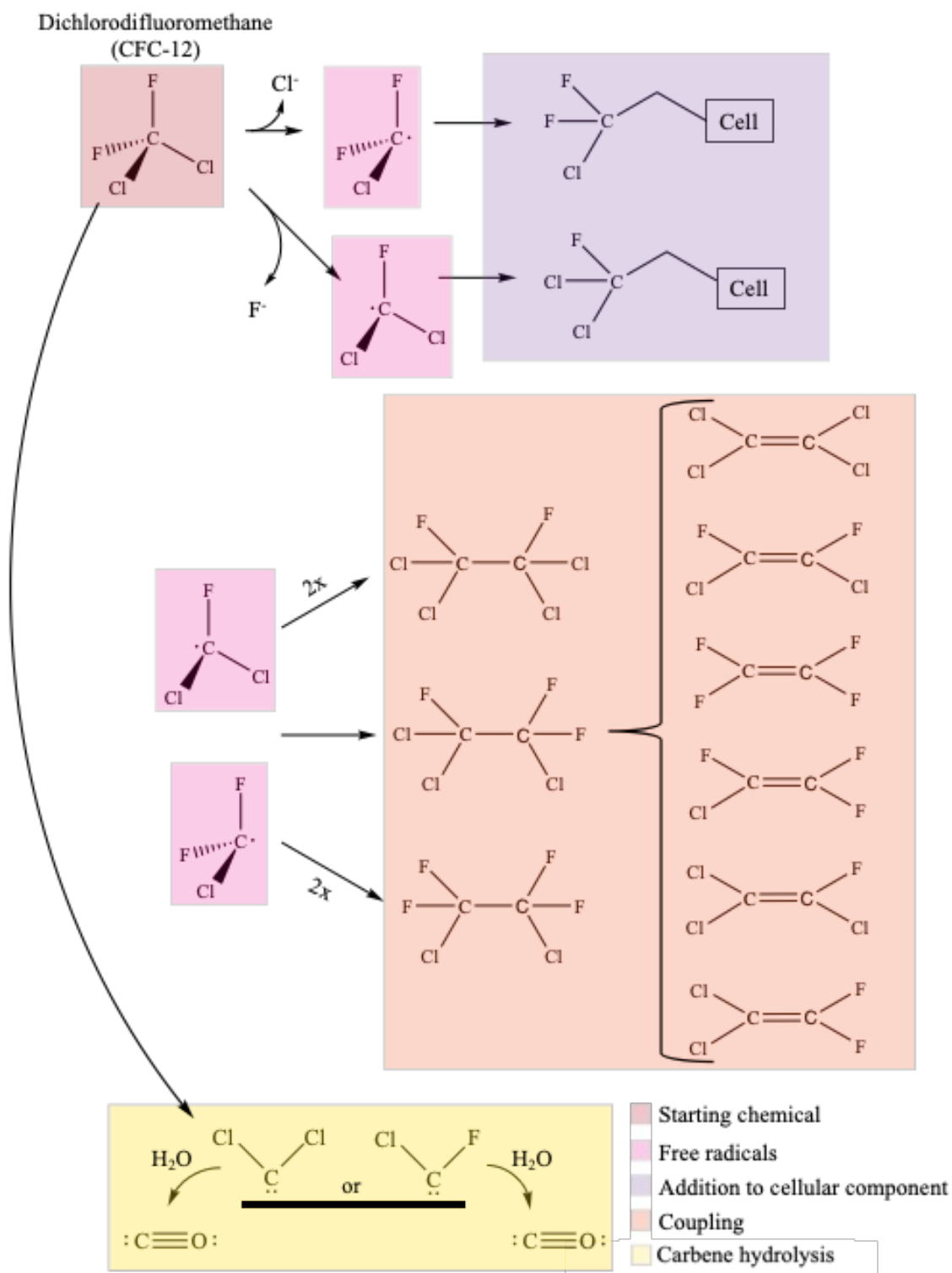


Figure 3. Dichlorodifluoromethane transformation pathways part 1

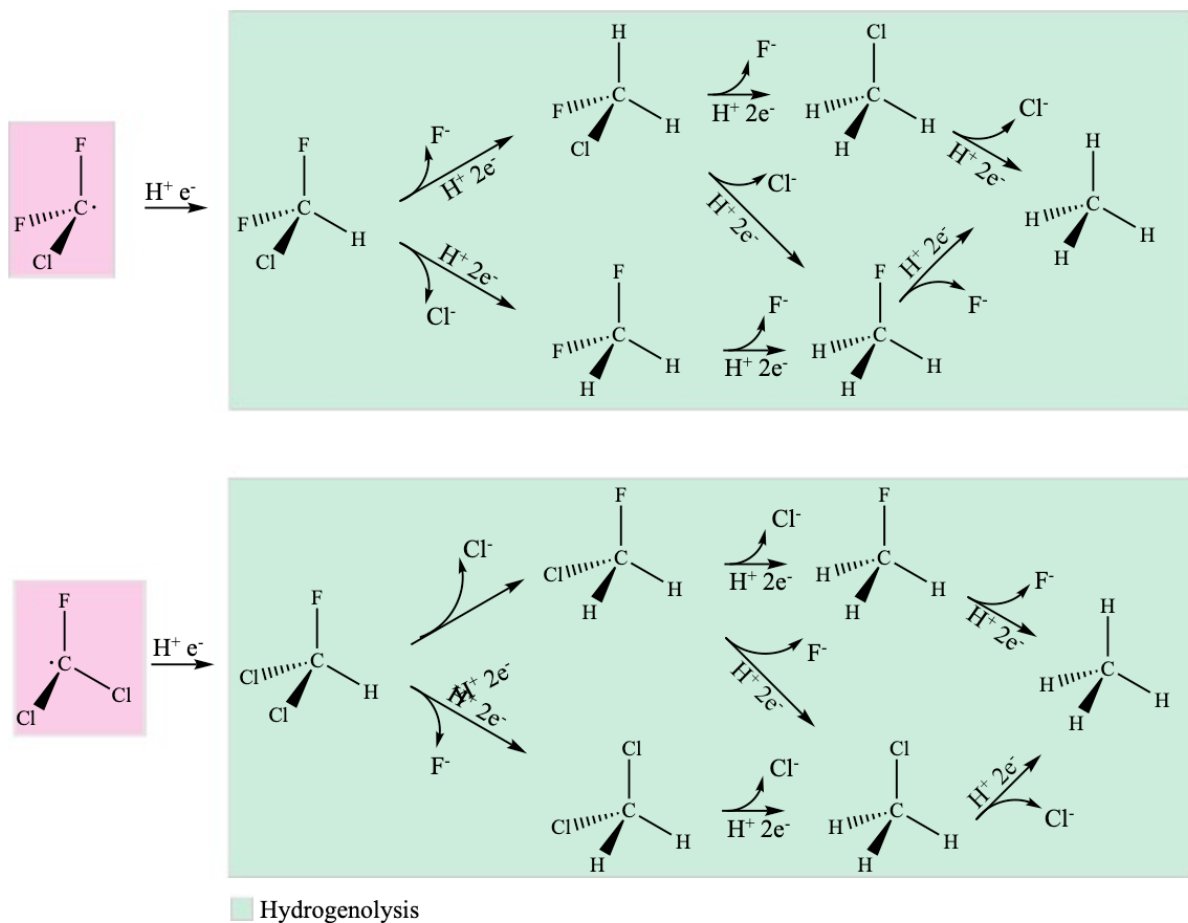
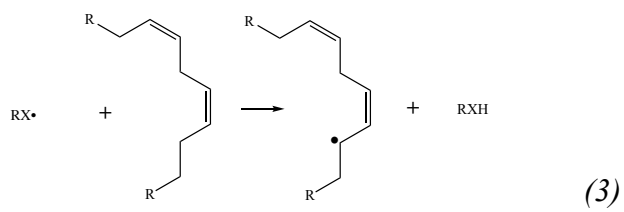
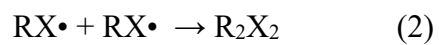
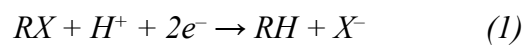


Figure 4. Dichlorodifluoromethane transformation pathways part 2

Reaction equations.



CHAPTER II

HIGH-RESOLUTION SITE CHARACTERIZATION AND LABORATORY- SCALE TREATABILITY STUDY ENABLING AQUIFER REMEDIATION AT AN INDUSTRIAL SITE.

Abstract

This study characterizes the contamination of an aquifer at an industrial site in South America and evaluates the effectiveness of monitored natural attenuation, anaerobic-enhanced in situ bioremediation (EISB), and enhanced abiotic degradation for treating specific contaminant of concern (PCE, 1,1-DCE, CT, CF, CFC-11, and CFC-12). The techniques used to characterize the site included membrane interface probe-hydraulic profiling tool logging, measurements of contaminants in the groundwater and sediment, and qPCR of DNA from groundwater samples to determine the presence and abundance of biomarker genes for organohalide-respiring bacteria. Microcosms containing aquifer material were monitored to a) assess the potential of natural attenuation as a remediation strategy; b) determine whether native microorganisms or commercially available bioaugmentation consortia can degrade target contaminants when provided lactate as an electron donor; c) evaluate the potential for enhanced abiotic CT degradation; and d) examine the degradation of CT when microcosms receive reactive iron species (ZVI, FeS, Fe₃O₄), commercially available bioaugmentation consortia, and lactate. The microcosm data suggests that bioaugmentation could be an effective remediation strategy for PCE, 1,1-DCE, and CF. Unfortunately, CT, CFC-11, and CFC-12 were not susceptible to degradation by the bioaugmentation culture. Furthermore, CT was degraded by reactive iron species, but more work needs to be done to determine an effective remediation strategy for CFC-11 and CFC-12.

Introduction

The industrial site was originally a Freon production plant, though Freon production has not occurred there for 26 years. Monitoring wells have identified various contaminants, including tetrachloroethene (PCE), trichloroethene (TCE), 1,1-dichloroethene (1,1-DCE), carbon tetrachloride (CT), chloroform (CF), trichlorofluoromethane (CFC-11), and dichlorodifluoromethane (CFC-12) in the groundwater at the site. An air stripper is currently being used to remove volatile organic compounds (VOCs) from the groundwater, but implementation of additional remediation strategies is desired to accelerate remediation efforts at the site. The area of interest for this study was a portion of the site referred to as Area A. Area A was chosen by the technical team as the area of interest. There is currently no industrial activity occurring at Area A.

Technologies were used to characterize Area A, include membrane interface probe-hydraulic profiling tool (MiHPT) pushes, measurements of volatile organic carbon in the groundwater and sediment, and measurements of dissolved iron. Four borehole locations were selected along a transect in Area A, and MiHPT pushes were performed at those locations. These pushes measure VOCs, soil electrical conductance, and soil permeability. Sampling intervals for sediment and groundwater were chosen based on the possible presence of contaminants and variation in soil permeability, as indicated by the MiHPT pushes. Portions of the collected aquifer material were sent off for measurements of contaminants and iron in the collected groundwater and sediment. The rest of the aquifer material was used to set up microcosms for the treatability study.

Laboratory-based bench-scale treatability studies are performed to assess the potential for various remediation strategies to meet site-specific cleanup goals, aid in selecting a remediation strategy, and inform pilot-scale testing or, in some cases, inform full-scale remediation design.¹⁴² Site-specific cleanup goals are selected to conform to regulatory standards. In the United States,

the Environmental Protection Agency (EPA) sets maximum contaminant levels (MCLs) for contaminants, and site-specific cleanup goals are often set to meet these MCLs. Monitored natural attenuation (MNA), enhanced abiotic degradation, and enhanced *in situ* bioremediation (EISB) technologies are commonly tested during treatability studies for sites impacted by chlorinated, volatile organic solvents.

EISB technologies utilize organohalide-respiring bacteria (OHRB), which use contaminants as energy sources.¹⁴³ In contaminated aquifers, OHRB are often limited by the availability of electron donors. The addition of fermentable substrate (*i.e.*, biostimulation) increases the flux of hydrogen (H₂), a key electron donor for OHRB. If OHRB are not present or active at a contaminated site, then contaminant-degrading microbial consortia containing OHRB are available, and their injection (*i.e.*, bioaugmentation) into a contaminated aquifer can increase contaminant degradation rates and extents.¹⁴⁴ Bioaugmentation is typically performed in combination with biostimulation (*i.e.*, the addition of fermentable substrates) to increase H₂ flux and to ensure efficient contaminant detoxification.

The presence and abundance of OHRB in an impacted aquifer can be determined by quantitative polymerase chain reaction (qPCR). qPCR assays targeting specific reductive dehalogenase (RDase) genes utilized by OHRB are available because the OHRB involved in the degradation of chlorinated solvents are reasonably well understood. For several contaminants, specific RDase enzyme systems have been identified.¹⁴⁵ Moreover, qPCR-based monitoring regimes can provide quantitative data about OHRB and provide information about bioremediation progress.¹⁴⁶

EISB technologies are not applicable to all contaminants. For example, OHRB capable of respiring CT, CFC-11, or CFC-12 have not been identified, though co-metabolic CT degradation has been documented.^{147,148} Microbial co-metabolism of CT is due to fortuitous reactions between CT and reduced transition metal co-factors, such as corrinoids (Krone et al., 1991). CT is also susceptible to degradation by magnetite (a reactive mixed ferrous/ferric iron mineral), and microbial processes generating magnetite can indirectly promote CT degradation.^{122,149} Furthermore, CT is susceptible to abiotic degradation by zero-valent iron (ZVI) and ferrous sulfide (FeS).^{150,151} No specific organisms have been identified that dehalogenate CFC-11 and CFC-12, but dechlorination has been observed under anoxic conditions.^{85,93}

This study uses MiHPT data, and VOC measurements to characterize the contaminant profile of Area A at the industrial site. DNA is extracted from Sterivex filters collected at the site to determine the presence and abundance of biomarker genes for OHRB via qPCR. Microcosms are also set up to determine the potential of biostimulation, bioaugmentation, and amendments with reactive iron species as remediation strategies at an industrial site.

Methods

Membrane Interface Probe-Hydraulic Profiling Tool (MiHPT) Logging.

MiHPT pushes were advanced with a Track Rig-TC10 drill rig along a transect in Area A. The MiHPT push locations were selected with an expectation of encountering zones of high and low contamination. The membrane interface probe (MIP) is 24 inches long and has a diameter of 1.5

inches. Components included on the MiHPT probe are a photoionization detector, a flame ionization detector, and a halogen-specific detector.¹⁵² The photoionization detector uses a 10.6 electron volt (eV) lamp and detects compounds with double or triple bonds, which have ionization energies below 10.6 eV. The flame ionization detector measures ions produced during combustion and detects any combustible compound. The halogen-specific detector detects halogens by measuring the current produced when halogens react with alkali atoms on the surface of electrically charged platinum beads. Checks of the MIP components were performed by testing known concentrations of TCE, CFC-11, CT, and 1,1-DCE on the MiHPT probe. The hydraulic profiling tool (HPT) portion of the MiHPT probe injects water at a rate of between 50 mL and 500 mL per minute and uses a downhole transducer to measure hydraulic pressure, and an electrical conductivity (EC) dipole measures electrical conductivity. The MiHPT probe was advanced at a rate of 12 inches per minute with a Track Rig-TC10 drill rig in boreholes formed by a cone penetration test (CPT) probe. The Track Rig-TC10 drill rig is a direct push machine that uses the vehicle's static weight and percussive pushes to advance tools and probes into the ground.

Dissipation tests were performed by stopping the injection of water from the HPT and recording hydrostatic pressure once the pressure had stabilized. Measurements of atmospheric pressure and the hydrostatic pressure throughout the borehole were used to calculate the depth of the water table and the corrected HPT pressure. Then, the HPT flow rate and the calculated corrected pressure were fed into an empirical model to estimate hydraulic conductivity (K).¹⁵³

Aquifer Material Sampling.

Sampling intervals were decided by the technical team, and the Löffler lab **Table 3**, Appendix B. Sampling intervals were selected to capture variation in lithological properties throughout the aquifer and variation in contaminant profile, as suggested by the MiHPT data. The depth and location of the four sampling intervals can be seen in **Table 3**, Appendix B. A Track Rig-TC10 drilling rig and a 30-cm Doris-tube piston samplers (Adara Systems, Richmond, BC) were used to collect aquifer material at the designated sampling intervals. Aquifer material was recovered in stainless steel sleeves, visually inspected for lithological properties, capped to limit atmospheric exposure, and labeled by members of the technical team. The aquifer material samples were sent to Pace Analytical laboratories for VOC analysis following the EPA method SW846-8260B, and to the Löffler laboratory for bench-scale treatability tests. The samples were shipped on wet ice to maintain a temperature of ~ 4 °C.

Groundwater Sampling.

Groundwater samples were taken at approximately the same sampling intervals as the aquifer material samples **Table 3**, Appendix B. Team members recommended the use of three groundwater sampling techniques due to the variable aquifer conditions. The Hydropunch technique was used in areas of the aquifer that had relatively high permeability, while temporary wells were set up in areas of the aquifer with relatively low permeability, and an existing monitoring well was used when the sampling interval was close to the aquitard. Estimates of aquifer permeability were based on hydraulic conductivity profiling provided by the CPT and MiHPT pushes.

Groundwater sampling at Borehole A1 (BHA1) used the hydropunch technique. The Hydropunch tool was advanced to the desired sampling depth by direct push using the Track Rig-TC10 drill rig. At the desired sampling depth, the Hydropunch's cone-tipped, 30-cm slotted piston was unsheathed, exposing the screen to the aquifer and allowing water to enter the direct pushrods, and a stainless steel retention valve and disposable tubing were used to verify that the aquifer was responding consistently during manual development. Micro-purging and low-flow sampling were conducted using a Geotech 0.675-inch outer diameter bladder pump.

Temporary wells were set up for groundwater sampling at Boreholes A3 and A4. Temporary wells were installed by advancing direct push rods containing a 0.75-inch inner diameter polyvinyl chloride (PVC) well screen and riser pipes to the desired sampling depths. The well screen was exposed to the aquifer by raising the direct pushrods a minimum of 1.5 m once the desired sampling depth was reached. Well rods were secured at the surface with clamps. A stainless steel retention valve and disposable tubing were used to verify that the aquifer was responding consistently during manual development. Micro-purging and low-flow sampling were conducted using a Geotech 0.675-inch outer diameter bladder pump.

Groundwater sampling from an existing monitoring well occurred near Borehole A3 (BHA3) to access groundwater near the aquitard. A Geotech 1.66-inch outer diameter bladder pump was used to conduct micro-purging and low-flow sampling inside of the existing monitoring well (Geotech, Denver, CO).

A Horiba U-50 multiparameter meter was used to measure the temperature, pH, oxidation-reduction potential (ORP), specific conductance, turbidity, dissolved oxygen, water level, and purge rate of the groundwater. Team members from the technical team, and the Löffler lab took turns monitoring the Horiba U-50 multiparameter meter parameters during the micro-purging of wells under low flow conditions. Groundwater samples were not taken until the parameters measured by the Horiba U-50 device were stable. Four types of groundwater samples were taken: groundwater for the treatability study microcosms was collected in 1-l bottles; groundwater for the total metals analysis was distributed (not-filtered) into 100-mL bottles containing 1% nitric acid; groundwater for VOC analysis was collected in 40-mL bottles; and groundwater was filtered through Sterivex filters for qPCR. These groundwater samples were stored and shipped on wet ice to maintain a temperature of ~4 °C.

Borehole Abandonment.

Team members used a Geoprobe grout pump to fill each borehole with cement-free bentonite slurry. Boreholes that were less than 20 m deep were grouted by connecting PVC pipes to the grout pump, and those greater than 20 m deep were grouted by connecting direct pushrods to the grout pump. Boreholes located in paved areas were cemented over at the surface once they had been grouted with bentonite slurry.

Volatile Organic Compounds (VOC) analysis.

Pace Analytical Laboratories performed VOC analysis of sediment and groundwater samples. The EPA method SW846-8260B was used to identify VOCs in the samples (Pace Analytical, Minneapolis, MN).

Total Iron Measurements.

Groundwater samples from each of the boreholes and monitoring well PZ2 were received for analysis of total iron by inductively coupled argon plasma optical emission spectrometry (ICP-OES). These samples were stored at 4 °C until they were given to the Water Quality Core Facility at the University of Tennessee, Knoxville, on January 7th. The ICP-OES method used by this facility is based on the EPA method 200.7.

Quantitative Polymerase Chain Reaction (qPCR).

Team members from the technical team, and the Löffler lab collaborated in obtaining field samples for qPCR at the groundwater sampling locations. The Sterivex-GP cartridges (Millipore, Billerica, MA) were identified by borehole location and shipped on wet ice to the Löffler lab. Upon arrival, the cartridges were immediately stored at -80 °C to preserve the collected biomass. They were then processed by members of the Löffler lab for DNA analysis. Following the manufacturer's recommendations, DNA was isolated from the Sterivex-GP cartridges using the MoBio PowerLyzer PowerSoil Kit (MoBio, Carlsbad, CA). Moreover, according to the manufacturer's manual, DNA concentrations were quantified using the Qubit dsDNA BR assay (Life Technologies, Grand Island, NY). DNA solutions were stored at -80 °C until analysis. For qPCR analysis, each sample was diluted to three different dilutions using nuclease-free water (*i.e.*, 1:10, 1:100, and 1:1000) to determine whether any inhibitors were present that would interfere with the qPCR analysis. The *General Bacteria* 16S rRNA gene-targeted qPCR assay was chosen to demonstrate any interference from unidentified compounds in each sample. Upon analysis of the qPCR results, if the results determined no interfering contaminants present, then the most diluted sample that exhibited the best fit within the template DNA standard curve was chosen for further qPCR analysis (*i.e.*, 1:10 dilution). Standard curves were prepared using synthetic linear DNA fragments obtained from GeneArt (Life Technologies, Grand Island, NY). The synthetic linear DNA fragment standard curves spanned a concentration range of approximately 10 to 10⁸ target gene copies/mL and were prepared using a 10-fold serial dilution series of template DNA. All standard curves had a total of eight calibration points. qPCR targets were the *Dehalococcoides mccartyi* 16S rRNA gene, the *Dehalobacter restrictus* 16S rRNA gene, and the *Dehalogenimonas* sp. 16S rRNA gene to determine whether the chlorinated compound-degrading bacteria were present at levels significant enough to observe degradation activity.^{154–156}

Microcosm Setup and Monitoring.

Sediment and groundwater samples were stored at 4 °C until microcosm setup which was performed by members of the Löffler lab and the technical team. All 60-mL glass serum bottles and black butyl rubber stoppers were cleaned and autoclaved before microcosm setup (Bellco Glass, Vineland, NJ, USA). Reduced anoxic mineral salts medium (RAMM) was prepared in 300-mL serum bottles with 200 mL of RAMM and 100 mL of headspace under anoxic conditions following established procedures.¹⁵⁷ Groundwater (600 mL) was aliquoted into 1-L glass serum bottles closed with butyl rubber stoppers and crimp caps, and it was flushed with oxygen-free nitrogen (N₂) for 30 min to remove any remaining VOCs associated with the groundwater. The compounds of concern, except CFC-11 and CFC-12, were added to individual 300-mL or 1-L bottles containing sterilized RAMM and groundwater, respectively, using dedicated Hamilton glass syringes **Table 4**, Appendix B. Additionally, the vessels containing RAMM received 5 mM lactate from a 1 M anoxic stock solution. The vessels were shaken at 60

rpm for 1–2 days at room temperature to achieve equilibration before the medium was dispensed inside the anoxic chamber into individual 60-mL serum bottles. This procedure ensured that all microcosms received the same amount of the respective contaminant of concern and an electron donor (RAMM microcosms only). Since CFC-11 and CFC-12 are highly volatile, these compounds were added directly into individual microcosms after setup **Table 4**, Appendix B. CFC-11 (liquid) or CFC-12 (gas) with a volume of 3 μ L or 0.3 mL, respectively, was directly injected into microcosms to achieve aqueous phase concentrations of 0.25 mM. The physical properties used to calculate the aqueous phase concentrations of each contaminant are reported in **Table 5**, Appendix B.

A sediment extrusion apparatus was designed and built to remove the aquifer material from the stainless-steel core casing **Figure 5**, Appendix B. Extruded aquifer material was collected in sterilized glass Mason jars under a constant flow of sterile N₂ gas to avoid air exposure. The Mason jars containing the aquifer material, the 1-L vessels with groundwater, the 1-L vessels with RAMM, the sterilized 60-mL glass serum bottles, and sterilized metal spatulas were transferred into an anoxic chamber containing 2–3% H₂ with a balance of N₂ **Figure 6**, Appendix B.

Following the transfer of all materials into the anoxic chamber, the aquifer material, groundwater, and RAMM were used to construct 60-mL batch microcosms containing ~6 g (wet weight) of aquifer material and 40 mL of associated groundwater or RAMM. The solid core aquifer materials were manually homogenized with a sterilized spatula inside the Mason jars prior to dispensing 6-g aliquots into 60-mL serum bottles. RAMM stocks containing 5 mM lactate were used for microcosms designated to receive 5 mM lactate to test the effects of biostimulation. Lactate was added by injecting 0.2 mL of a 1 M lactate stock solution with 1-mL plastic syringes for biostimulation microcosms prepared with groundwater.

Following microcosm setup, all glass serum bottles were immediately capped with butyl rubber stoppers, removed from the anoxic chamber, and sealed with aluminum crimp caps. All microcosms were equilibrated overnight, and negative control microcosms were autoclaved on two consecutive days.

The following day, each microcosm was measured for initial concentrations of chlorinated compounds in the headspace using a gas chromatograph equipped with a flame ionization detector (GC-FID). To quantify lower CF concentrations, microcosms containing CF were analyzed using a gas chromatograph equipped with a micro-electron capture detector (GC- μ ECD). The GC-FID (Agilent GC 7890A) was equipped with a DB-624 column (60-m length, 320- μ m diameter, and 1.8- μ m film thickness) and used the Agilent OpenLab (Rev. C.01.06[61]) software (Agilent, Santa Clara, CA). The GC- μ ECD (Agilent GC 7890A) was equipped with an Agilent HP-PLOT/Q column (30-m length, 320- μ m diameter, 20- μ m film thickness) and used Agilent ChemStation version G1701EA MSD. Headspace samples were collected with gastight Hamilton #1725 syringes fitted with a Luer Lock adapter to allow for the use of sterile needles (25G 7/8). Before sampling, the Hamilton syringes were flushed with sterile N₂ gas. Once inserted into the sample bottle, the syringe was flushed three times with headspace gas before 100 μ L of headspace sample was collected. The gas sample was manually injected into the GC. Once all samples had been run, the peak area for each chlorinated compound present was

recorded. Standard curves were applied to calculate each contaminant's concentrations in a given sample based on the raw peak area data. Detailed instrument settings used for the analysis of headspace samples with gas chromatography are summarized in **Table 6**, Appendix B and **Table 7**, Appendix B. To distinguish methane from ethene in the PCE or 1,1-DCE microcosms, 100- μ L headspace measurements were made using a GC-FID (Agilent GC 7890B) equipped with a J&W HP-PlotQ column (30-m length, 535- μ m diameter, and 40- μ m film thickness) and used the Agilent OpenLab (Rev. C.01.06[61]) software (Agilent, Santa Clara, CA).

Additionally, samples were taken from each microcosm to measure the initial lactate concentrations using high-performance liquid chromatography (HPLC). A volume of 0.6 mL of liquid was removed from each microcosm with a 1-mL, N₂-flushed, sterile plastic syringe and a 25G 7/8" needle. The samples were filtered through a 0.2- μ m membrane into 1.5-mL plastic Eppendorf tubes and frozen at -20 °C until measurements could be taken. Preparation of samples for measurement on the HPLC required the samples to be thawed and centrifuged at 10,000 x g for 5 min. Then, 300 μ L from each sample was transferred to an HPLC vial, acidified with 2 μ L 1 M sulfuric acid, and placed in the HPLC autosampler rack where it was injected into the HPLC.

To ensure that reducing conditions had been established in the microcosms, an additional 0.2 mM Na₂S was added to all microcosms from a 100 mM sterilized stock solution. Resazurin, a redox indicator, was added to the RAMM medium to monitor the redox condition in the microcosms visually. Resazurin is pink when the redox potential is above -110 mV, and it turns clear when the redox potential is below -110 mV. Once the medium had turned clear, the bioaugmentation consortia were added to the designated microcosms **Table 9**, Appendix B. Microcosms containing PCE or 1,1-DCE were inoculated with 1.2 mL (3% v/v) of the SDC-9 consortium (APTIM), and microcosms containing CT or CF were inoculated with 1.2 mL (3% v/v) of the KB-1 Plus consortium (SiREM). Dechlorination of CFC-11 and CFC-12 by sulfate-reducing bacteria was observed.¹⁵⁸ To stimulate the growth of sulfate-reducing bacteria and possibly enhance the degradation of CFC-11 and CFC-12, sulfate was added to some CFC-11- and CFC-12-containing microcosms **Table 9**, Appendix B. Slurries (0.5 g/mL) of FeS, ZVI, and Fe₃O₄ were prepared inside the glove box by weighing out the minerals and transferring them to separate 60-mL glass serum bottles and adding anoxic water using a 25-mL plastic pipette. While dispensing the three aforementioned compounds to individual microcosms, the bottles with the stock slurries were gently mixed while the suspension was withdrawn with a 3-mL syringe with an 18-gauge needle. Then 2 mL of the slurry was immediately dispensed into the designated microcosm **Table 9**, Appendix B. Each microcosm received 1 g of the designated reactive iron mineral, and all microcosms were incubated upside down in the dark under static conditions at room temperature with periodic measurements, and the results were recorded in Excel spreadsheets.

A summary of the microcosms established as part of this treatability study is provided in **Table 9**, Appendix B. All microcosms were set up in duplicate, except for some heat-killed controls, which consisted of one heat-killed microcosm containing groundwater and a second heat-killed microcosm containing RAMM. Each microcosm received a sample ID. Each sample ID was assigned a letter a–e, with the letter representing the aquifer material used to set up the microcosms as follows: (a) microcosms containing aquifer material from BHA1-12 m, (b)

microcosms containing aquifer material from BHA3-7 m, (c) microcosms containing aquifer material from BHA3-15 m, (d) microcosms containing aquifer material from BHA3-24 m, and (e) microcosms containing aquifer material from BHA4-10 m. The numerical portion of the sample ID is an identifier used to log information about that microcosm. Three microcosms were mislabeled during setup and are identified as b-15-2nd, b-27-2nd, and b80-2nd.

Standard Curve Preparation of Gas Chromatograph Equipped with a Flame Ionization Detector (GC-FID) and Gas Chromatograph Equipped with a Micro-Electron Capture Detector (GC- μ ECD).

Standard curves for each chlorinated compound of interest and their predicted degradation products were established **Table 8**, Appendix B. Stock solutions of PCE, TCE, *c*DCE, 1,1-DCE, CT, and CF were prepared in ice-cold methanol and shaken vigorously. To prepare individual standards with the desired amounts of PCE, TCE, *c*DCE, 1,1-DCE, CT, and CF, designated volumes of the stock solutions were transferred to 60-mL glass serum bottles containing 40 mL of anoxic Milli-Q water. The bottles were shaken at room temperature for at least 24 h prior to sampling. Stocks for VC and ethene were prepared using 125-mL glass serum bottles filled with N₂ at 1 atmosphere. VC and ethene were added with 3-mL plastic syringes, and serial dilutions of the VC and ethene stock bottles were performed in 60-mL bottles to achieve the desired amounts for the standards. All bottles containing gases without liquid received approximately 3–5 glass beads so that the gasses could be efficiently mixed by shaking.

CFC-11 has a boiling point of 23.82 °C at 1 atmosphere of pressure, and the stock solution of CFC-11 at a concentration of 10 mg/mL was prepared gravimetrically in ice-cold methanol. Standards of CFC-11 at different concentrations were prepared in 60-mL serum bottles containing 40 mL of MilliQ-H₂O and 20 mL of headspace. Different volumes (1 μ L to 1,000 μ L) of the CFC-11 stock solution (10 mg/mL) were injected into the 60-mL serum bottles to achieve the desired amounts. Furthermore, CFC-12 has a boiling point of -29.79 °C and is a gas at room temperature. Standards of CFC-12 were also prepared in 60-mL serum bottles with 40 mL of MilliQ-H₂O and 20 mL of headspace. Different volumes of CFC-12 gas, ranging from 10 μ L to 2,000 μ L, were injected into serum bottles to achieve the desired amounts for standard curve preparation.

Standards for PCE, TCE, *c*DCE, and 1,1-DCE, VC, ethene, CT, CF, DCM, methane, CFC-11, and CFC-12 were measured using the GC-FID, and CF, DCM, and CM standards were measured using the GC- μ ECD. Standard curves for the compounds of concern and possible degradation products are depicted in **Figure 7**, Appendix B and **Figure 8**, Appendix B.

Data Management.

Data generated during the treatability studies (*i.e.*, HPLC, qPCR and GC measurements) were manually entered into Excel spreadsheets. Calculations of contaminant, methane, ethene and organic acid concentrations were performed in Excel, and graphs were generated. A CSV spreadsheet containing data on the percentage of actively dehalogenating microcosms was uploaded into R and used to create the heat maps **Figure 13**, Appendix B and **Figure 14**, Appendix B. The code used to generate these figures can be found in the appendix. The qPCR data was generated by the QuantStudio 12K Flex system (Applied Biosystems) in CSV format. The quantity of each sample was imported in to Excel and then used to calculate the gene

copies/mL.¹⁴⁶ The qPCR data is shown in **Table 18**, Appendix B. The MiHPT push data were shared with the Löffler lab as PDF files. In addition, dissolved iron data were generated by the Water Quality Core Facility at the University of Tennessee, Knoxville, and shared with the Löffler lab in the form of an Excel spreadsheet. Finally, groundwater and sediment VOC data were generated by Pace Analytical and shared with the Löffler lab in the form of Excel spreadsheets.

Results

MiHPT Overview.

Six separate graphs are presented in **Figure 9**, Appendix B through **Figure 12**, Appendix B for each MiHPT push location. The Y axis for all graphs is depth, which is reported in meters below ground surface (mbgs). From left to right, the graphs display EC dipole data, followed by the PID, FID, and XSD graphs. The HPT graph illustrates average pressure in black and HPT flow in red, and finally, estimated values for K are graphed in the last panel. EC is reported in (mS/m); coarse grained particles such as sand are indicated by low EC values, and fine grained particles such as silts and clays are indicated by high EC values. The PID, FID, and XSD data is all reported in ($\mu\text{V} \times 10^5$), so that comparisons between boreholes can be made. Furthermore, HPT pressure is reported in (kPa), and HPT flow is reported in (mL/min). The rule of thumb for HPT data is that a lower pressure and a higher flow rate indicate greater permeability in the aquifer material, while a higher pressure and a lower flow rate indicate lower permeability in the aquifer material.

Borehole A1 (BHA1)—MiHPT Data.

The PID, FID, and XSD signals from the BHA1 MiHPT push suggest the presence of a combustible compound or compounds containing double or triple bonds, and a low degree of halogenation present between 12.50 mbgs and 14 mbgs **Figure 9**, Appendix B. The compound of interest that fits these data best is 1,1-DCE, or the degradation product VC. The PID, FID, and XSD peaks at 18.50 mbgs also suggest the presence of compounds containing double or triple bonds, and a low degree of halogenation, possibly 1,1-DCE or VC. Sediment and groundwater samples were taken between 12.25 mbgs and 13.15 mbgs for microcosm setup and VOC analysis. The HPT data revealed an average pressure of ~200 kPa between 8.5 mbgs and 18.5 mbgs. These pressure data indicate an interval of lower permeability materials and a potential storage zone for contaminants in the aquifer. The measurements from the EC dipole suggest the presence of silt in the interval spanning 0–12 mbgs and a transition to clay in the interval spanning 12–19 mbgs. Dissipation tests could not be performed in BHA1 due to failure of the HPT pump, so the depth of the water table and hydraulic conductivity were not estimated in BHA1.

Borehole A2 (BHA2)—MiHPT Data.

The lack of any response from the PID, FID, and XSD detectors during the BHA2 push suggests that no contaminants of interest were present at BHA2 **Figure 10**, Appendix B. Therefore, no sediment or groundwater samples were taken from this location. The HPT data indicates that in BHA2, the average pressure from 8.25 to 11.25 mbgs was ~200 kPa, thereby implying a zone of lower permeability. In BHA2 from 11.50 mbgs to 20 mbgs, the average pressure was ~750 kPa;

the high pressure in this interval highlights an area with low permeability and a potential storage zone for contaminants. Furthermore, the measurements from the EC dipole suggest the presence of silt in the interval from 8.25 mbgs to 11.25 mbgs and clay in the interval from 11.50 mbgs to 20 mbgs. The depth of the water table was estimated to be 8.5 mbgs at BHA2. Estimates for hydraulic conductivity report K being as low as 0 mL per day and as high as 9 mL per day in zones containing fine-grained, lower-permeability materials and courser-grained, higher-permeability materials, respectively.

Borehole A3 (BHA3)—MiHPT Data.

The PID, FID, and XSD signals from the BHA3 MiHPT push suggest the presence of a noncombustible, highly halogenated compound between 7.00 mbgs and 8.00 mbgs **Figure 11**. The compounds of interest that these signals best represent are CT, CFC-11, and CFC-12. The PID, FID, and XSD peaks between 14.00 mbgs and 15.00 mbgs indicate the presence of a compound or compounds containing double or triple bonds, and a medium degree of halogenation, possibly TCE, 1,1-DCE, or the degradation product VC. The PID, FID, and XSD peaks between 24.25 mbgs and 26.00 mbgs point to the presence of a combustible compound or compounds containing double or triple bonds. The lack of halogenation suggests that the compound or compounds are not contaminants of interest. These signals demonstrate the presence of hydrocarbons or possibly the degradation product ethene. Moreover, the HPT data indicates that the average pressure in BHA3 from 8.00 mbgs to 27.00 mbgs was ~750 kPa. This interval of high pressure implies an area with low permeability, and a potential storage zone for contaminants. The EC measurements in BHA3 from 8.00 mbgs to 27.00 mbgs were consistently above 100 mS/m, thus suggesting the presence of clay materials. Additionally, the water table depth at BHA3 was estimated to be 6.5 mbgs. Finally, estimates for hydraulic conductivity report K being as low as 0 mL per day and as high as 45 mL per day in zones containing fine-grained. Lower-permeability materials and in pockets containing courser-grained, higher-permeability materials, respectively.

Borehole A4 (BHA4)—MiHPT Data.

The PID, FID, and XSD signals from the BHA4 MiHPT push suggest the presence of a compound containing double or triple bonds, and a low degree of halogenation, possibly TCE, 1,1-DCE, or the degradation product VC **Figure 12**, Appendix B. The HPT data indicates that the average pressure in BHA4 from 4.5 mbgs to 9.00 mbgs was ~750 kPa. The high pressure in this interval demonstrates an area with lower permeability. In BHA4 from 9.00 mbgs to 11.50 mbgs, the average pressure was ~300 kPa; this pressure points to an area of higher permeability. Furthermore, the water table was estimated to begin at ~8.75 mbgs. EC measurements greater than 100 mS/m and high HPT pressure indicate the presence of a non-porous material such as clay in the area between the ground surface and the water table. The low HPT pressure and EC measurements around 60 mS/m between 9 mbgs and 12 mbgs suggest an area containing silt. Additionally, the K estimations suggest that there is some hydraulic conductivity throughout the interval from 9 mbgs and 12 mbgs. K values in the interval between 9 m and 12 m vary between 0 mL and 20 mL per day.

Sediment and Groundwater VOC Data.

Table 10, Appendix B shows that no contaminants were detected in the sediment samples from BHA1 (12.85–13.00 mbgs) or BHA3 (7.40–7.55 mbgs). CT and CF were detected in the

sediment sample from BHA3 (14.55–14.75 mbgs), while CF was the only contaminant detected in BHA3 (24.50–24.75 mbgs). Moreover, PCE was detected in the sediment sample from BHA4 (10.55–10.75 mbgs). The VOC analysis of groundwater from BHA1 (12.25–12.50 mbgs) and BHA3 (12.80–14.50 mbgs) identified CT, CF, and PCE **Table 11**, Appendix B. The amounts of contaminants detected was higher in the samples from BHA3 (12.80–14.50 mbgs) than in samples from BHA1 (12.25–12.50 mbgs). The highest amounts of CT, CF, CFC-11, CFC-12, PCE, and 1,1-DCE were detected in BHA3 (19.70–24.70 mbgs). No contaminants were detected in the groundwater sample from BHA4 (9.95–11.30 mbgs).

Total Iron Data.

Table 12, Appendix B reports the values of total iron in the groundwater samples identified by ICPOES. Notably, the groundwater sample from BHA4 has a higher concentration of iron than the other samples.

Overview of Treatability Study Microcosm Performance.

To generate, **Figure 13**, Appendix B and **Figure 14**, Appendix B microcosm performance was deemed successful if contaminant degradation occurred, and unsuccessful if degradation of the target contaminant did not occur. Percentages were determined based on the number of successful microcosms from each borehole that received the same treatment. Microcosms tested for the same remediation strategy, but set up with different aqueous phases (*i.e.*, groundwater or RAMM) were considered to be part of the same group when determining the percent of successful microcosms. VOC concentrations over time for each contaminant, borehole, and remediation strategy can be seen in **Figure 15**, Appendix B through **Figure 45**, Appendix B. All plots showing VOC concentrations over time show data from one representative microcosm, replicate microcosms behaved similarly, and are mentioned in the text but are not included in **Figure 15**, Appendix B through **Figure 45**, Appendix B.

Degradation of 1,1-DCE, CT, CF, CFC-11, or CFC-12 was not observed in any microcosms where MNA was tested as a remediation strategy **Figure 13**, Appendix B. In contrast, degradation of PCE and the formation of TCE, cDCE, VC, and ethene was observed under MNA conditions 1 year after initial set up in all microcosms set up with aquifer material from BHA3-7 m, BHA3-15 m, and BHA4-10 m. Furthermore, no activity was observed in any heat-killed microcosms. In addition, biostimulation was not a successful remediation strategy in microcosms that contained 1,1-DCE, CT, CF, CFC-11, or CFC-12. Some transformation of PCE was observed in biostimulation microcosms set up with aquifer material from BHA3-15 m, BHA3-7 m, and BHA4-10 m. Degradation was observed in all PCE and 1,1-DCE microcosms bioaugmented with SDC-9 and biostimulated with lactate. Degradation of CF was observed in microcosms set up with aquifer material from BHA1-12 m, BHA3-15 m, and BHA3-24 m that were bioaugmented with KB-1 Plus and biostimulated with lactate. CF degradation was observed in bioaugmented microcosms set up with sediment and RAMM from BHA3-7m and BHA4-10 m, but not in microcosms where groundwater was the liquid phase. It is possible that redox conditions were not favorable for reductive dechlorination in the microcosms containing groundwater from BHA3-7 m and BHA4-10 m, but this observation is ambiguous as a redox indicator was not added to the microcosm set up with groundwater.

Additional remediation strategies were tested for CT **Figure 14**, Appendix B. Transformation of CT to CF was observed in all microcosms that were amended with ZVI or FeS. Amending CT-containing microcosms with Fe₃O₄ was successful in microcosms set up with aquifer material from BHA4-10 m. Similarly, combining reactive iron species, biostimulation, and bioaugmentation followed the same pattern of activity as the reactive iron species alone. However, in microcosms where reactive iron species, biostimulation, and bioaugmentation were combined, CT was degraded to non-halogenated end products **Figure 18**, **Figure 19**, **Figure 25**, **Figure 31**, **Figure 37**, are all found in Appendix B.

Lactate Fermentation and Microcosm Performance: Borehole A1 at 12-m depth (BHA1-12 m).

Lactate fermentation was observed under biostimulation and bioaugmentation conditions in all microcosms that contained PCE, 1,1-DCE, or CF **Table 13**, Appendix B. In microcosms that contained CT, lactate fermentation occurred under bioaugmentation conditions, but not under biostimulation conditions. **Table 13**, Appendix B lists the percentage of microcosms in which lactate fermentation occurred under those conditions for microcosms that contained PCE, 1,1-DCE, CT, and CF.

PCE (BHA1-12 m).

No PCE degradation products including TCE, DCEs, VC, and ethene were observed in killed control microcosms (a1, a2; **Figure 15**, Appendix B). PCE degradation products were also not observed in microcosms established with aquifer material from BHA1-12 m under natural attenuation (a3) and biostimulation (a4, a5 a9, a10) conditions, regardless of whether groundwater or RAMM was used for microcosm setup. The degradation of PCE and the formation of ethene were only observed in the microcosms that received lactate and were bioaugmented with the SDC-9 consortium (a6, a7 and a11, a12). The killed control vessels were heat inactivated, and some PCE loss occurred during the autoclave cycle, which explains the lower initial PCE amounts in these vessels (*e.g.*, a2). Some loss of PCE occurred over time, as evidenced in vessels without transformation product formation (*e.g.*, a3, a4, a9). Additional measurements were taken for these microcosms 1 year after the initial setup, and no PCE degradation was apparent.

1,1-DCE (BHA1-12 m).

No 1,1-DCE degradation products including VC and ethene were observed in killed control microcosms (a13, a14) established with BHA1-12 m aquifer material. 1,1-DCE degradation products were also not observed in microcosms mimicking natural attenuation (a15) or biostimulation (a16, a17 and a21, a22) conditions, regardless of whether groundwater or RAMM was used for microcosm setup **Figure 16**, Appendix B. The formation of VC and ethene was only observed in microcosms amended with 5 mM of lactate and the SDC-9 consortium (a18, a19 and a23, a24). Additional measurements were taken for these microcosms 1 year after the initial setup, and no 1,1-DCE degradation was seen.

CF (BHA1-12 m).

The degradation of CF and the formation of DCM were observed in bioaugmentation microcosms established with aquifer material from BHA1-12 m for microcosms established with groundwater (a30, a31) or RAMM (a35, a36), as illustrated in **Figure 17**, Appendix B. No CF

degradation was observed in microcosms without the addition of the bioaugmentation consortium KB-1 Plus. Active microcosms a30, a31, a35, and a36 were re-spiked with an additional 12 $\mu\text{mol/bottle}$ of CF on Day 112; this is indicated by a star in **Figure 17**, Appendix B. The killed control vessels were heat inactivated, and some CF loss occurred during the autoclave cycle (*e.g.*, a25). Some loss of CF occurred over time, as evidenced in vessels without transformation product formation (*e.g.*, a34). Additional measurements were taken for these microcosms 1 year after the initial setup, and no CF degradation was observed.

CT (BHA1-12 m).

Microcosms a55–a62 were set up without aquifer material but received the KB-1 Plus culture to test whether this culture alone could mediate CT transformation **Figure 18**, Appendix B. Moreover, microcosms a55 and a56 were set up without additional reactive iron minerals to determine whether the FeS present in the KB-1 Plus consortium was sufficient to degrade the amount of CT added to the microcosms. The potential degradation products of CT (*i.e.*, CF, DCM, CM, and methane) were not observed in microcosms a55 and a56. Microcosms a57–a62 were then amended with KB-1 Plus and a reactive iron species. Microcosms a57 and a58 contained ZVI, while microcosms a59 and a60 contained additional FeS, and microcosms a61 and a62 contained Fe₃O₄ (magnetite). Degradation of CT was observed in microcosms a57, a58, a59, and a60, but not in microcosms a61 and a62. Unexpectedly, the magnetite used in these experiments (50–100-nm particles purchased from Sigma-Aldrich) was not active toward CT.

In microcosms established with aquifer material from BHA1-12m, CT degradation products including CF, DCM, CM, and methane were not observed in killed control microcosms (a37, a38) or natural attenuation microcosms (a63, a64), as illustrated in **Figure 19**, Appendix B. CT degradation products were also not observed in biostimulation (a39, a40, a41, a42) and bioaugmentation (a47, a48) microcosms, independent of whether groundwater or RAMM was used as the liquid phase. CT was transformed to CF in microcosms amended with ZVI (a43, a44) or FeS (a83, a84). However, degradation products were not observed in microcosms amended with Fe₃O₄ (a85, a86). In microcosms amended with KB-1 Plus and additional ZVI (a49, a50) or FeS (a51, a52), the degradation products CF and DCM were observed. Degradation products were also not observed in microcosms amended with KB-1 Plus and Fe₃O₄ (a53, a54). Furthermore, in heat-killed control vessels, some CT loss occurred during the autoclave cycle (*e.g.*, a38). Some loss of CT occurred over time, as evidenced in vessels without transformation product formation (*e.g.*, a46, a56, a64, a86). Active microcosms (a43, a44, a83, a84, a49, a50, a51, a52, a57, a58, a59, a60) were re-spiked with an additional 10 $\mu\text{mol/bottle}$ of CT on Day 118, as indicated by a star in **Figure 19**, Appendix B. Additional measurements were taken for these microcosms 1 year after the initial setup, and no CT degradation was observed.

CFC-11 (BHA1-12 m).

During a 7-month monitoring period, no degradation of CFC-11 was observed in any of the microcosms **Figure 20**, Appendix B. Slight decreases in CFC-11 concentrations were observed during the first 2 month of incubation.

CFC-12 (BHA1-12 m).

CFC-12 was recalcitrant in all microcosms. During the 7-month monitoring period, no degradation of CFC-12 was observed in microcosms without additions (natural attenuation),

biostimulation with lactate, biostimulation with sulfate, or biostimulation with lactate combined with bioaugmentation with the KB-1 Plus consortium **Figure 21**, Appendix B.

Lactate Fermentation and Microcosm Performance: Borehole A3 at 7-m Depth (BHA3-7 m).

Lactate fermentation was observed under biostimulation conditions in all microcosms that contained PCE or CF **Table 14**, Appendix B. In microcosms that contained CT, lactate fermentation under biostimulation conditions was observed in microcosms that contained RAMM as the liquid phase (b43, b44), but not in microcosms that contained groundwater as the liquid phase (b41, b42). In addition, in microcosms containing 1,1-DCE lactate, fermentation occurred under biostimulation conditions in microcosms b16, b17, and b22, but not in b21, which contained RAMM as the liquid phase. Lactate fermentation was observed under bioaugmentation conditions in all microcosms that contained PCE, 1,1-DCE, CF, or CT **Table 14**, Appendix B.

PCE (BHA3-7 m).

No PCE degradation products including TCE, DCEs, VC, and ethene were observed in killed control microcosms (b1, b2). PCE degradation products were also not observed in microcosms established with aquifer material from BHA3-7 m under natural attenuation (b3, b3-2nd) and biostimulation (b4, b5 b9, b10) conditions, independent of whether groundwater or RAMM was used for microcosm setup **Figure 22**, Appendix B. Degradation of PCE and the formation of ethene were only observed in the microcosms that received lactate and were bioaugmented with the SDC-9 consortium (b6, b7 and b11, b12). The killed control vessels were heat inactivated, and some PCE loss occurred during the autoclave cycle (e.g., b2). Some loss of PCE occurred over time, as evidenced in vessels without transformation product formation (e.g., b3, b4, b9). Additional measurements were taken for these microcosms 1 year after the initial setup, and no additional PCE degradation was seen, with the exception of one microcosm (b10) initially established in RAMM and biostimulated with 5 mM lactate. Further investigation is required to confirm degradation.

1,1-DCE (BHA3-7 m).

No 1,1-DCE degradation products including VC and ethene were observed in killed control microcosms (b13, b14) established with BHA3-7 m aquifer material. 1,1-DCE degradation products were also not observed in microcosms mimicking natural attenuation (b15, b15-2nd) or biostimulation (b16, b17 and b21, b22) conditions, regardless of whether groundwater or RAMM was used for microcosm setup **Figure 23**, Appendix B. The formation of VC and ethene was only observed in microcosms amended with 5 mM lactate and the SDC-9 consortium (b18, b19 and b23, b24). Additional measurements were taken for these microcosms 1 year after the initial setup, and no additional 1,1-DCE degradation was seen.

CF (BHA3-7 m).

The degradation of CF and the formation of DCM were observed in bioaugmentation microcosms established with aquifer material from BHA3-7 m for microcosms established with RAMM (b35, b36), as illustrated in **Figure 24**, Appendix B. No CF degradation was observed in groundwater microcosms with or without bioaugmentation with consortium KB-1 Plus. In contrast, CF degradation was observed in microcosms established with RAMM and bioaugmented with consortium KB-1 Plus (b35, b36). Active microcosms were re-spiked with an

additional 12 μmol /bottle of CF on Day 102, as indicated by a star in **Figure 24**, Appendix B. Some loss of CF occurred over time, as evidenced in vessels without transformation product formation (e.g., b29). Additional measurements were taken for these microcosms 1 year after the initial setup, and no additional CF degradation was observed.

CT (BHA3-7 m).

In microcosms established with aquifer material from BHA3-7 m, CT degradation products including CF, DCM, CM, and methane were not observed in killed control microcosms (b39, b40), as illustrated in **Figure 25**, Appendix B. CT degradation products were also not observed in biostimulation (b41, b42, b43, b44) and bioaugmentation (b51, b52, b53, b54) conditions, regardless of whether groundwater or RAMM was used as the liquid phase. However, CT was degraded to CF in near stoichiometric ratios when ZVI (b45, b46) or FeS (b47, b48) were added to the system. Furthermore, degradation products were not observed in microcosms amended with Fe_3O_4 (b49, b50), whereas in microcosms amended with KB-1 Plus and ZVI (b55, b56) or FeS (b57, b58), the degradation products CF and DCM were observed. Degradation products were not observed in microcosms amended with KB-1 Plus and Fe_3O_4 (b59, b60). Active microcosms (b45, b46, b47, b48, b55, b56, b57, b58) were re-spiked with an additional 10 μmol /bottle of CT on Day 102, as indicated by a star in **Figure 25**, Appendix B. Some loss of CT occurred over time, as evidenced in vessels without transformation product formation (e.g., b51, b59). The obvious CT concentration decline in the heat-killed controls (b39, b40) was likely due to improper handling—these microcosms were accidentally incubated in an upright position, causing increased sorption to the rubber stopper. Additional measurements were taken for these microcosms 1 year after the initial setup, and no additional CT degradation was observed.

CFC-11 (BHA3-7 m).

During the 7-month monitoring period, no degradation was observed in microcosms that tested natural attenuation capacity or that were biostimulated with either lactate, sulfate, or lactate combined with bioaugmentation with consortium KB-1 Plus **Figure 26**, Appendix B. Figure 26 illustrates the CFC-11 microcosm performance for Borehole A3-7 m.

CFC-12 (BHA3-7 m).

During the 7-month monitoring period, no degradation of CFC-12 was observed in microcosms testing natural attenuation capacity. Degradation was also not observed in microcosms that were biostimulated with either lactate, sulfate, or lactate combined with bioaugmentation with consortium KB-1 Plus **Figure 27**, Appendix B. Figure 27 illustrates the CFC-11 microcosm performance for Borehole A3-7 m.

Lactate Fermentation and Microcosm Performance: Borehole A3 at 15-m Depth (BHA3-15 m).

Lactate fermentation was observed under biostimulation and bioaugmentation conditions in all microcosms that contained PCE, 1,1-DCE, CF, or CT **Table 15**, Appendix B. Table 15 lists the percentage of microcosms in which lactate fermentation occurred under those two conditions.

PCE (BHA3-15 m).

No PCE degradation products including TCE, DCEs, VC, and ethene were observed in killed control microcosms (c1, c2), nor were such products observed in microcosms established with

aquifer material from BHA3-15 m under natural attenuation (c3) and biostimulation (c4, c5 c9, c10) conditions, independent of whether groundwater or RAMM was used for microcosm setup **Figure 28**, Appendix B. Degradation of PCE and the formation of ethene were only observed in the microcosms that received lactate and were bioaugmented with the SDC-9 consortium (c6, c7 and c11, c12). Furthermore, the killed control vessels were heat inactivated, and some PCE loss occurred during the autoclave cycle (*e.g.*, c2). Some loss of PCE also occurred over time, as evidenced in vessels without transformation product formation (*e.g.*, c3, c4, c9). As with all previously mentioned experiments, additional measurements were taken for these microcosms 1 year after the initial setup. Potential additional PCE degradation was seen under certain conditions, namely, natural attenuation (c3) and biostimulation with 5 mM lactate (c4, c5, c10). However, further investigation is required to confirm degradation.

1,1-DCE (BHA3-15 m).

No 1,1-DCE degradation products including VC and ethene were observed in killed control microcosms (c13, c14) established with BHA3-15 m aquifer material. 1,1-DCE degradation products were also not observed in microcosms mimicking natural attenuation (c15) or biostimulation (c16, c17 and c21, c22) conditions, regardless of whether groundwater or RAMM was used for microcosm setup **Figure 29**, Appendix B. The formation of VC and ethene was only observed in microcosms amended with 5 mM lactate and the SDC-9 consortium (c18, c19 and c23, c24). Additional measurements were taken for these microcosms 1 year after the initial setup, and no additional 1,1-DCE degradation was seen.

CF (BHA3-15 m).

Degradation of CF and the formation of DCM were observed in bioaugmentation microcosms established with aquifer material from BHA3-15 m established with groundwater (c30, c31) or RAMM (c35, c36), as illustrated in **Figure 30**, Appendix B. In contrast, no CF degradation was observed in microcosms without the addition of the bioaugmentation consortium KB-1 Plus. Active microcosms c30, c31, c35, and c36 were re-spiked with an additional 12 µmol/bottle of CF on Day 105, as indicated by a star in **Figure 30**, Appendix B. The killed control vessels were heat inactivated, and some CF loss occurred during the autoclave cycle, which explains the lower initial CF amounts in these vessels (*e.g.*, c26). Some loss of CF also occurred over time, as evidenced in vessels without transformation product formation (*e.g.*, c27, c28, c33). Additional measurements were taken for these microcosms 1 year after the initial setup, and no additional CF degradation was observed.

CT (BHA3-15 m).

Degradation of CT was not observed in heat-killed controls and in biostimulation or bioaugmentation microcosms. In addition, in microcosms established with aquifer material from BHA3-15 m, potential CT degradation products including CF, DCM, CM, and methane were not observed in killed control microcosms (c37, c38), as illustrated in **Figure 31**, Appendix B. CT degradation products were also not observed in biostimulation (c39, c40, c41, c42) and bioaugmentation (c49, c50, c51, c52) microcosms, independent of whether groundwater or RAMM was used as the liquid phase. However, CT was degraded to CF in near stoichiometric ratios when ZVI (c43, c44) or FeS (c45, c46) was added to the system. Moreover, degradation products were not observed in microcosms amended with Fe₃O₄ (c47, c48), but in microcosms amended with KB-1 Plus and additional ZVI (c53, c54) or FeS (c55, c56), the degradation

products CF and DCM were observed. Degradation products were also not observed in microcosms amended with KB-1 Plus and Fe_3O_4 (c57, c58). Active microcosms (c43, c44, c45, c46, c53, c54, c55, c56) were re-spiked with an additional 10 $\mu\text{mol/bottle}$ of CT on Day 106, as indicated by a star in **Figure 31**, Appendix B. The killed control vessels were heat inactivated, and some CT loss occurred during the autoclave cycle (e.g., c38). Furthermore, some loss of CT occurred over time, as evidenced in vessels without transformation product formation (e.g., c36, c48, c49, c58). Additional measurements were taken for these microcosms 1 year after the initial setup, and no additional CT degradation was observed.

CFC-11 (BHA3-15 m).

During the 7-month monitoring period, no degradation of CFC-11 was observed in microcosms testing natural attenuation capacity, or microcosms that were biostimulated with either lactate, sulfate, or lactate combined with bioaugmentation with consortium KB-1 Plus **Figure 32**, Appendix B. Figure 32 illustrates the CFC-11 microcosm performance for Borehole A3-15 m.

CFC-12 (BHA3-15 m).

During the 7-month monitoring period. No degradation of CFC-12 was observed in microcosms testing natural attenuation capacity or in microcosms that were biostimulated with either lactate, sulfate, lactate combined with bioaugmentation with consortium KB-1 Plus **Figure 33**, Appendix B. Figure 33 illustrates the CFC-12 microcosm performance for Borehole A3-15 m.

Lactate Fermentation and Microcosm Performance: Borehole A3 at 24-m Depth (BHA3-24 m).

Lactate fermentation was observed under biostimulation conditions in all microcosms that contained PCE, CT, or CF. In microcosms that contained 1,1-DCE, lactate fermentation under biostimulation conditions was observed in microcosms that contained RAMM as the liquid phase (d21, d22), but not in microcosms that contained groundwater as the liquid phase (d15, d16).

lists the percentage of microcosms in which lactate fermentation occurred under those two conditions for microcosms that contained PCE, 1,1-DCE, CT, and CF.

PCE (BHA3-24 m).

No PCE degradation products including TCE, DCEs, VC, and ethene were observed in killed control microcosms (d1, d2). PCE degradation products were also not observed in microcosms established with aquifer material from BHA3-24 m under natural attenuation (d3) and biostimulation (d4, d5 d9, d10) conditions, regardless of whether groundwater or RAMM was used for microcosm setup **Figure 34**, Appendix B. Degradation of PCE and the formation of ethene were only observed in the microcosms that received lactate and were bioaugmented with the SDC-9 consortium (d6, d7 and d11, d12). The killed control vessels were heat inactivated, and some PCE loss occurred during the autoclave cycle (e.g., d2). In addition, some loss of PCE occurred over time, as evidenced in vessels without transformation product formation (e.g., d3, d4, d9). Additional measurements were taken for these microcosms 1 year after the initial setup, and no additional PCE degradation was seen.

1,1-DCE (BHA3-24 m).

No 1,1-DCE degradation products including VC and ethene were observed in killed control microcosms (d13, d14) established with BHA3-24 m aquifer material, nor were those

degradation products observed in microcosms mimicking natural attenuation (d15) or biostimulation (d16, d17 and d21, d22) conditions, independent of whether groundwater or RAMM was used for microcosm setup **Figure 35**, Appendix B. The formation of VC and ethene was only observed in microcosms amended with 5 mM lactate and the SDC-9 consortium (d18, d19 and d23, d24). Additional measurements were taken for these microcosms 1 year after the initial setup, and no additional 1,1-DCE degradation was seen.

CF (BHA3-24 m).

Degradation of CF was observed in bioaugmentation microcosms established with aquifer material from BHA3-24 m for microcosms established with groundwater (d30, d31) or RAMM (d35, d36), as illustrated in **Figure 36**, Appendix B. Furthermore, the production of DCM was observed in microcosms d30 and d31, but not in microcosms d35 and d36. DCM was presumably further degraded in microcosms d35 and d36. Moreover, no CF degradation was observed in microcosms without the addition of the bioaugmentation consortium KB-1 Plus. Active microcosms d30, d31, d35, and d36 were re-spiked with an additional 12 μmol /bottle of CF on Day 112, as indicated by a star in **Figure 36**, Appendix B. Some loss of CF occurred over time, as evidenced in vessels without transformation product formation (*e.g.*, d29). Additional measurements were taken for these microcosms 1 year after the initial setup; degradation of CF in bioaugmented microcosms that contained groundwater (d30 and d31) has concluded, and no chlorinated methanes were identified in these microcosms.

CT (BHA3-24 m).

Degradation of CT was not observed in heat-killed controls, nor in biostimulation and bioaugmentation microcosms. Moreover, in microcosms established with aquifer material from BHA3-24 m, CT degradation products including CF, DCM, CM, and methane were not observed in killed control microcosms (d37, d38), as **Figure 37**, Appendix B illustrates. CT degradation products were also not observed in biostimulation (d39, d40, d41, d42) and bioaugmentation (d49, d50, d51, d52) microcosms, regardless of whether groundwater or RAMM was used as the liquid phase. However, CT was degraded to CF in near stoichiometric ratios when ZVI (d43, d44) or FeS (d45, d46) was added to the system. Furthermore, degradation products were not observed in microcosms amended with Fe_3O_4 (d47, d48), whereas in microcosms amended with KB-1 Plus and ZVI (d53, d54) or FeS (d55, d56), the degradation products CF and DCM were observed. Degradation products were also not observed in microcosms amended with KB-1 Plus and Fe_3O_4 (d57, d58). Active microcosms (d43, d44, d45, d46, d53, d54, d55, d56) were re-spiked with an additional 10 μmol /bottle of CT on Day 113, as indicated by a star in **Figure 37**, Appendix B. Some loss of CT occurred over time, as evidenced in vessels without transformation product formation (*e.g.*, d39, d41, d48, d50, d51, d60). Additional measurements were taken for these microcosms 1 year after the initial setup, and no additional CT degradation was observed.

CFC-11 (BHA3-24 m).

During the 7-month monitoring period, no degradation of CFC-11 was observed in microcosms testing natural attenuation capacity or in microcosms that were biostimulated with either lactate, sulfate, or lactate combined with bioaugmentation with consortium KB-1 Plus, as illustrated in **Figure 38**, Appendix B. Measurements after an extended incubation period of around 1 year

revealed no significant degradation of CFC-11 in all microcosms, and CFC-11 remained persistent in the microcosms.

CFC-12 (BHA3-24 m).

During the 7-month monitoring period at BHA3-24 m, no degradation of CFC-12 was observed in any of the microcosms (*i.e.*, those that tested natural attenuation capacity or that were biostimulated with either lactate, sulfate, or lactate combined with bioaugmentation with consortium KB-1 Plus; **Figure 39**, Appendix B. Measurements after an extended incubation period of approximately 1 year showed no significant degradation of CFC-12 in all microcosms, and CFC-12 remained persistent in the microcosms.

Lactate Fermentation and Microcosm Performance: Borehole A4 at 10-m Depth (BHA4-10 m).

Lactate fermentation was observed under biostimulation and bioaugmentation conditions in all microcosms that contained PCE, 1,1-DCE, CF, or CT **Table 17**, Appendix B. Table 17 presents the percentage of microcosms in which lactate fermentation occurred under those two conditions for the aforementioned microcosms.

PCE (BHA4-10 m).

No PCE degradation products including TCE, DCEs, VC, and ethene were observed in killed control microcosms (e1, e2). PCE degradation products were also not observed in microcosms established with aquifer material from BHA4-10 m under natural attenuation (e3, e4) and biostimulation (e4, e5 e9, e10) conditions, independent of whether groundwater or RAMM was used for microcosm setup **Figure 40**, Appendix B in the first 9 months of observation. Degradation of PCE and the formation of ethene were only observed in the microcosms that received lactate and were bioaugmented with the SDC-9 consortium (e6, e7 and e11, e12). The killed control vessels were heat inactivated, and some PCE loss occurred during the autoclave cycle (*e.g.*, e2). In addition, some loss of PCE occurred over time, as evidenced in vessels without transformation product formation (*e.g.*, e3, e4, e9). Additional measurements were taken for these microcosms 1 year after the initial setup. Potential additional PCE degradation was seen under biostimulated conditions in microcosms created with groundwater (e5, e6) as well as those created with RAMM (e9, e10). However, further investigation is required to confirm degradation.

1,1-DCE (BHA4-10 m).

No 1,1-DCE degradation products including VC and ethene were observed in killed control microcosms (e13, e14) established with BHA4-10 m aquifer material. Such products were also not observed in microcosms mimicking natural attenuation (e15, e16) or biostimulation (e17, e18 and e21, e22) conditions, regardless of whether groundwater or RAMM was used for microcosm setup **Figure 41**, Appendix B. The formation of VC and ethene was only observed in microcosms amended with 5 mM lactate and the SDC-9 consortium (e18, e19 and e23, e24). Additional measurements were taken for these microcosms 1 year after the initial setup. No additional 1,1-DCE degradation was seen.

CF (BHA4-10 m).

Degradation of CF and the formation of DCM were observed in bioaugmentation microcosms established with aquifer material from BHA4-10 m for microcosms established with RAMM (e35, e36), depicted in **Figure 42**, Appendix B. However, no CF degradation was observed in microcosms established with KB-1 Plus and groundwater, or without the addition of the bioaugmentation consortium KB-1 Plus. Active microcosms e35 and e36 were re-spiked with an additional 12 μmol /bottle of CF on Day 102, as indicated by a star in **Figure 42**, Appendix B. The killed control vessels were heat inactivated, and some CF loss occurred during the autoclave cycle (*e.g.*, e25). Some loss of CF also occurred over time, as evidenced in vessels without transformation product formation (*e.g.*, e27, e30). Additional measurements were taken for these microcosms 1 year after initial setup, and no additional CF degradation was observed.

CT (BHA4-10 m).

In microcosms established with aquifer material from BHA4-10 m, potential CT degradation products including CF, DCM, CM, and methane were not observed in killed control microcosms (e37, e38), as illustrated in **Figure 43**, Appendix B. CT degradation products were also not observed in biostimulation (e39, e40, e41, e42) and bioaugmentation (e49, e50, e51, e52) microcosms, independent of whether groundwater or RAMM was used as the liquid phase. Furthermore, CT was degraded to CF in near stoichiometric ratios when ZVI (e43, e44), FeS (e45, e46), or Fe_3O_4 (e47, e48) was added to the system. In microcosms amended with KB-1 Plus and additional ZVI (e53, e54), FeS (e55, e56), or Fe_3O_4 (e57, e58), the degradation products CF and DCM were observed. Some loss of CT occurred over time, as evidenced in vessels without transformation product formation (*e.g.*, e38, e41, e50, e52). By accident, all of the microcosms established with BHA4-10 m aquifer material were incubated with the stopper up and the headspace in contact with the stopper between Day 60 and Day 94, resulting in increased CT loss due to sorption. Active microcosms and microcosms that experienced CT loss (e37, e38, e41, e42, e43, e44, e45, e56, e47, e48, e49, e51, e52, e53, e54, e55, e56, e57, e58) were re-spiked with an additional 10 μmol /bottle of CT on Day 104, as indicated by a star in **Figure 43**, Appendix B. Additional measurements were taken for these microcosms 1 year after the initial setup, and no additional CT degradation was observed.

CFC-11 (BHA4-10 m).

Degradation of CFC-11 was not observed in any microcosms during the 7-month monitoring period—neither in microcosms testing natural attenuation capacity nor in microcosms that were biostimulated with either lactate, sulfate, or lactate combined with bioaugmentation with consortium KB-1 Plus **Figure 44**, Appendix B. Figure 44 depicts the CFC-11 microcosm performance at BHA4-10 m.

CFC-12 (BHA4-10 m).

During the 7-month monitoring period, no degradation of CFC-12 was observed in any microcosms (*i.e.*, not in microcosms testing natural attenuation capacity, nor in microcosms that were biostimulated with one of the following: lactate, sulfate, or lactate combined with bioaugmentation with consortium KB-1 Plus) **Figure 45**, Appendix B. Figure 45 illustrates the CFC-12 microcosm performance at BHA4-10 m.

qPCR Data from Sterivex Filters.

qPCR analysis was used to determine the abundance of OHRB (*i.e.*, *Dhc*, *Dhb*, and *Dhgm*) in the groundwater from Sterivex cartridges collected at the site. The abundance of reductive dechlorination biomarkers (*i.e.*, *tceA*, *vcrA*, *bvcA*, and *cerA* genes) is typically used to demonstrate reductive dechlorination activity in groundwater; however, to reliably determine the abundance of those biomarkers, sufficient quantities of OHRB must be present (Clark et al., 2018). A cell titer of 10^7 *Dhc* cell per liter is regarded as a minimum amount of cells needed to sustain useful rates of reductive dechlorination at a contaminated site. Due to the low abundance of *Dhc*, *Dhb*, and *Dhgm* 16S rRNA genes in the groundwater, RDase genes were not quantified **Table 18**, Appendix B. The qPCR analysis demonstrated that the *Dhc* 16S rRNA gene was not detected in any of the borehole locations that were sampled in this study. The qPCR analysis also revealed that the *Dhb* 16S rRNA gene was present in boreholes BER-RT1-BHA4-SF1 (ST-5A), BER-RT1-BHA4-SF5 (ST-17A), and BER-RT1-BHA4-SF6 (ST-6B) at an abundance of approximately 10^2 cells per liter. The *Dhb* 16S rRNA gene was not detected in any other borehole. Finally, based on the qPCR analysis, the *Dhgm* 16S rRNA gene was not detected in any of the boreholes.

Discussion

Data from the MiHPT pushes and VOC analysis of the sediment and groundwater provide information on contaminant distribution in Area A. Data from the MiHPT pushes and groundwater samples analyzed for VOCs identified a broader range of contaminants and higher contaminant concentrations at sampling locations BHA1 and BHA3 than in samples from BHA2 or BHA4. These data suggest that additional sampling near the diagonal southern boundary of Area A could be useful to further characterize the site.

The microcosm treatability study evaluated the potential for anaerobic microbial degradation of the target contaminants in microcosms established with site material collected from three borehole locations in Area A. The treatability study explored contaminant transformation and degradation under anoxic conditions because prior research has demonstrated that the target contaminants (*e.g.*, PCE, 1,1-DCE, and CF) are susceptible to anaerobic degradation. The study tested the potential of native microbes (*i.e.*, microorganisms associated with the aquifer materials and the groundwater collected from the site) to degrade the target contaminants (*i.e.*, natural attenuation potential). First, microcosms amended with lactate tested whether the addition of an electron donor would have a positive effect on the degradation of the target contaminants by native microorganisms (*i.e.*, biostimulation). Second, bioaugmentation combined with biostimulation tested the potential for initiating biodegradation of the target contaminants following the injection of biodegrading microbial consortia (*i.e.*, consortium SDC-9 and consortium KB-1 Plus). Third, additional microcosms tested the potential for abiotic contaminant transformation (*i.e.*, ZVI, FeS, and Fe₃O₄).

The microbial reductive dehalogenation process requires anoxia and low redox conditions. To facilitate the establishment of redox conditions conducive to microbial reductive dehalogenation, all microcosms received 0.2 mM of sodium sulfide as a reductant. The chemical resazurin changes color from pink to clear at a redox potential of -110 mV and serves as a visual indicator of whether reducing conditions have been established in the vessels. Resazurin was present in the

microcosms with RAMM, and a single dose of 0.2 mM sulfide was observed to be insufficient to lower the redox potential in all of the microcosms, as was indicated by the pink sheen of the liquid phase. Additional sulfide was added to establish reducing conditions in the microcosms. The microcosm setup followed stringent procedures that avoided oxygen exposure and intrusion, and the observations indicate that the aquifer material was oxidizing with a positive redox potential. Groundwater samples should be analyzed for dissolved oxygen, methane, and ferrous iron to obtain additional information about the redox state of the aquifer. The keystone bacterial population involved in the degradation of the target compounds are strict anaerobes. The qPCR analysis of biomass collected from Area A groundwater samples provided little to no evidence of measurable biomarker genes of OHRB known to degrade chlorinated solvents, indicating that the site aquifer in Area A most likely does not harbor bacteria capable of degrading the target contaminants under anoxic conditions. The low abundance of known OHRB could be due to oxic aquifer conditions.

Following the addition of extra reductant (*i.e.*, sulfide), reducing conditions were established in all microcosms included in the treatability study. The degradation of target contaminants was not observed under MNA or biostimulation conditions in microcosms contain any of the other target contaminants after a 7-month incubation period. Some degradation was observed in a subset of PCE-containing microcosms set up under MNA or biostimulation conditions from BHA3-7 m, BHA3-15 m, and BHA4-10 m after a 1-year incubation period. These microcosms have been transferred, and further monitoring is underway. These observations suggest that OHRB capable of degrading the target contaminants may be present in low abundances and are heterogeneously distributed throughout Area A aquifer.

A decline in the concentration of PCE, CT, CF, and CFC-11 without the formation of transformation products was observed in microcosms from all sampling locations and conditions. This decline in concentration is attributed to sorption onto the aquifer material or the stopper. It does not indicate degradation as it occurs in the heat-killed controls and transformation products were not observed.

The degradation of PCE and 1,1-DCE was observed in microcosms that received the bioaugmentation consortium SDC-9 and lactate as an electron donor. Ethene was observed as a dechlorination product, indicating that complete reductive dechlorination occurred and a non-toxic and environmentally benign product was formed. Furthermore, the degradation of CF was observed in microcosms amended with the bioaugmentation consortium KB-1 Plus. DCM was observed as a transformation product, and it was further degraded to innocuous products. These results indicate that the aquifer material does not contain inhibitory chemicals that would inhibit the growth of OHRB. Additionally, bioaugmentation combined with biostimulation can establish conditions conducive to reductive dechlorination and lead to the degradation and detoxification of chlorinated ethenes and CF.

CT is a strong inhibitor of bacteria involved in the degradation of the other target contaminants. Therefore, CT removal should be the first step in a remediation strategy and implemented prior to biological remedies for the other contaminants. CT is susceptible to abiotic degradation and readily reacts with ZVI and FeS. The microcosm study demonstrated rapid transformation of CT following the addition of ZVI and FeS, and CF was observed as the transformation product. Data

from the bioaugmentation microcosms indicate that the amount of FeS present in KB-1 Plus is insufficient to transform the CT present in the microcosms vessels. Transformation of CT to nonhalogenated products was observed in microcosms where ZVI or FeS was added to combined bioaugmentation and biostimulation conditions. CT was also reported to be susceptible to degradation mediated by Fe₃O₄; however, the experiments performed in this treatability study failed to demonstrate CT degradation with Fe₃O₄ in microcosms set up with aquifer material from all sampling locations except for BHA4-10 m. The elevated dissolved iron concentration observed in samples from BHA4 could explain why CT was transformed to CF in microcosms amended with Fe₃O₄ and aquifer material from BHA4-10 m. Some literature suggests that surface-associated Fe(II) may be the principal reductant of CT in the presence of Fe₃O₄. Several reports in the literature suggest that the reactivity of Fe₃O₄ toward chlorinated solvents depends on the availability of ferrous iron in the system.^{159–162}

In microcosms with CT that were bioaugmented with consortium KB-1 Plus and amended with ZVI or FeS, CF and DCM were observed as transformation products. Both CF and DCM were degraded, and complete removal of chlorinated methanes was achieved. This is a relevant finding and suggests that a combined abiotic/biotic treatment can remove CT and chlorinated transformation products (*i.e.*, CF and DCM).

Conclusions

This research aimed to characterize the contaminant profile in Area A, determine the presence and abundance of biomarker genes for OHRB at sampling locations, and determine the potential of biostimulation, bioaugmentation, and amendments with reactive iron species as remediation strategies. The MiHPT, and VOC data suggests that the region of highest contamination in Area A is along the diagonal southern boundary of Area A. Performance of the MNA and biostimulation microcosms along with the qPCR data suggest that known OHRBs are absent or present in low abundances in Area A. Bioaugmentation combined with biostimulation is a promising treatment approach to detoxify chlorinated ethenes as well as CF and DCM. However, CT removal should be the first step if the goal is the subsequent implementation of an *in situ* remedial strategy. The microcosm study suggested that *in situ* CT removal can occur via ZVI treatment. The treatability study provided no evidence of CFC-11 or CFC-12 degradation, and more research is needed to determine an effective remediation strategy for these contaminants.

Appendix B

Table 3. Sampling depth intervals for aquifer material collected in Area A.

Borehole ID	Depth (mbgs)
BHA1	12.25 – 13.15
BHA3	7.55 – 8.60
BHA3	13.90 – 14.90
BHA3	24.20 – 25.00
BHA4	10.10 – 11.20

mbgs: meters below ground surface

Table 4. Halogenated compounds added to microcosms.

Chlorinated compound	Volume (μL) added	Target aqueous concentration in microcosms (mM)
PCE	• 6 μL to 300-mL vessels containing 200 mL of RAMM • 20 μL to 1-L vessels containing 600 mL of groundwater	0.2
1,1-DCE	• 6 μL to 300-mL vessels containing 200 mL RAMM • 20 μL to 1-L vessels containing 600 mL of groundwater	0.2
CFC-11	• 3 μL of neat CFC-11 liquid was directly injected into individual microcosms	0.25
CFC-12	• 0.3 mL of neat CFC-12 gas was directly injected into individual microcosms	0.25
CT	• 4 μL to 300-mL vessels containing 200 mL of RAMM • 12 μL to 1-L vessels containing 600 mL of groundwater	0.12
CF	• 2 μL to 300-mL vessels containing 200 mL RAMM • 6 μL to 1-L vessels containing 600 mL of groundwater	0.11

Table 5. Physical properties of target contaminants and projected transformation products.

Chemical	Molecular Weight (g/mol)¹⁶³	Density (g/mL)¹⁶⁴	Aqueous Solubility (mg/L)¹⁶⁴	K^oH (mol/kg*bar)¹⁶³
Tetrachloroethene	165.833	1.62	206	0.057
Trichloroethene	131.388	1.4642	1,280	0.11
<i>cis</i> -Dichloroethene	96.943	1.2837	3500	0.27
Vinyl chloride	62.498	0.9106	2700	0.038
Ethene	28.0532	– ^g	131	0.0047
Carbon Tetrachloride	153.823	1.59	793	0.033
Chloroform	119.378	1.4788	7950	0.28
Dichloromethane	84.933	1.325	13,200	0.47
Chloromethane	50.488	0.911	5040	0.12
Methane	16.0425	– ^g	22	0.0013
Trichlorofluoromethane	37.368	1.49	1,100	0.0012
Dichlorodifluoromethane	120.914	1.35	280	0.0025

(–^g) these chemicals are gasses at environmentally relevant conditions. K^o_H = Henry's law constant for solubility in water at 298.15 K

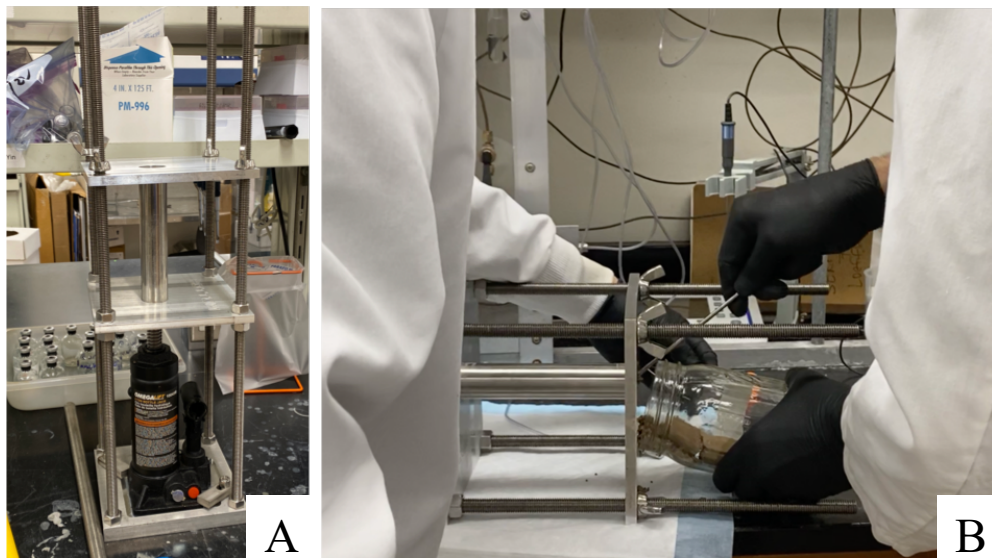


Figure 5. Aquifer material extraction from stainless steel cores.

(A) The custom-build stainless steel core extruder apparatus. (B) Aquifer material being extruded from the stainless steel core with constant flow of sterile N_2 and placed inside a sterilized Mason jar for transfer into the anoxic chamber.

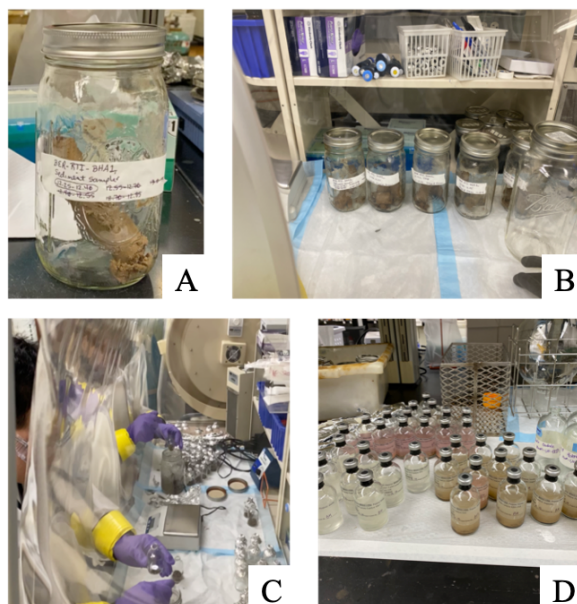


Figure 6. Microcosm setup inside an anoxic chamber.

(A) A Mason jar containing an extruded aquifer core. (B) Mason jars with aquifer material transferred inside the anoxic chamber. (C) Aquifer material (6 g) being distributed into 60-mL glass serum bottles using sterilized spatulas and a balance. (D) Labeled microcosms containing aquifer material ready for incubation and monitoring.

Table 6. Detailed instrument settings used for the analysis of headspace samples with gas chromatography - FID.

Chlorinated compounds	Inlet	Inlet/Detector Gases	(FID) Detector	Program	Column
PCE, 1,1-DCE, CFC-11, CFC-12, CT	Heater = 200 °C Pressure = 23.193 psi Septum Purge flow = 1.5 mL/min Split ratio of 50:1 w/Split flow of 150 mL/min	He = inlet N ₂ = detector	Heater = 200 °C Air Flow = 400 mL/min H ₂ Fuel flow = 30 mL/min	Initial Rate °C/min = 0 Initial Value °C = 60 Initial Hold Time min = 2 Initial Run Time min = 2 Ramp 1 Rate °C/min = 25 Ramp 1 Value °C = 200 Ramp 1 Hold Time min = 1 Ramp 1 Run Time min = 8.6	DB-624 (60 m length, 320 µm diameter, and a 1.8 µm film thickness.)
Methane, Ethene	Heater = 250 °C Pressure = 10 psi Septum Purge flow = 4 mL/min Split ratio of 9:1 w/Split flow of 78.511 mL/min	He = inlet N ₂ = detector	Heater = 200 °C Air Flow = 400 mL/min H ₂ Fuel flow = 30 mL/min	Initial Rate °C/min = 0 Initial Value °C = 60 Initial Hold Time min = 4 Initial Run Time min = 4 Ramp 1 Rate °C/min = 50 Ramp 1 Value °C = 240 Ramp 1 Hold Time min = 1 Ramp 1 Run Time min = 9.5	HP-PLOT/Q column (30 m length, 320 µm diameter, and a 20 µm film thickness.)

Table 7. Detailed instrument settings used for the analysis of headspace samples with gas chromatography - μ ecd detector.

Chlorinated compounds	Inlet	Inlet/Detector Gases	(μ ECD) Detector	Program	Column
CF, DCM, CM	Heater = 200 °C Pressure = 32.45 psi Septum Purge flow = 3 mL/min Split ratio of 100:1 w/Split flow of 400 mL/min	He = inlet N2 = detector	Heater = 250 °C N2 Flow = 15 mL/min	Initial Rate °C/min = 0 Initial Value °C = 180 Initial Hold Time min = 6 Initial Run Time min = 6	HP-PLOT/Q column (30 m length, 320 μ m diameter, and a 20 μ m film thickness.)

Table 8. Chlorinated compounds of concern and expected degradation products.

Chlorinated Compound	Expected Degradation Products
PCE	TCE, cDCE, VC, ethene
1,1-DCE	VC, ethene
CFC-11	Dichlorofluoromethane, chlorofluoromethane, fluoromethane, CF, DCM, CM, methane
CFC-12	Difluorochloromethane, difluoromethane, dichlorofluoromethane, chlorofluoromethane, DCM, CM, methane
CT	CF, DCM, CM, methane
CF	DCM, CM, methane

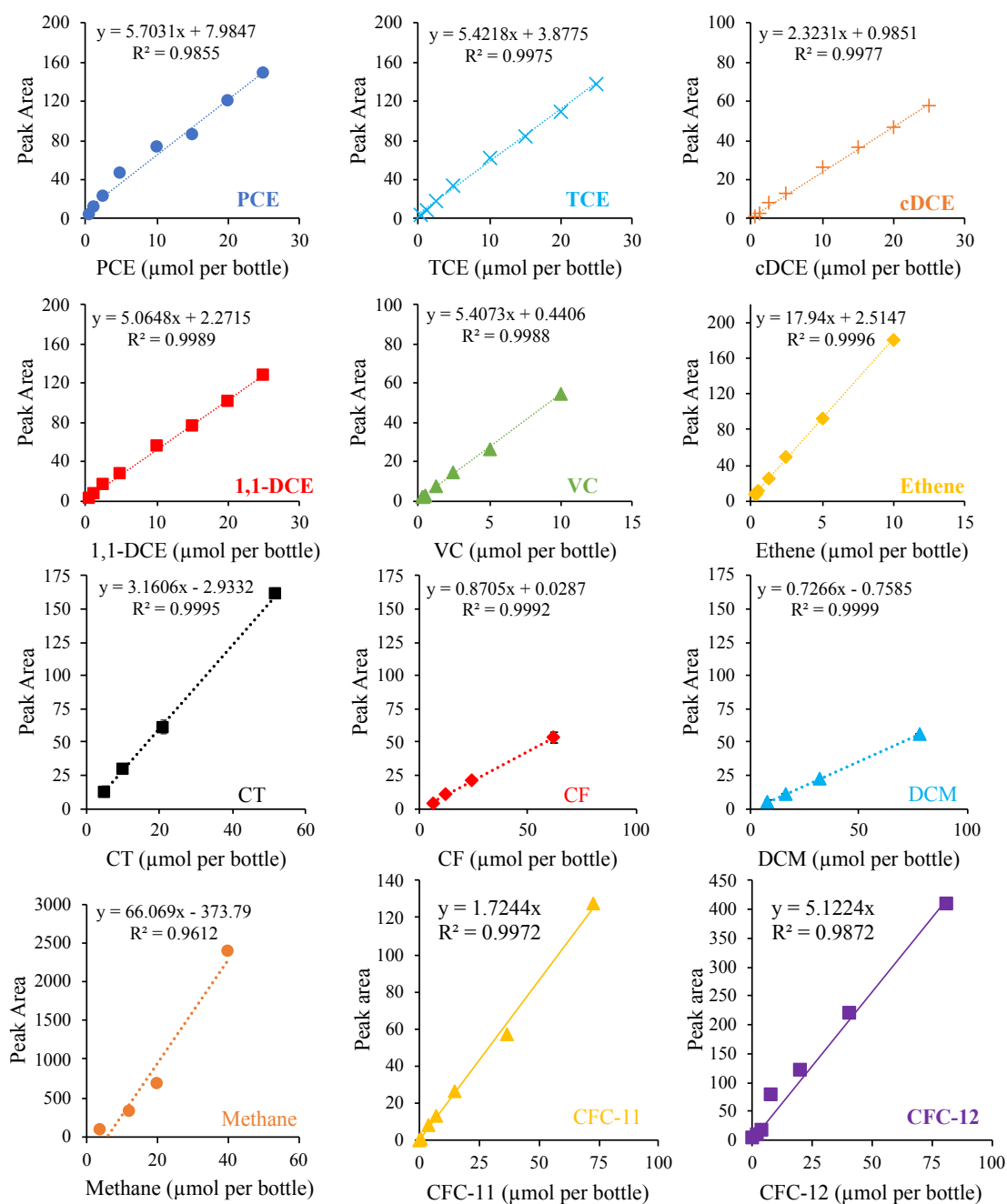


Figure 7. Standard curves for target contaminants and degradation products (GC-FID).

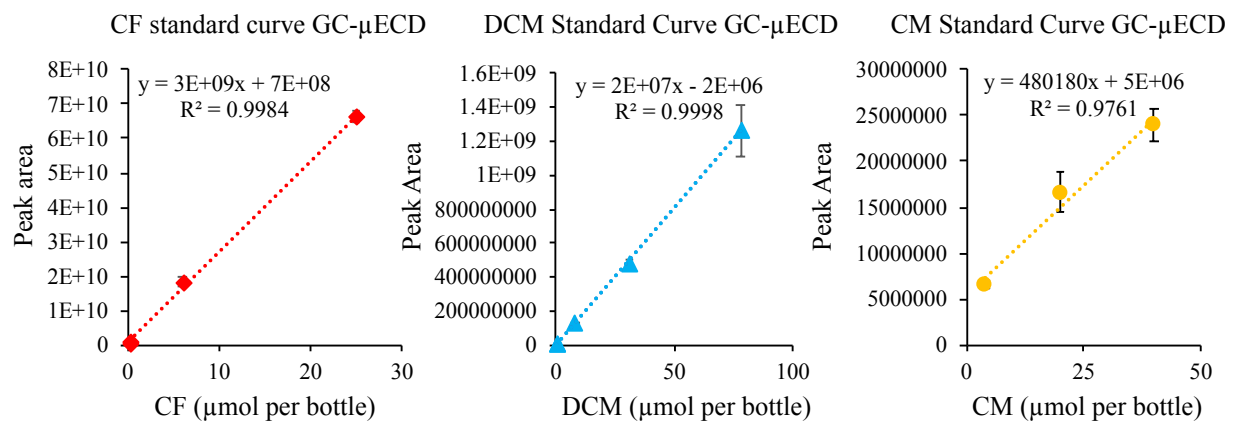


Figure 8. Standard curves for target contaminants and degradation products (GC-μECD).

Table 9. Microcosms established with aquifer core materials to assess target contaminant degradation.

Purpose	Sample ID	Aquifer Material	Liquid Phase	Compound of Concern	Methane Formation
heat kill control	a1	BHA1-12 m	GW	PCE	No
heat kill control	a2	BHA1-12 m	GW	PCE	No
natural attenuation	a3	BHA1-12 m	GW	PCE	Yes
biostimulated	a4	BHA1-12 m	GW	PCE	Yes
biostimulated	a5	BHA1-12 m	GW	PCE	Yes
bioaugmented	a6	BHA1-12 m	GW	PCE	Yes
bioaugmented	a7	BHA1-12 m	GW	PCE	Yes
heat kill control	a8	BHA1-12 m	RAMM	PCE	No
biostimulated	a9	BHA1-12 m	RAMM	PCE	Yes
biostimulated	a10	BHA1-12 m	RAMM	PCE	Yes
bioaugmented	a11	BHA1-12 m	RAMM	PCE	Yes
bioaugmented	a12	BHA1-12 m	RAMM	PCE	Yes
heat kill	a13	BHA1-12 m	GW	1,1-DCE	No
heat kill	a14	BHA1-12 m	GW	1,1-DCE	No
natural attenuation	a15	BHA1-12 m	GW	1,1-DCE	No
biostimulated	a16	BHA1-12 m	GW	1,1-DCE	No
biostimulated	a17	BHA1-12 m	GW	1,1-DCE	No
bioaugmented	a18	BHA1-12 m	GW	1,1-DCE	Yes
bioaugmented	a19	BHA1-12 m	GW	1,1-DCE	Yes
heat kill	a20	BHA1-12 m	RAMM	1,1-DCE	No
biostimulated	a21	BHA1-12 m	RAMM	1,1-DCE	No
biostimulated	a22	BHA1-12 m	RAMM	1,1-DCE	No
bioaugmented	a23	BHA1-12 m	RAMM	1,1-DCE	Yes
bioaugmented	a24	BHA1-12 m	RAMM	1,1-DCE	Yes
heat kill	a25	BHA1-12 m	GW	CF	No
heat kill	a26	BHA1-12 m	GW	CF	No
natural attenuation	a27	BHA1-12 m	GW	CF	No
biostimulated	a28	BHA1-12 m	GW	CF	No
biostimulated	a29	BHA1-12 m	GW	CF	No
bioaugmented	a30	BHA1-12 m	GW	CF	Yes
bioaugmented	a31	BHA1-12 m	GW	CF	Yes
heat kill	a32	BHA1-12 m	RAMM	CF	No
biostimulated	a33	BHA1-12 m	RAMM	CF	No
biostimulated	a34	BHA1-12 m	RAMM	CF	No
bioaugmented	a35	BHA1-12 m	RAMM	CF	Yes
bioaugmented	a36	BHA1-12 m	RAMM	CF	Yes
heat kill	a37	BHA1-12 m	GW	CT	No
heat kill	a38	BHA1-12 m	GW	CT	No
biostimulated	a39	BHA1-12 m	GW	CT	No
biostimulated	a40	BHA1-12 m	GW	CT	No
biostimulated	a41	BHA1-12 m	RAMM	CT	No

Table 9. Continued.

Purpose	Sample ID	Aquifer Material	Liquid Phase	Compound of Concern	Methane Formation
biostimulated	a42	BHA1-12 m	RAMM	CT	No
abiotic	a43	BHA1-12 m	GW	CT	Yes
abiotic	a44	BHA1-12 m	GW	CT	Yes
abiotic	a83	BHA1-12 m	GW	CT	No
abiotic	a84	BHA1-12 m	GW	CT	No
abiotic	a85	BHA1-12 m	GW	CT	No
abiotic	a86	BHA1-12 m	GW	CT	No
bioaugmented	a45	BHA1-12 m	GW	CT	Yes
bioaugmented	a46	BHA1-12 m	GW	CT	Yes
bioaugmented	a47	BHA1-12 m	RAMM	CT	Yes
bioaugmented	a48	BHA1-12 m	RAMM	CT	Yes
bioaugmented	a49	BHA1-12 m	GW	CT	Yes
bioaugmented	a50	BHA1-12 m	GW	CT	Yes
bioaugmented	a51	BHA1-12 m	GW	CT	Yes
bioaugmented	a52	BHA1-12 m	GW	CT	Yes
bioaugmented	a53	BHA1-12 m	GW	CT	Yes
bioaugmented	a54	BHA1-12 m	GW	CT	Yes
Positive control	a55	none	RAMM	CT	Yes
Positive control	a56	none	RAMM	CT	Yes
Positive control	a57	none	RAMM	CT	Yes
Positive control	a58	none	RAMM	CT	Yes
Positive control	a59	none	RAMM	CT	Yes
Positive control	a60	none	RAMM	CT	Yes
Positive control	a61	none	RAMM	CT	No
Positive control	a62	none	RAMM	CT	No
natural attenuation	a63	BHA1-12 m	GW	CT	No
natural attenuation	a64	BHA1-12 m	GW	CT	No
heat kill	a65	BHA1-12 m	GW	CFC-11	No
heat kill	a66	BHA1-12 m	RAMM	CFC-11	No
natural attenuation	a67	BHA1-12 m	GW	CFC-11	No
biostimulated	a68	BHA1-12 m	GW	CFC-11	No
biostimulated	a69	BHA1-12 m	GW	CFC-11	No
biostimulated	a70	BHA1-12 m	RAMM	CFC-11	No
biostimulated	a71	BHA1-12 m	RAMM	CFC-11	No
bioaugmented	a72	BHA1-12 m	RAMM	CFC-11	Yes
bioaugmented	a73	BHA1-12 m	RAMM	CFC-11	Yes
heat kill	a74	BHA1-12 m	GW	CFC-12	No
heat kill	a75	BHA1-12 m	RAMM	CFC-12	No
natural attenuation	a76	BHA1-12 m	GW	CFC-12	No
biostimulated	a77	BHA1-12 m	GW	CFC-12	No
biostimulated	a78	BHA1-12 m	GW	CFC-12	No
biostimulated	a79	BHA1-12 m	RAMM	CFC-12	No

Table 9. Continued.

Purpose	Sample ID	Aquifer Material	Liquid Phase	Compound of Concern	Methane Formation
biostimulated	a80	BHA1-12 m	RAMM	CFC-12	No
bioaugmented	a81	BHA1-12 m	RAMM	CFC-12	Yes
bioaugmented	a82	BHA1-12 m	RAMM	CFC-12	Yes
heat kill	b1	BHA3-7 m	GW	PCE	No
heat kill	b2	BHA3-7 m	GW	PCE	No
natural attenuation	b3	BHA3-7 m	GW	PCE	No
natural attenuation	b3	BHA3-7 m	GW	PCE	No
biostimulated	b4	BHA3-7 m	GW	PCE	No
biostimulated	b5	BHA3-7 m	GW	PCE	No
bioaugmented	b6	BHA3-7 m	GW	PCE	Yes
bioaugmented	b7	BHA3-7 m	GW	PCE	Yes
biostimulated	b9	BHA3-7 m	RAMM	PCE	No
biostimulated	b10	BHA3-7 m	RAMM	PCE	No
bioaugmented	b11	BHA3-7 m	RAMM	PCE	Yes
bioaugmented	b12	BHA3-7 m	RAMM	PCE	Yes
heat kill	b13	BHA3-7 m	GW	1,1-DCE	No No No No No No Yes Yes No No Yes Yes
heat kill	b14	BHA3-7 m	GW	1,1-DCE	
natural attenuation	b15	BHA3-7 m	GW	1,1-DCE	
natural attenuation	b15-2	BHA3-7 m	GW	1,1-DCE	
biostimulated	b16	BHA3-7 m	GW	1,1-DCE	
biostimulated	b17	BHA3-7 m	GW	1,1-DCE	
bioaugmented	b18	BHA3-7 m	GW	1,1-DCE	
bioaugmented	b19	BHA3-7 m	GW	1,1-DCE	
biostimulated	b21	BHA3-7 m	RAMM	1,1-DCE	
biostimulated	b22	BHA3-7 m	RAMM	1,1-DCE	
bioaugmented	b23	BHA3-7 m	RAMM	1,1-DCE	
bioaugmented	b24	BHA3-7 m	RAMM	1,1-DCE	
heat kill	b25	BHA3-7 m	GW	CF	
heat kill	b26	BHA3-7 m	GW	CF	
natural attenuation	b27	BHA3-7 m	GW	CF	
natural attenuation	b27-2	BHA3-7 m	GW	CF	
biostimulated	b28	BHA3-7 m	GW	CF	
biostimulated	b29	BHA3-7 m	GW	CF	
bioaugmented	b30	BHA3-7 m	GW	CF	
bioaugmented	b31	BHA3-7 m	GW	CF	
biostimulated	b33	BHA3-7 m	RAMM	CF	
biostimulated	b34	BHA3-7 m	RAMM	CF	
bioaugmented	b35	BHA3-7 m	RAMM	CF	
bioaugmented	b36	BHA3-7 m	RAMM	CF	
heat kill	b39	BHA3-7 m	GW	CT	No
heat kill	b40	BHA3-7 m	GW	CT	No
biostimulated	b41	BHA3-7 m	GW	CT	No

Table 9. Continued.

Purpose	Sample ID	Aquifer Material	Liquid Phase	Compound of Concern	Methane Formation
biostimulated	b42	BHA3-7 m	GW	CT	No
biostimulated	b43	BHA3-7 m	RAMM	CT	No
biostimulated	b44	BHA3-7 m	RAMM	CT	No
abiotic	b45	BHA3-7 m	GW	CT	Yes
abiotic	b46	BHA3-7 m	GW	CT	Yes
abiotic	b47	BHA3-7 m	GW	CT	No
abiotic	b48	BHA3-7 m	GW	CT	No
abiotic	b49	BHA3-7 m	GW	CT	No
abiotic	b50	BHA3-7 m	GW	CT	No
bioaugmented	b51	BHA3-7 m	GW	CT	Yes
bioaugmented	b52	BHA3-7 m	GW	CT	Yes
bioaugmented	b53	BHA3-7 m	RAMM	CT	Yes
bioaugmented	b54	BHA3-7 m	RAMM	CT	Yes
bioaugmented	b55	BHA3-7 m	GW	CT	Yes
bioaugmented	b56	BHA3-7 m	GW	CT	Yes
bioaugmented	b57	BHA3-7 m	GW	CT	Yes
bioaugmented	b58	BHA3-7 m	GW	CT	Yes
bioaugmented	b59	BHA3-7 m	GW	CT	Yes
bioaugmented	b60	BHA3-7 m	GW	CT	Yes
heat kill	b71	BHA3-7 m	GW	CFC-11	No
heat kill	b72	BHA3-7 m	RAMM	CFC-11	No
natural attenuation	b73	BHA3-7 m	GW	CFC-11	No
natural attenuation	b74	BHA3-7 m	GW	CFC-11	No
biostimulated	b75	BHA3-7 m	GW	CFC-11	No
biostimulated	b76	BHA3-7 m	GW	CFC-11	No
biostimulated	b77	BHA3-7 m	RAMM	CFC-11	No
biostimulated	b78	BHA3-7 m	RAMM	CFC-11	No
bioaugmented	b79	BHA3-7 m	RAMM	CFC-11	No
bioaugmented	b80	BHA3-7 m	RAMM	CFC-11	No
heat kill	b80-2	BHA3-7 m	GW	CFC-12	No
heat kill	b81	BHA3-7 m	RAMM	CFC-12	No
natural attenuation	b82	BHA3-7 m	GW	CFC-12	No
natural attenuation	b83	BHA3-7 m	GW	CFC-12	No
biostimulated	b84	BHA3-7 m	GW	CFC-12	No
biostimulated	b85	BHA3-7 m	GW	CFC-12	No
biostimulated	b86	BHA3-7 m	RAMM	CFC-12	No
biostimulated	b87	BHA3-7 m	RAMM	CFC-12	No
bioaugmented	b88	BHA3-7 m	RAMM	CFC-12	No
bioaugmented	b89	BHA3-7 m	RAMM	CFC-12	No
heat kill	c1	BHA3-15 m	GW	PCE	No
heat kill	c2	BHA3-15 m	GW	PCE	No
natural attenuation	c3	BHA3-15 m	GW	PCE	No

Table 9. Continued.

Purpose	Sample ID	Aquifer Material	Liquid Phase	Compound of Concern	Methane Formation
biostimulated	c4	BHA3-15 m	GW	PCE	No
biostimulated	c5	BHA3-15 m	GW	PCE	No
bioaugmented	c6	BHA3-15 m	GW	PCE	Yes
bioaugmented	c7	BHA3-15 m	GW	PCE	Yes
heat kill	c8	BHA3-15 m	RAMM	PCE	No
biostimulated	c9	BHA3-15 m	RAMM	PCE	No
biostimulated	c10	BHA3-15 m	RAMM	PCE	No
bioaugmented	c11	BHA3-15 m	RAMM	PCE	Yes
bioaugmented	c12	BHA3-15 m	RAMM	PCE	Yes
heat kill	c13	BHA3-15 m	GW	1,1-DCE	No
heat kill	c14	BHA3-15 m	GW	1,1-DCE	No
natural attenuation	c15	BHA3-15 m	GW	1,1-DCE	No
biostimulated	c16	BHA3-15 m	GW	1,1-DCE	No
biostimulated	c17	BHA3-15 m	GW	1,1-DCE	No
bioaugmented	c18	BHA3-15 m	GW	1,1-DCE	Yes
bioaugmented	c19	BHA3-15 m	GW	1,1-DCE	Yes
heat kill	c20	BHA3-15 m	RAMM	1,1-DCE	No
biostimulated	c21	BHA3-15 m	RAMM	1,1-DCE	No
biostimulated	c22	BHA3-15 m	RAMM	1,1-DCE	No
bioaugmented	c23	BHA3-15 m	RAMM	1,1-DCE	Yes
bioaugmented	c24	BHA3-15 m	RAMM	1,1-DCE	Yes
heat kill	c25	BHA3-15 m	GW	CF	No
heat kill	c26	BHA3-15 m	GW	CF	No
natural attenuation	c27	BHA3-15 m	GW	CF	No
biostimulated	c28	BHA3-15 m	GW	CF	No
biostimulated	c29	BHA3-15 m	GW	CF	No
bioaugmented	c30	BHA3-15 m	GW	CF	Yes
bioaugmented	c31	BHA3-15 m	GW	CF	Yes
heat kill	c32	BHA3-15 m	RAMM	CF	No
biostimulated	c33	BHA3-15 m	RAMM	CF	No
biostimulated	c34	BHA3-15 m	RAMM	CF	No
bioaugmented	c35	BHA3-15 m	RAMM	CF	Yes
bioaugmented	c36	BHA3-15 m	RAMM	CF	Yes
heat kill	c37	BHA3-15 m	GW	CT	No
heat kill	c38	BHA3-15 m	GW	CT	No
biostimulated	c39	BHA3-15 m	GW	CT	No
biostimulated	c40	BHA3-15 m	GW	CT	No
biostimulated	c41	BHA3-15 m	RAMM	CT	No
biostimulated	c42	BHA3-15 m	RAMM	CT	No
abiotic	c43	BHA3-15 m	GW	CT	Yes
abiotic	c44	BHA3-15 m	GW	CT	Yes
abiotic	c45	BHA3-15 m	GW	CT	Yes

Table 9. Continued.

Purpose	Sample ID	Aquifer Material	Liquid Phase	Compound of Concern	Methane Formation
abiotic	c46	BHA3-15 m	GW	CT	Yes
abiotic	c47	BHA3-15 m	GW	CT	No
abiotic	c48	BHA3-15 m	GW	CT	No
bioaugmented	c49	BHA3-15 m	GW	CT	Yes
bioaugmented	c50	BHA3-15 m	GW	CT	Yes
bioaugmented	c51	BHA3-15 m	RAMM	CT	Yes
bioaugmented	c52	BHA3-15 m	RAMM	CT	Yes
bioaugmented	c53	BHA3-15 m	GW	CT	Yes
bioaugmented	c54	BHA3-15 m	GW	CT	Yes
bioaugmented	c55	BHA3-15 m	GW	CT	Yes
bioaugmented	c56	BHA3-15 m	GW	CT	Yes
bioaugmented	c57	BHA3-15 m	GW	CT	Yes
bioaugmented	c58	BHA3-15 m	GW	CT	Yes
natural attenuation	c59	BHA3-15 m	GW	CT	
natural attenuation	c60	BHA3-15 m	GW	CT	
heat kill	c61	BHA3-15 m	GW	CFC-11	No
heat kill	c62	BHA3-15 m	RAMM	CFC-11	No
natural attenuation	c63	BHA3-15 m	GW	CFC-11	No
biostimulated	c64	BHA3-15 m	GW	CFC-11	No
biostimulated	c65	BHA3-15 m	GW	CFC-11	No
biostimulated	c66	BHA3-15 m	RAMM	CFC-11	No
biostimulated	c67	BHA3-15 m	RAMM	CFC-11	No
bioaugmented	c68	BHA3-15 m	RAMM	CFC-11	No
bioaugmented	c69	BHA3-15 m	RAMM	CFC-11	No
heat kill	c70	BHA3-15 m	GW	CFC-12	No
heat kill	c71	BHA3-15 m	RAMM	CFC-12	No
natural attenuation	c72	BHA3-15 m	GW	CFC-12	No
biostimulated	c73	BHA3-15 m	GW	CFC-12	No
biostimulated	c74	BHA3-15 m	GW	CFC-12	No
biostimulated	c75	BHA3-15 m	RAMM	CFC-12	No
biostimulated	c76	BHA3-15 m	RAMM	CFC-12	No
bioaugmented	c77	BHA3-15 m	RAMM	CFC-12	No
bioaugmented	c78	BHA3-15 m	RAMM	CFC-12	No
heat kill	d1	BHA3-24 m	GW	PCE	No
heat kill	d2	BHA3-24 m	GW	PCE	No
natural attenuation	d3	BHA3-24 m	GW	PCE	No
biostimulated	d4	BHA3-24 m	GW	PCE	No
biostimulated	d5	BHA3-24 m	GW	PCE	No
bioaugmented	d6	BHA3-24 m	GW	PCE	Yes
bioaugmented	d7	BHA3-24 m	GW	PCE	Yes
heat kill	d8	BHA3-24 m	RAMM	PCE	No
biostimulated	d9	BHA3-24 m	RAMM	PCE	No

Table 9. Continued.

Purpose	Sample ID	Aquifer Material	Liquid Phase	Compound of Concern	Methane Formation
biostimulated	d10	BHA3-24 m	RAMM	PCE	No
bioaugmented	d11	BHA3-24 m	RAMM	PCE	Yes
bioaugmented	d12	BHA3-24 m	RAMM	PCE	Yes
heat kill	d13	BHA3-24 m	GW	1,1-DCE	No
heat kill	d14	BHA3-24 m	GW	1,1-DCE	No
natural attenuation	d15	BHA3-24 m	GW	1,1-DCE	No
biostimulated	d16	BHA3-24 m	GW	1,1-DCE	No
biostimulated	d17	BHA3-24 m	GW	1,1-DCE	No
bioaugmented	d18	BHA3-24 m	GW	1,1-DCE	Yes
bioaugmented	d19	BHA3-24 m	GW	1,1-DCE	Yes
heat kill	d20	BHA3-24 m	RAMM	1,1-DCE	No
biostimulated	d21	BHA3-24 m	RAMM	1,1-DCE	No
biostimulated	d22	BHA3-24 m	RAMM	1,1-DCE	No
bioaugmented	d23	BHA3-24 m	RAMM	1,1-DCE	Yes
bioaugmented	d24	BHA3-24 m	RAMM	1,1-DCE	Yes
heat kill	d25	BHA3-24 m	GW	CF	No
heat kill	d26	BHA3-24 m	GW	CF	No
natural attenuation	d27	BHA3-24 m	GW	CF	No
biostimulated	d28	BHA3-24 m	GW	CF	No
biostimulated	d29	BHA3-24 m	GW	CF	No
bioaugmented	d30	BHA3-24 m	GW	CF	Yes
bioaugmented	d31	BHA3-24 m	GW	CF	Yes
heat kill	d32	BHA3-24 m	RAMM	CF	No
biostimulated	d33	BHA3-24 m	RAMM	CF	No
biostimulated	d34	BHA3-24 m	RAMM	CF	No
bioaugmented	d35	BHA3-24 m	RAMM	CF	Yes
bioaugmented	d36	BHA3-24 m	RAMM	CF	Yes
heat kill	d37	BHA3-24 m	GW	CT	No
heat kill	d38	BHA3-24 m	GW	CT	No
biostimulated	d39	BHA3-24 m	GW	CT	No
biostimulated	d40	BHA3-24 m	GW	CT	No
biostimulated	d41	BHA3-24 m	RAMM	CT	No
biostimulated	d42	BHA3-24 m	RAMM	CT	No
abiotic	d43	BHA3-24 m	GW	CT	Yes
abiotic	d44	BHA3-24 m	GW	CT	Yes
abiotic	d45	BHA3-24 m	GW	CT	Yes
abiotic	d46	BHA3-24 m	GW	CT	Yes
abiotic	d47	BHA3-24 m	GW	CT	No
abiotic	d48	BHA3-24 m	GW	CT	Yes
bioaugmented	d49	BHA3-24 m	GW	CT	Yes
bioaugmented	d50	BHA3-24 m	GW	CT	Yes
bioaugmented	d51	BHA3-24 m	RAMM	CT	Yes

Table 9. Continued.

Purpose	Sample ID	Aquifer Material	Liquid Phase	Compound of Concern	Methane Formation
bioaugmented	d52	BHA3-24 m	RAMM	CT	Yes
bioaugmented	d53	BHA3-24 m	GW	CT	Yes
bioaugmented	d54	BHA3-24 m	GW	CT	Yes
bioaugmented	d55	BHA3-24 m	GW	CT	Yes
bioaugmented	d56	BHA3-24 m	GW	CT	Yes
bioaugmented	d57	BHA3-24 m	GW	CT	Yes
bioaugmented	d58	BHA3-24 m	GW	CT	Yes
natural attenuation	d59	BHA3-24 m	GW	CT	No
natural attenuation	d60	BHA3-24 m	GW	CT	No
heat kill	d61	BHA3-24 m	GW	CFC-11	No
heat kill	d62	BHA3-24 m	RAMM	CFC-11	No
natural attenuation	d63	BHA3-24 m	GW	CFC-11	No
biostimulated	d64	BHA3-24 m	GW	CFC-11	No
biostimulated	d65	BHA3-24 m	GW	CFC-11	No
biostimulated	d66	BHA3-24 m	RAMM	CFC-11	No
biostimulated	d67	BHA3-24 m	RAMM	CFC-11	No
bioaugmented	d68	BHA3-24 m	RAMM	CFC-11	No
bioaugmented	d69	BHA3-24 m	RAMM	CFC-11	No
heat kill	d70	BHA3-24 m	GW	CFC-12	No
heat kill	d71	BHA3-24 m	RAMM	CFC-12	No
natural attenuation	d72	BHA3-24 m	GW	CFC-12	No
biostimulated	d73	BHA3-24 m	GW	CFC-12	No
biostimulated	d74	BHA3-24 m	GW	CFC-12	No
biostimulated	d75	BHA3-24 m	RAMM	CFC-12	No
biostimulated	d76	BHA3-24 m	RAMM	CFC-12	No
bioaugmented	d77	BHA3-24 m	RAMM	CFC-12	No
bioaugmented	d78	BHA3-24 m	RAMM	CFC-12	No
heat kill	e1	BHA4-10 m	GW	PCE	No
heat kill	e2	BHA4-10 m	GW	PCE	No
natural attenuation	e3	BHA4-10 m	GW	PCE	Yes
natural attenuation	e4	BHA4-10 m	GW	PCE	Yes
biostimulated	e5	BHA4-10 m	GW	PCE	Yes
biostimulated	e6	BHA4-10 m	GW	PCE	Yes
bioaugmented	e7	BHA4-10 m	GW	PCE	Yes
bioaugmented	e8	BHA4-10 m	GW	PCE	Yes
biostimulated	e9	BHA4-10 m	RAMM	PCE	Yes
biostimulated	e10	BHA4-10 m	RAMM	PCE	Yes
bioaugmented	e11	BHA4-10 m	RAMM	PCE	Yes
bioaugmented	e12	BHA4-10 m	RAMM	PCE	Yes
heat kill	e13	BHA4-10 m	GW	1,1-DCE	No
heat kill	e14	BHA4-10 m	GW	1,1-DCE	No
natural attenuation	e15	BHA4-10 m	GW	1,1-DCE	No

Table 9. Continued.

Purpose	Sample ID	Aquifer Material	Liquid Phase	Compound of Concern	Methane Formation
natural attenuation	e16	BHA4-10 m	GW	1,1-DCE	No
biostimulated	e17	BHA4-10 m	GW	1,1-DCE	No
biostimulated	e18	BHA4-10 m	GW	1,1-DCE	No
bioaugmented	e19	BHA4-10 m	GW	1,1-DCE	Yes
bioaugmented	e20	BHA4-10 m	GW	1,1-DCE	Yes
biostimulated	e21	BHA4-10 m	RAMM	1,1-DCE	No
biostimulated	e22	BHA4-10 m	RAMM	1,1-DCE	No
bioaugmented	e23	BHA4-10 m	RAMM	1,1-DCE	Yes
bioaugmented	e24	BHA4-10 m	RAMM	1,1-DCE	Yes
heat kill	e25	BHA4-10 m	GW	CF	No
heat kill	e26	BHA4-10 m	GW	CF	No
natural attenuation	e27	BHA4-10 m	GW	CF	No
natural attenuation	e28	BHA4-10 m	GW	CF	No
biostimulated	e29	BHA4-10 m	GW	CF	No
biostimulated	e30	BHA4-10 m	GW	CF	No
bioaugmented	e31	BHA4-10 m	GW	CF	No
bioaugmented	e32	BHA4-10 m	GW	CF	No
biostimulated	e33	BHA4-10 m	RAMM	CF	No
biostimulated	e34	BHA4-10 m	RAMM	CF	No
bioaugmented	e35	BHA4-10 m	RAMM	CF	Yes
bioaugmented	e36	BHA4-10 m	RAMM	CF	Yes
heat kill	e37	BHA4-10 m	GW	CT	No
heat kill	e38	BHA4-10 m	GW	CT	No
biostimulated	e39	BHA4-10 m	GW	CT	No
biostimulated	e40	BHA4-10 m	GW	CT	No
biostimulated	e41	BHA4-10 m	RAMM	CT	Yes
biostimulated	e42	BHA4-10 m	RAMM	CT	Yes
abiotic	e43	BHA4-10 m	GW	CT	Yes
abiotic	e44	BHA4-10 m	GW	CT	Yes
abiotic	e45	BHA4-10 m	GW	CT	Yes
abiotic	e46	BHA4-10 m	GW	CT	Yes
abiotic	e47	BHA4-10 m	GW	CT	Yes
abiotic	e48	BHA4-10 m	GW	CT	Yes
bioaugmented	e49	BHA4-10 m	GW	CT	Yes
bioaugmented	e50	BHA4-10 m	GW	CT	Yes
bioaugmented	e51	BHA4-10 m	RAMM	CT	Yes
bioaugmented	e52	BHA4-10 m	RAMM	CT	Yes
bioaugmented	e53	BHA4-10 m	GW	CT	Yes
bioaugmented	e54	BHA4-10 m	GW	CT	Yes
bioaugmented	e55	BHA4-10 m	GW	CT	Yes
bioaugmented	e56	BHA4-10 m	GW	CT	Yes
bioaugmented	e57	BHA4-10 m	GW	CT	Yes
bioaugmented	e58	BHA4-10 m	GW	CT	Yes

Table 9. Continued.

Purpose	Sample ID	Aquifer Material	Liquid Phase	Compound of Concern	Methane Formation
natural attenuation	e59	BHA4-10 m	GW	CT	No
natural attenuation	e60	BHA4-10 m	GW	CT	No
heat kill	e61	BHA4-10 m	GW	CFC-11	No
heat kill	e62	BHA4-10 m	RAMM	CFC-11	No
natural attenuation	e63	BHA4-10 m	GW	CFC-11	No
natural attenuation	e64	BHA4-10 m	GW	CFC-11	No
biostimulated	e65	BHA4-10 m	GW	CFC-11	No
biostimulated	e66	BHA4-10 m	GW	CFC-11	No
biostimulated	e67	BHA4-10 m	RAMM	CFC-11	No
biostimulated	e68	BHA4-10 m	RAMM	CFC-11	No
bioaugmented	e69	BHA4-10 m	RAMM	CFC-11	No
bioaugmented	e70	BHA4-10 m	RAMM	CFC-11	No
heat kill	e71	BHA4-10 m	GW	CFC-12	No
heat kill	e72	BHA4-10 m	RAMM	CFC-12	No
natural attenuation	e73	BHA4-10 m	GW	CFC-12	No
natural attenuation	e74	BHA4-10 m	GW	CFC-12	No
biostimulated	e75	BHA4-10 m	GW	CFC-12	No
biostimulated	e76	BHA4-10 m	GW	CFC-12	No
biostimulated	e77	BHA4-10 m	RAMM	CFC-12	No
biostimulated	e78	BHA4-10 m	RAMM	CFC-12	No
bioaugmented	e79	BHA4-10 m	RAMM	CFC-12	No
bioaugmented	e80	BHA4-10 m	RAMM	CFC-12	No

Libraries and R code used to make Figure 13 and Figure 14

Libraries used: dplyr, tidyverse, ggplot2, gridExtra.

```
#read in file
CT <- read_csv("/Users/file-path")
CT_long <- gather(CT, contaminants, Percent, 'CT', factor_key = TRUE)

#plot heatmaps
plot <- test_long %>% ggplot(., aes(x= Site, y= Treatment, fill = Percent)) +
  geom_tile(color='white', size=0.3) + facet_grid(~contaminants) + theme_classic() +
  theme(axis.text.x = element_text(angle = 90, vjust = .5)) + scale_fill_gradientn(colors =
c("gray89", "midnightblue"))

ggsave("/Users/ file-path.pdf", plot=last_plot(), width = 9.36, height = 5.79, units = "in", limitsize
= FALSE)

grid.arrange(nrow=1, ncol=2, test_long %>% ggplot(., aes(x= Site, y= Treatment, fill = Percent))
+ geom_tile(color='white', size=0.3) + facet_grid(~contaminants) + theme_classic() +
theme(axis.text.x = element_text(angle = 90, vjust = .5)) + scale_fill_gradientn(colors =
c("gray89", "midnightblue")),

CT_long %>% ggplot(., aes(x= Site, y= Treatment, fill = Percent)) + geom_tile(color='white',
size=0.35) + facet_grid(~contaminants) + theme_classic() + theme(axis.text.x =
element_text(angle = 90, vjust = .5)) + scale_fill_gradientn(colors =
c("gray89", "midnightblue")) ggsave("/file-path", plot=last_plot(), width = 4.68, height = 5.79,
units = "in", limitsize = FALSE)
```

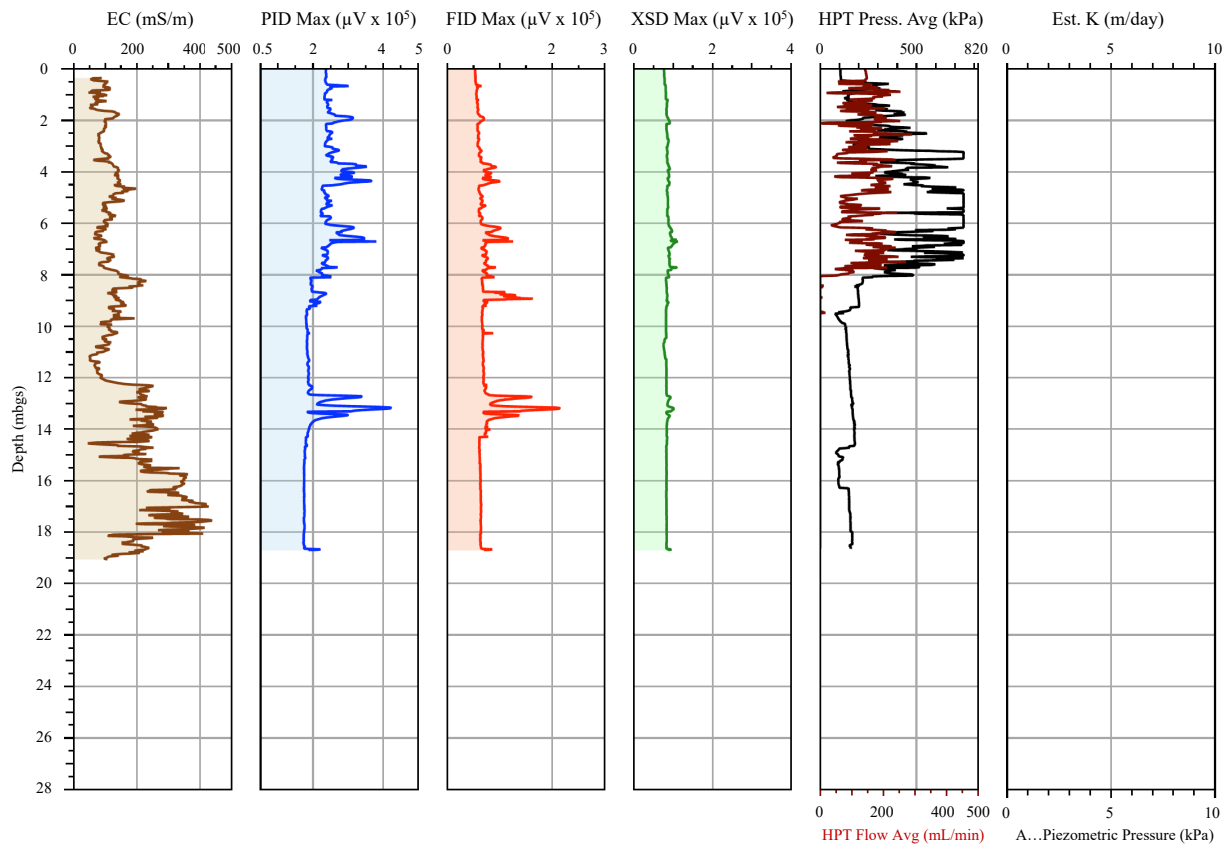


Figure 9. Membrane interface probe-hydraulic profiling tool data from Borehole 1.

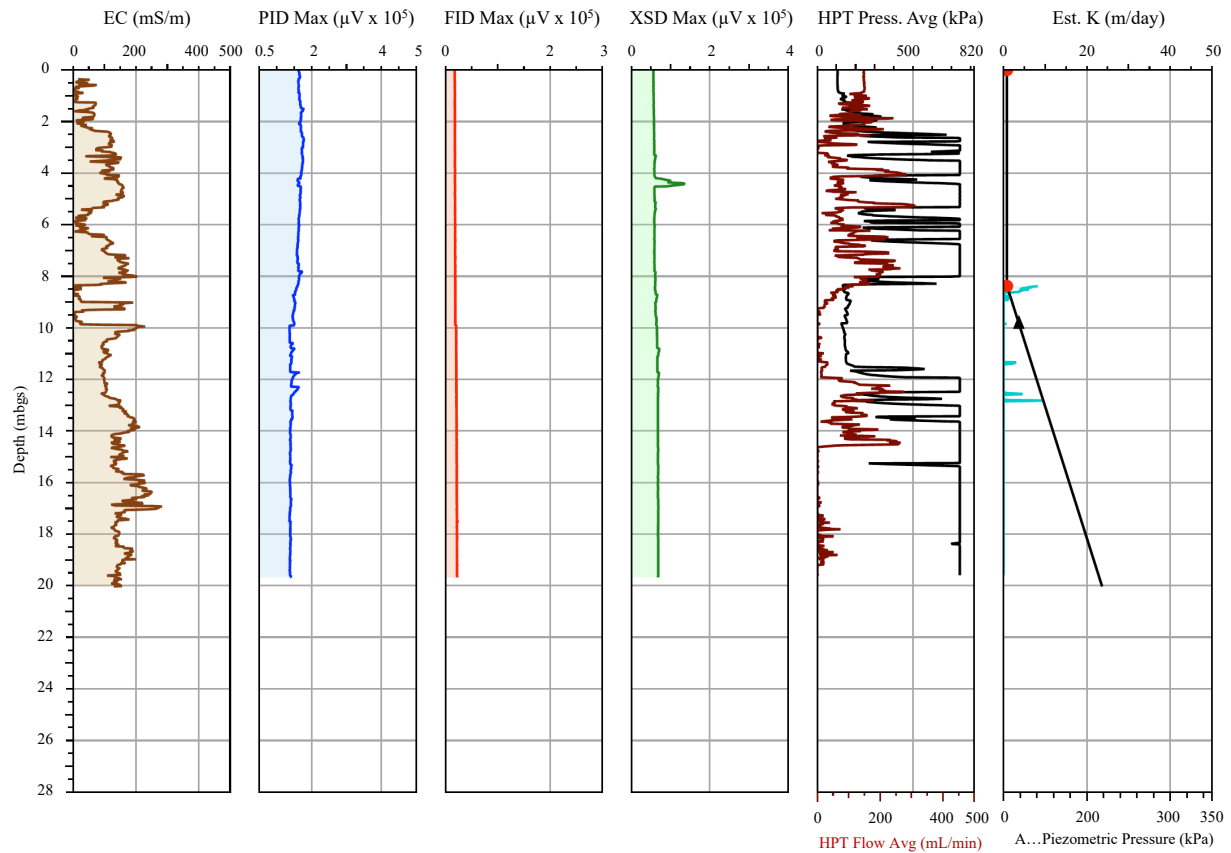


Figure 10. Membrane interface probe-hydraulic profiling tool data from Borehole 2.

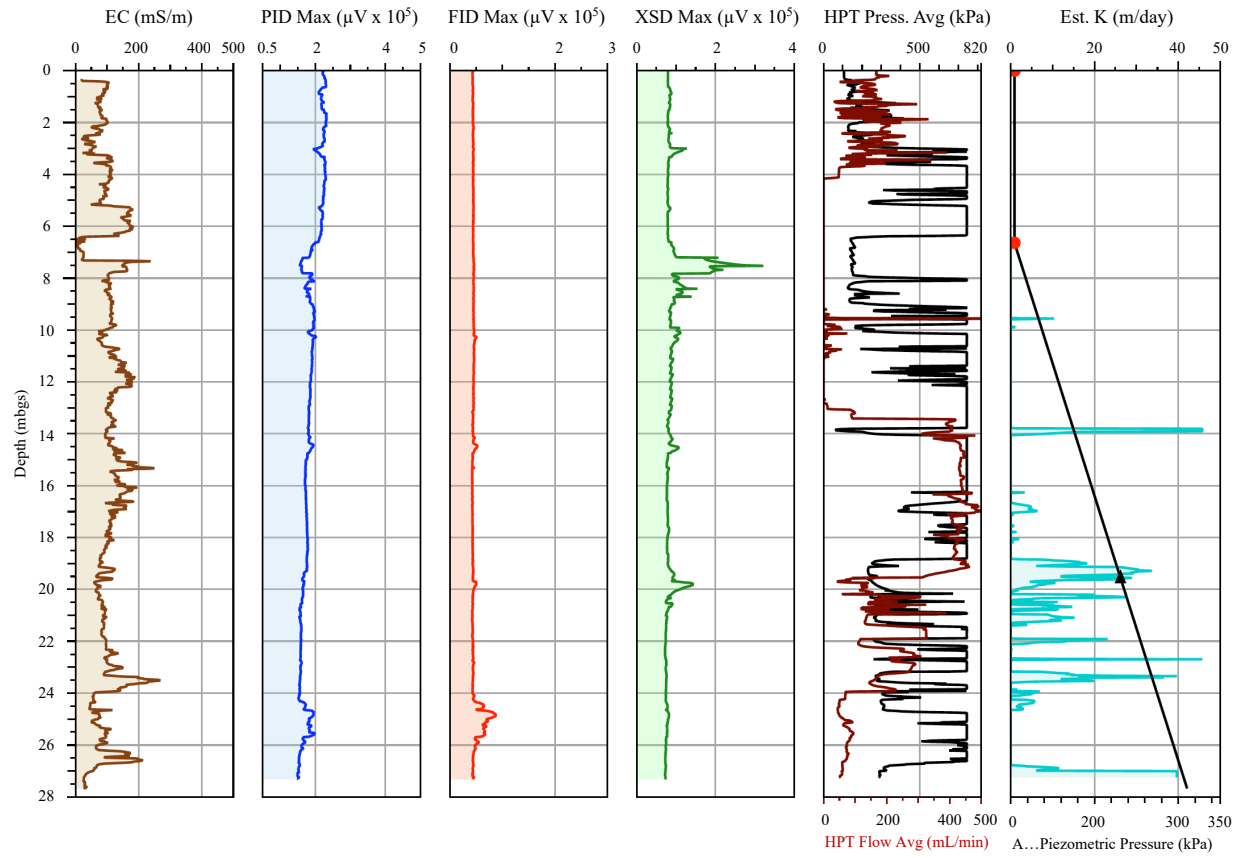


Figure 11. Membrane interface probe-hydraulic profiling tool data from Borehole 3.

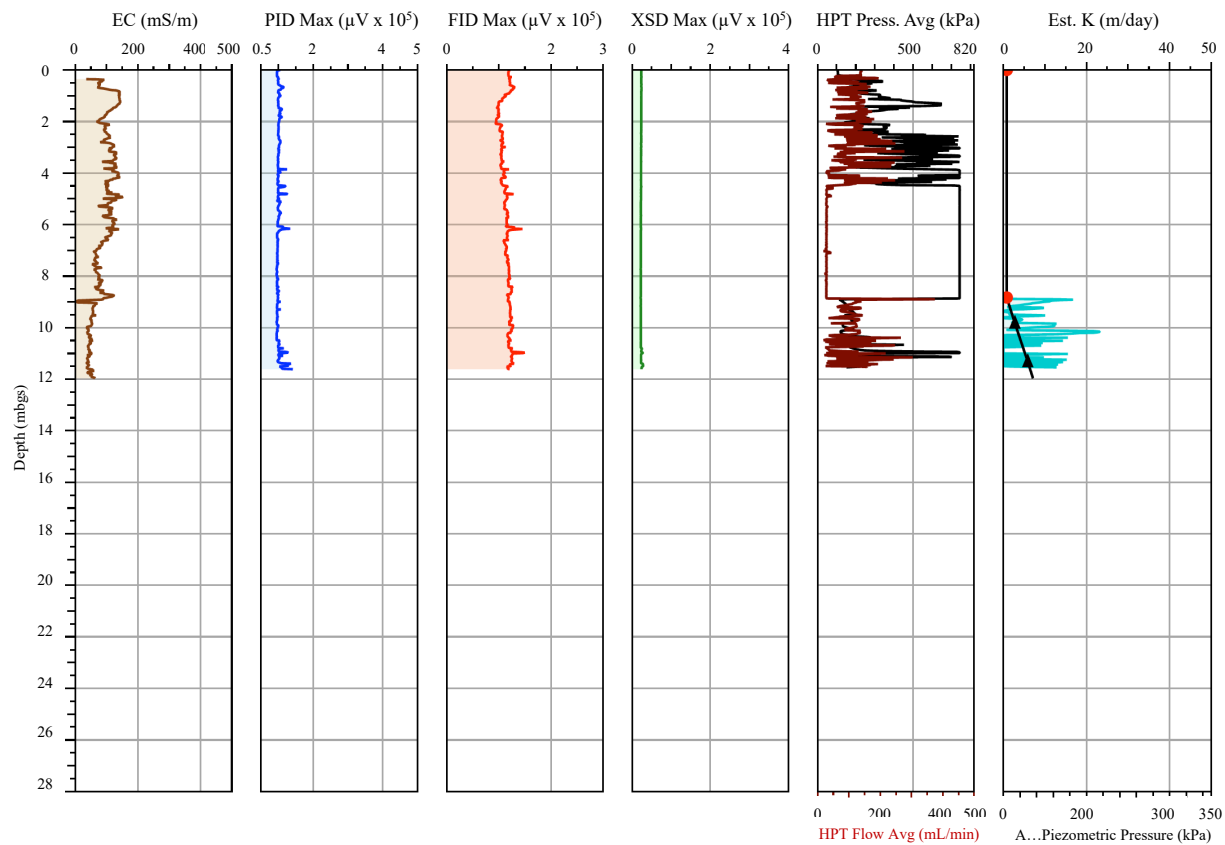


Figure 12. Membrane interface probe-hydraulic profiling tool data from Borehole 4.

Table 10. Amounts of chlorinated VOCs associated with aquifer materials collected in four different boreholes Area A.

Sampling Location and Depth (mbgs)	BHA1 12.85–13.00	BHA3 7.40–7.55	BHA3 14.55–14.75	BHA3 24.50–24.75	BHA4 10.55–10.75
CT (mg/kg)	ND	ND	0.268	ND	ND
CF (mg/kg)	ND	ND	0.118	0.0179	ND
CFC-11 (mg/kg)	ND	ND	ND	ND	ND
CFC-12 (mg/kg)	ND	ND	ND	ND	ND
PCE (mg/kg)	ND	ND	ND	ND	0.00608
1,1-DCE (mg/kg)	ND	ND	ND	ND	ND

Table 11. Groundwater Volatile Organic Compound Data.

Sampling Location and Depth (mbgs)	BHA1 12.25–12.50	BHA3 12.80–14.50	BHA3 19.70–24.70	BHA4 9.95–11.30
CT (µg/L)	5.77	488	4570	ND
CF (µg/L)	11.5	530	1100	ND
CFC-11 (µg/L)	ND	19.1	97.4	ND
CFC-12 (µg/L)	ND	ND	5.47	ND
PCE (µg/L)	1.44	6.38	10.4	ND
1,1-DCE (µg/L)	ND	ND	1.34	ND

Table 12. Total iron in groundwater samples.

Sample Location	Total Fe (mg/L)
BHA1	2.2
BHA3	1.3
BHA4	33.3
PZ2	0.1

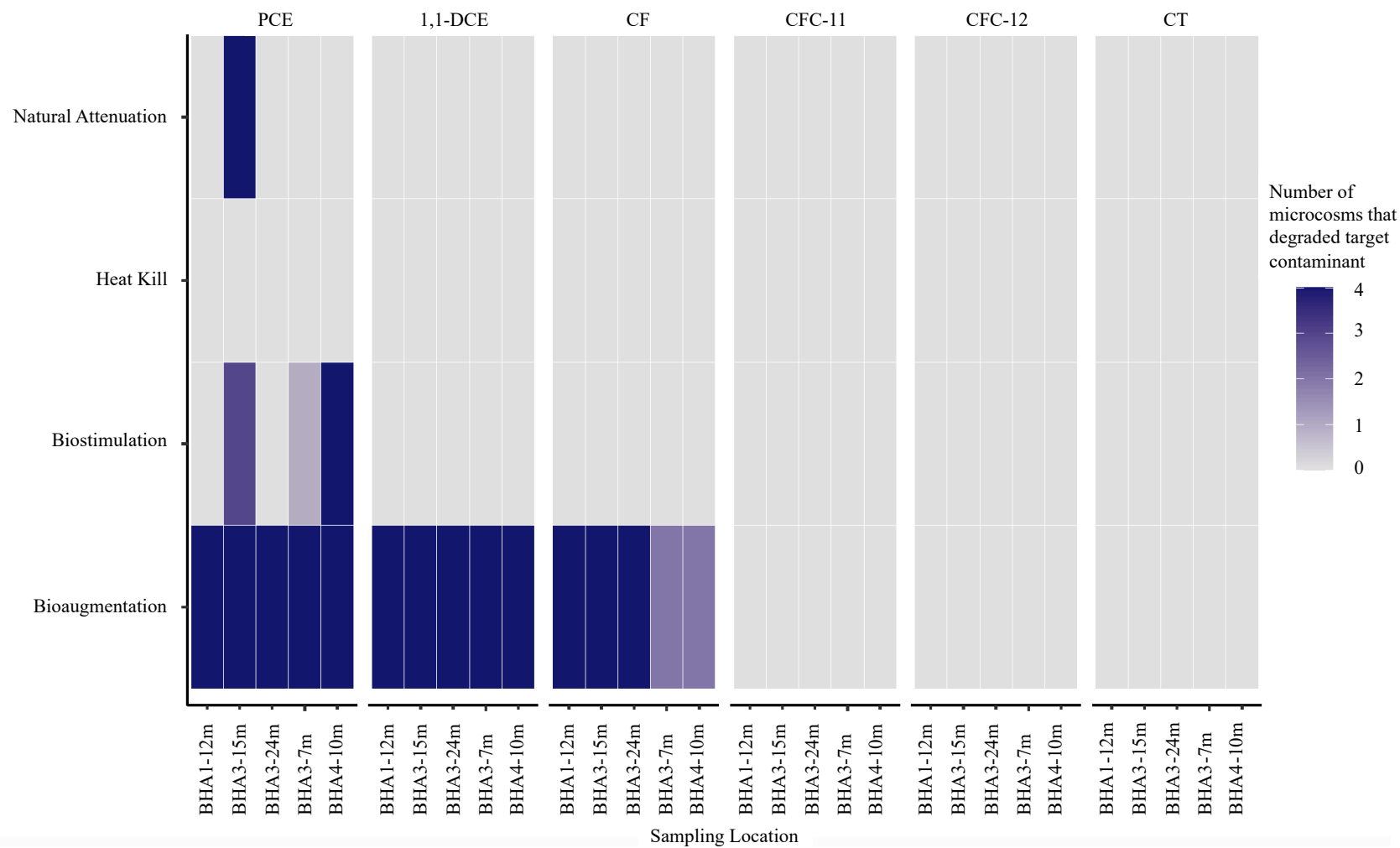


Figure 13. Performance of monitored natural attenuation, heat kill, biostimulation, and bioaugmentation microcosms.

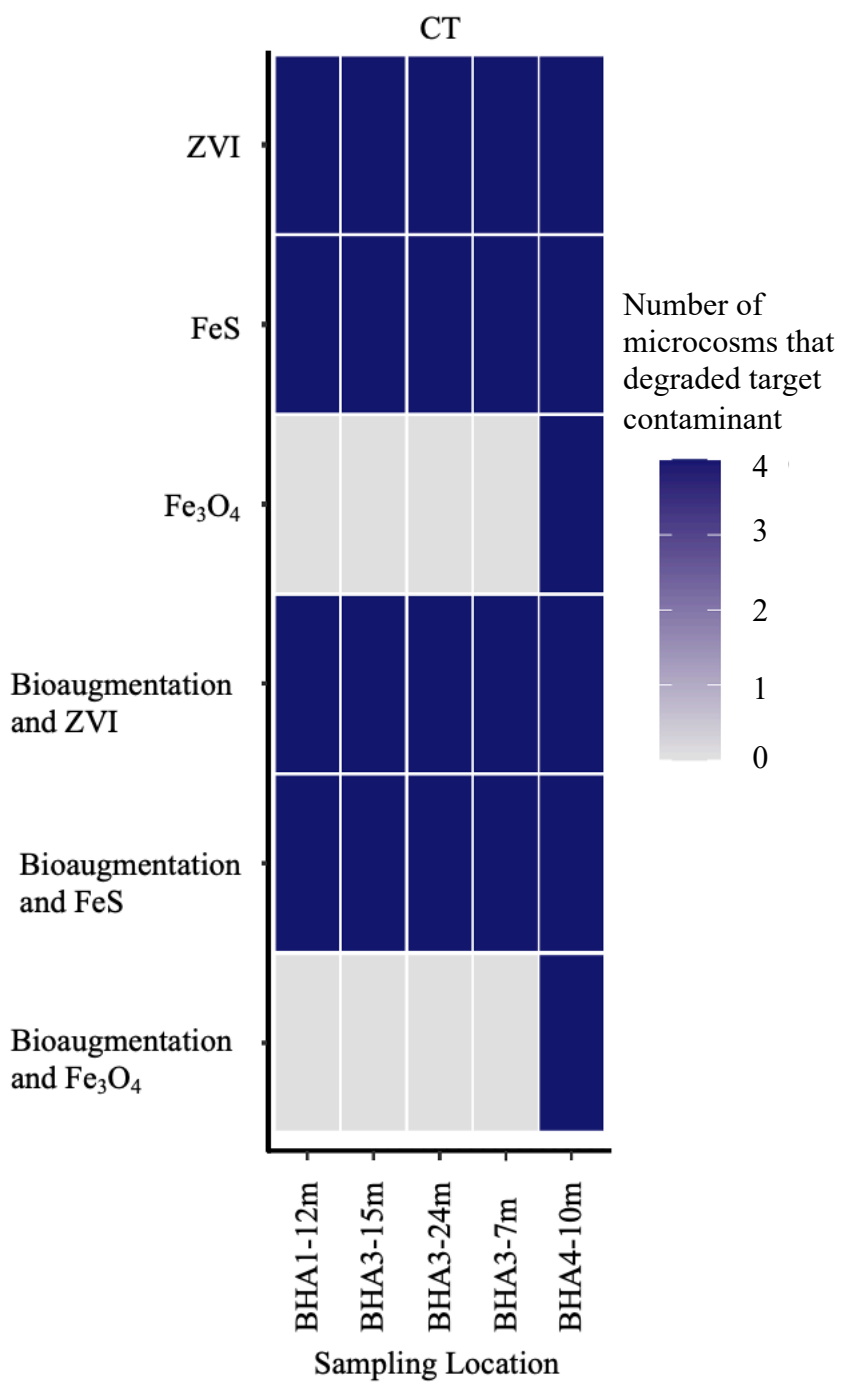


Figure 14. Performance of microcosms amended with reactive iron species, and combined bioaugmentation with reactive iron species.

Table 13. Borehole A1-12.25 m lactate fermentation performance

BHA1 - 12.25 m	PCE	1,1-DCE	CT	CF	CFC-11	CFC-12
Bioaugmentation	(4/4)	(4/4)	(4/4)	(4/4)	(4/4)	(4/4)
Biostimulation	(4/4)	(4/4)	(0/4)	(4/4)	(0/4)	(3/4)

The ratio of bottles in which lactate fermentation was observed in biostimulated and bioaugmented microcosms for each contaminant at borehole A1-12.25 m.

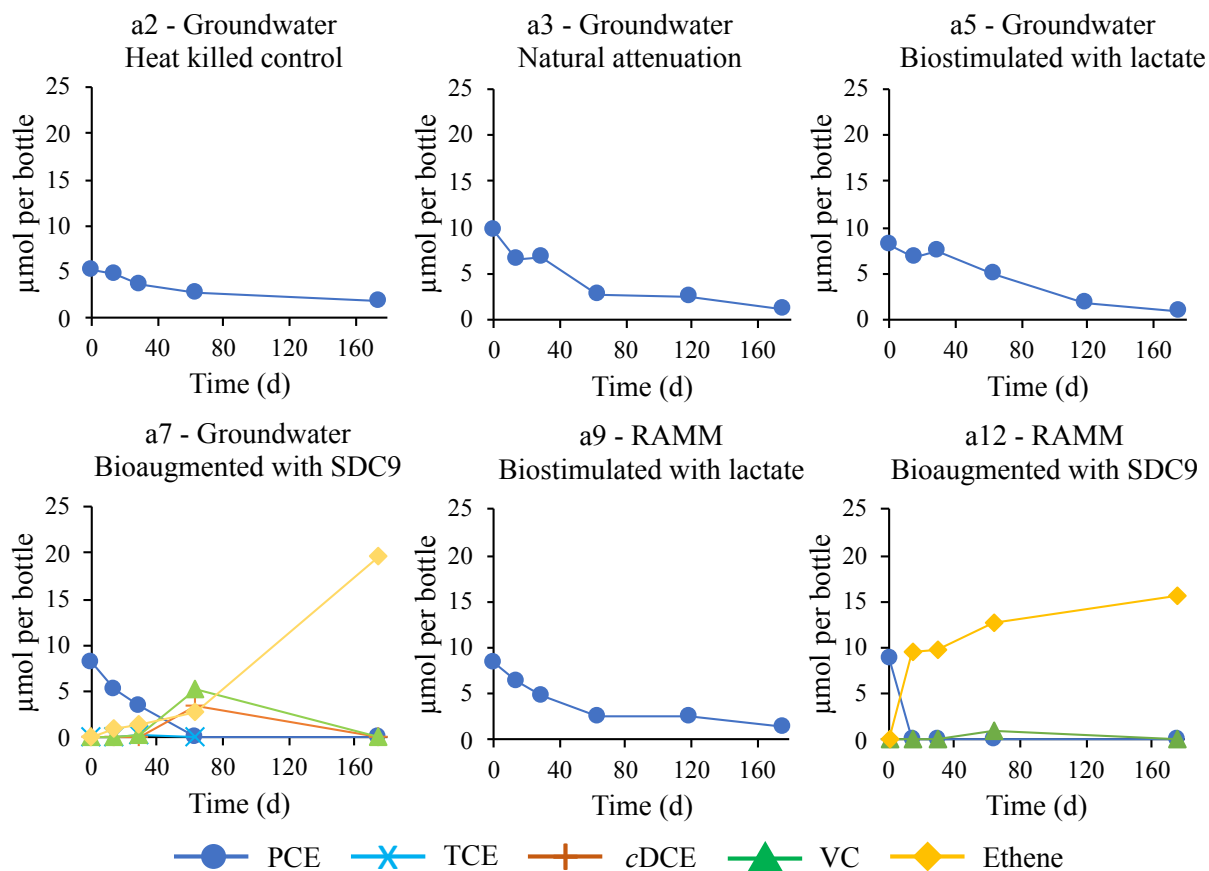


Figure 15. Borehole A1-12 m microcosm performance (PCE).

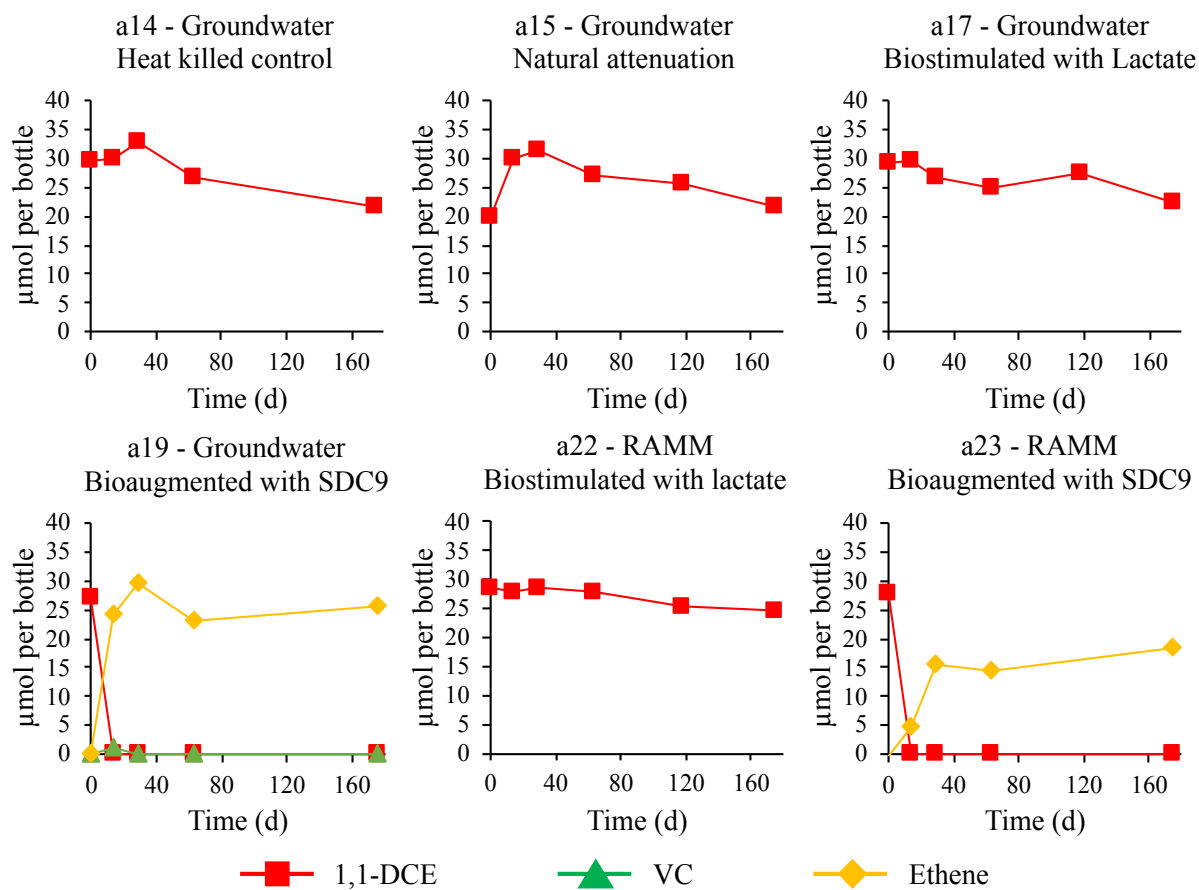


Figure 16. Borehole A1-12 m microcosm performance (1,1-DCE).

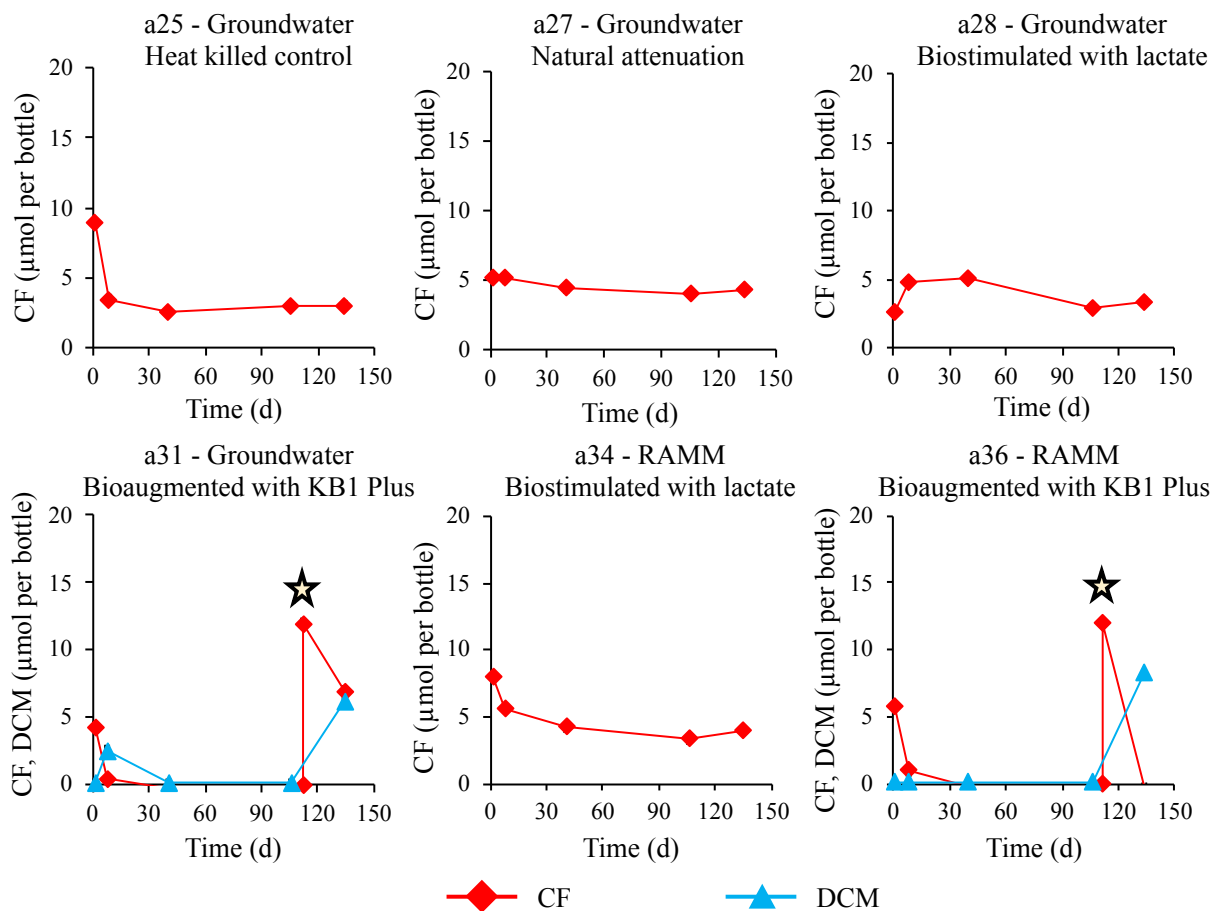


Figure 17. Borehole A1-12 m microcosm performance (CF).

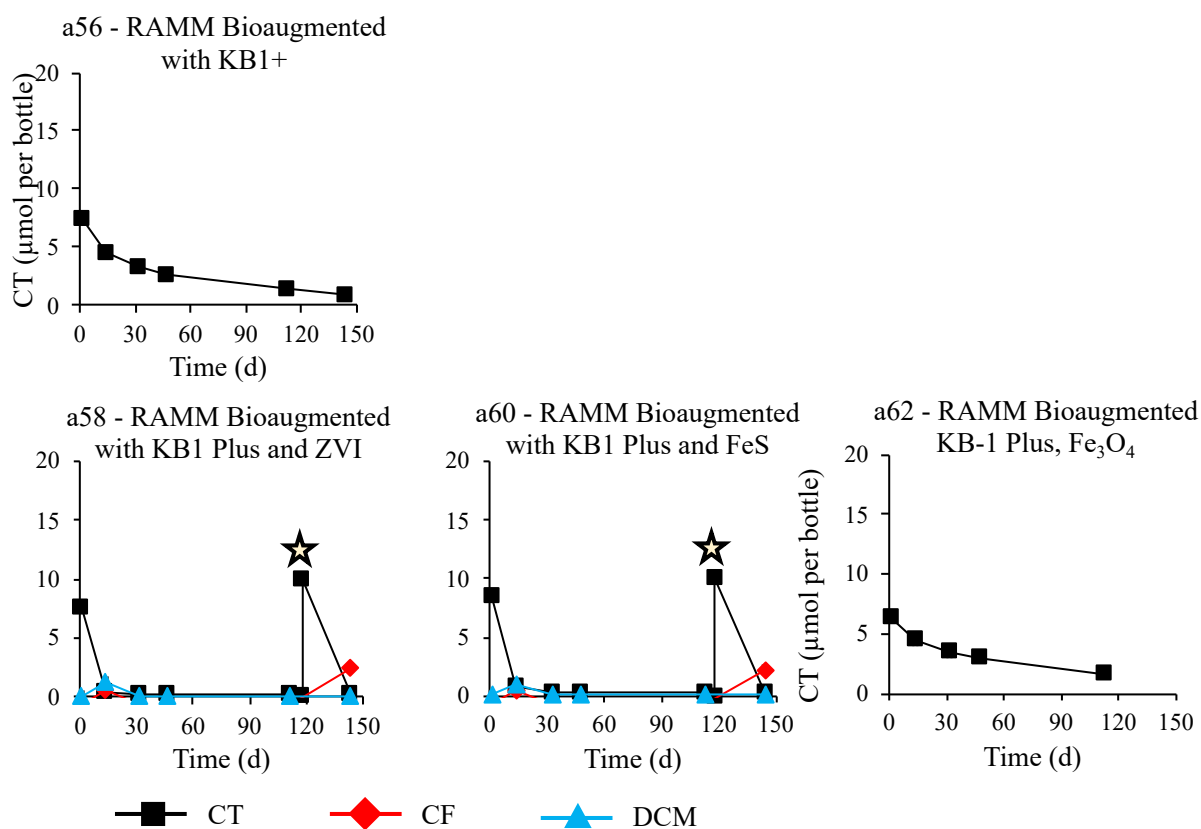


Figure 18. Microcosm setup to test the activity of KB-1 Plus on CT in the absence of aquifer material.

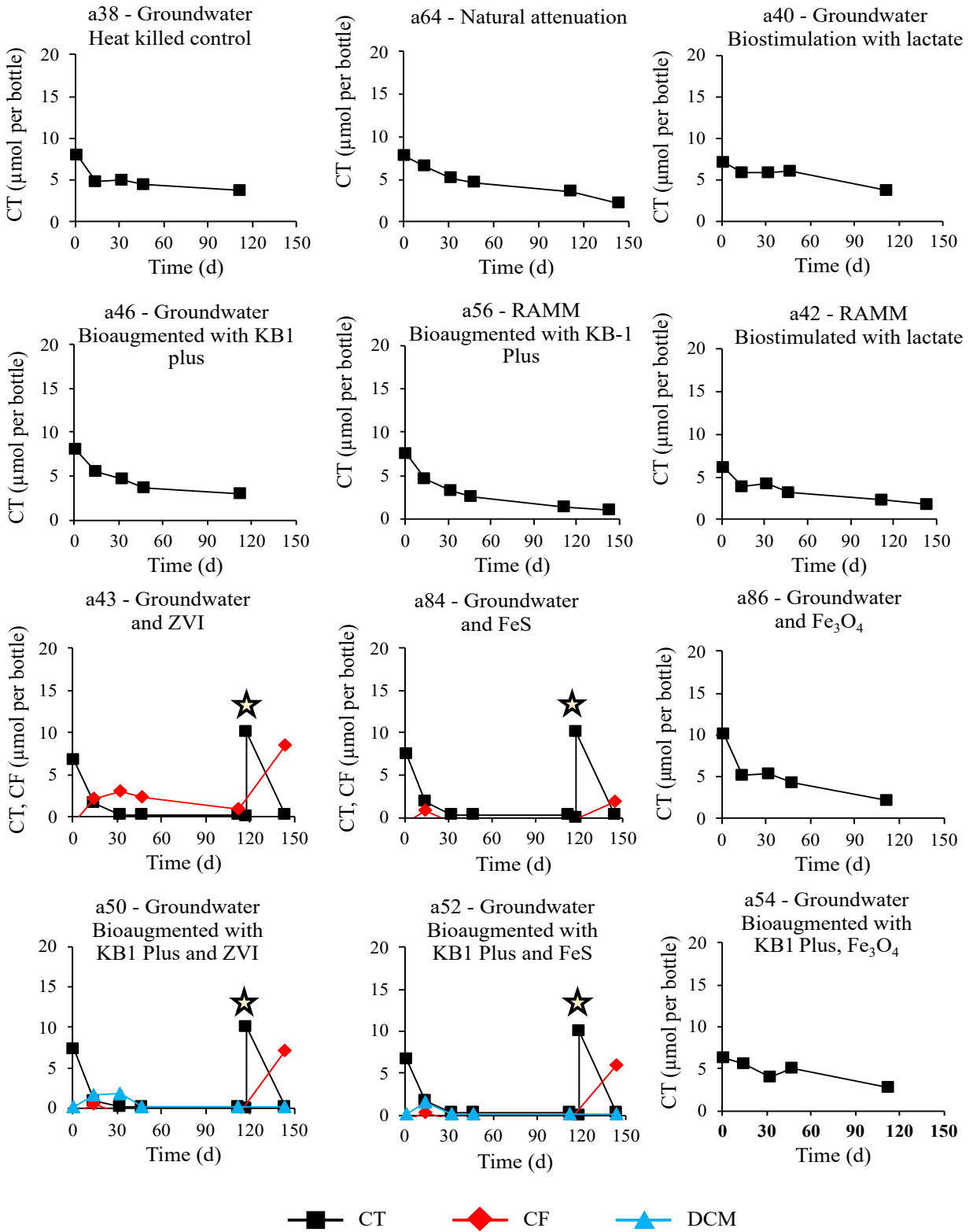


Figure 19. Borehole A1-12 m microcosm performance (CT).

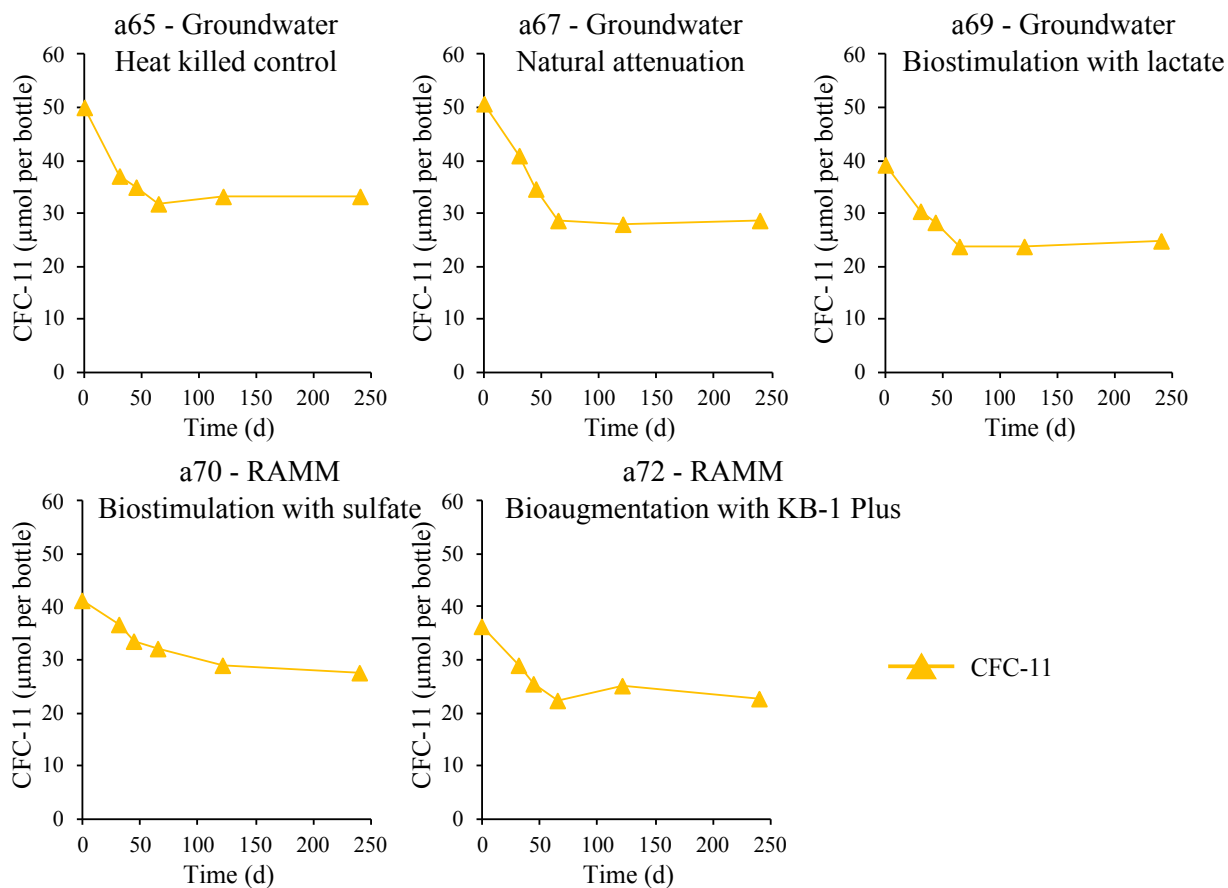


Figure 20. Borehole A1-12 m microcosm performance (CFC-11).

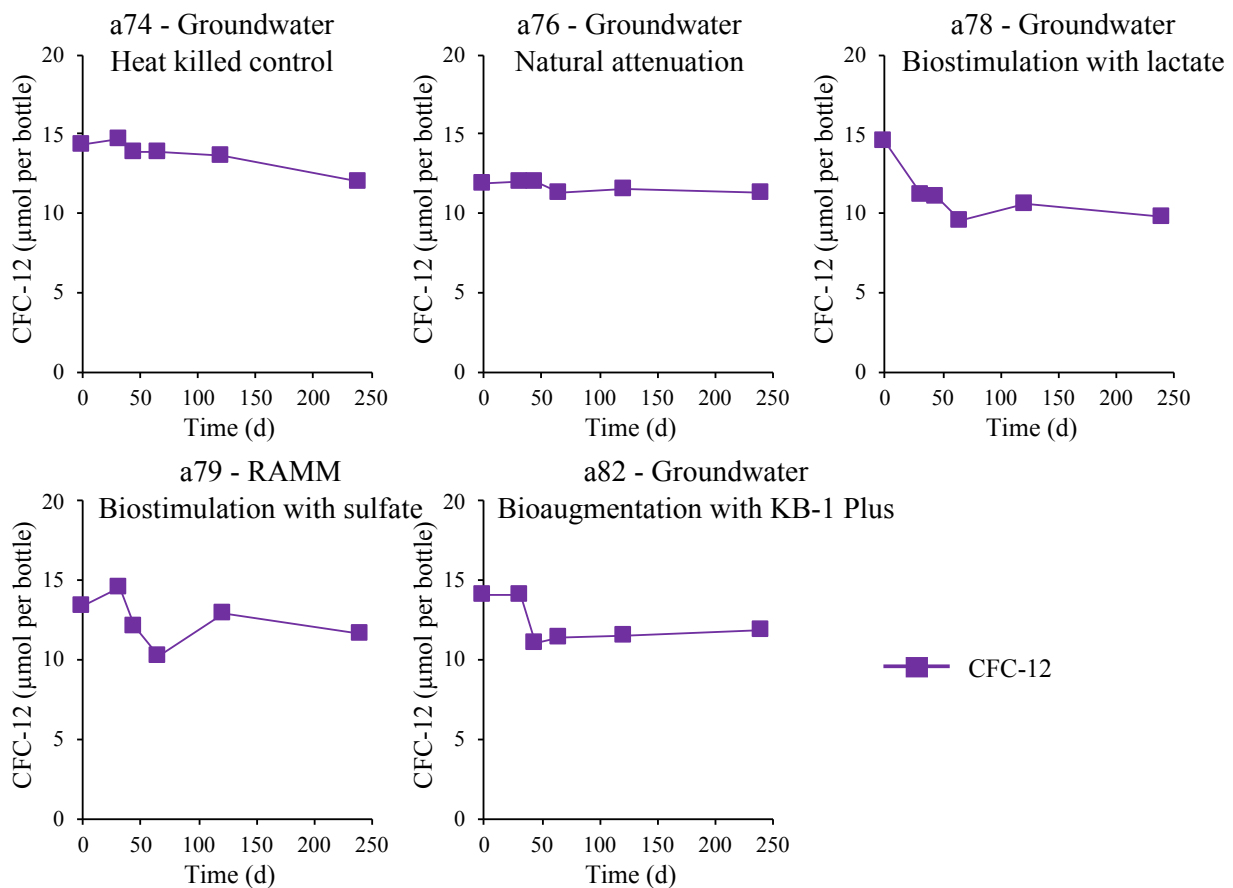


Figure 21. Borehole A1-12 m microcosm performance (CFC-12).

Table 14. Borehole A3-7.55 m lactate fermentation performance.

BHA3 - 7.55 m	PCE	1,1-DCE	CT	CF	CFC-11	CFC-12
Bioaugmentation	(4/4)	(4/4)	(4/4)	(4/4)	(4/4)	(4/4)
Biostimulation	(4/4)	(3/4)	(2/4)	(4/4)	(0/4)	(0/4)

The ratio of bottles in which lactate fermentation was observed in biostimulated and bioaugmented microcosms for each contaminant at borehole A3-7.55 m.

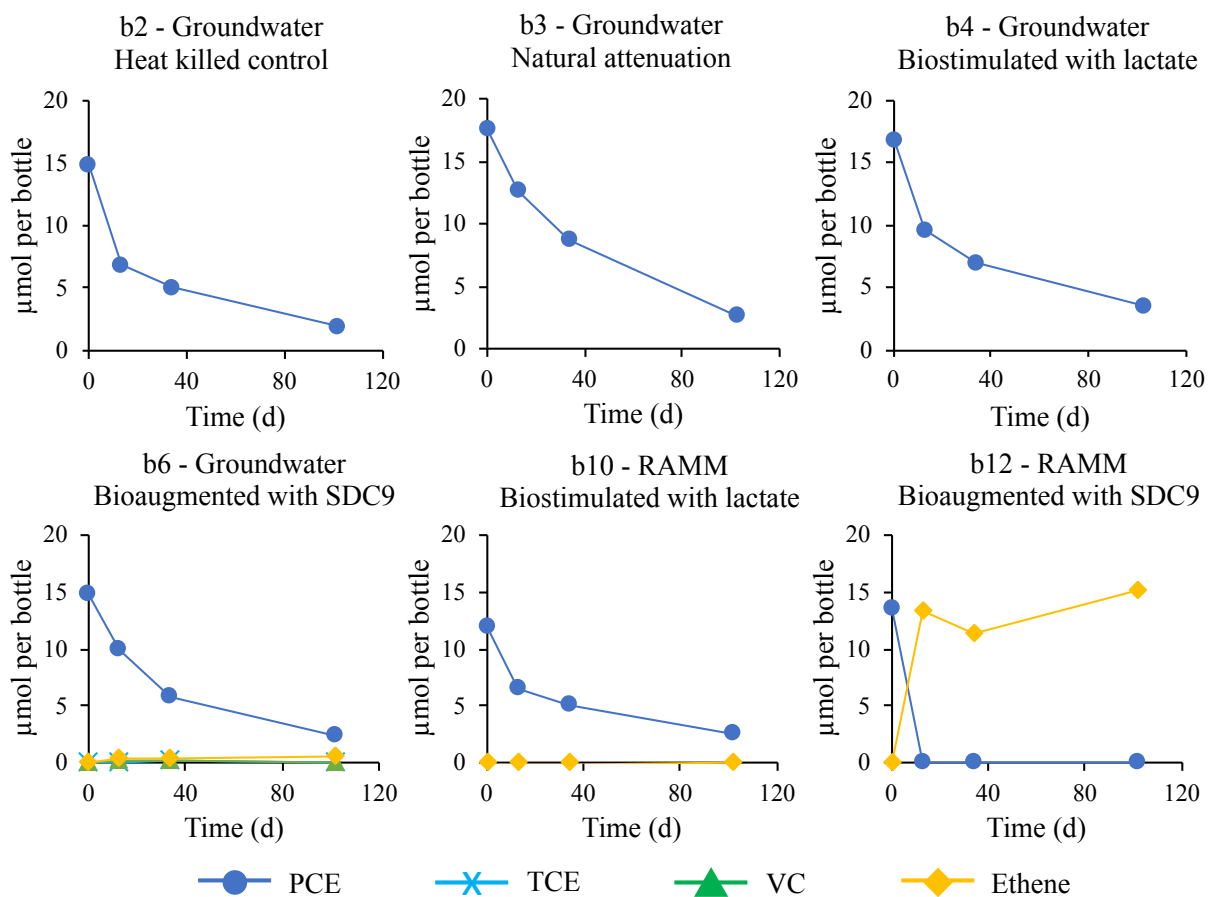


Figure 22. Borehole A3-7 m microcosm performance (PCE).

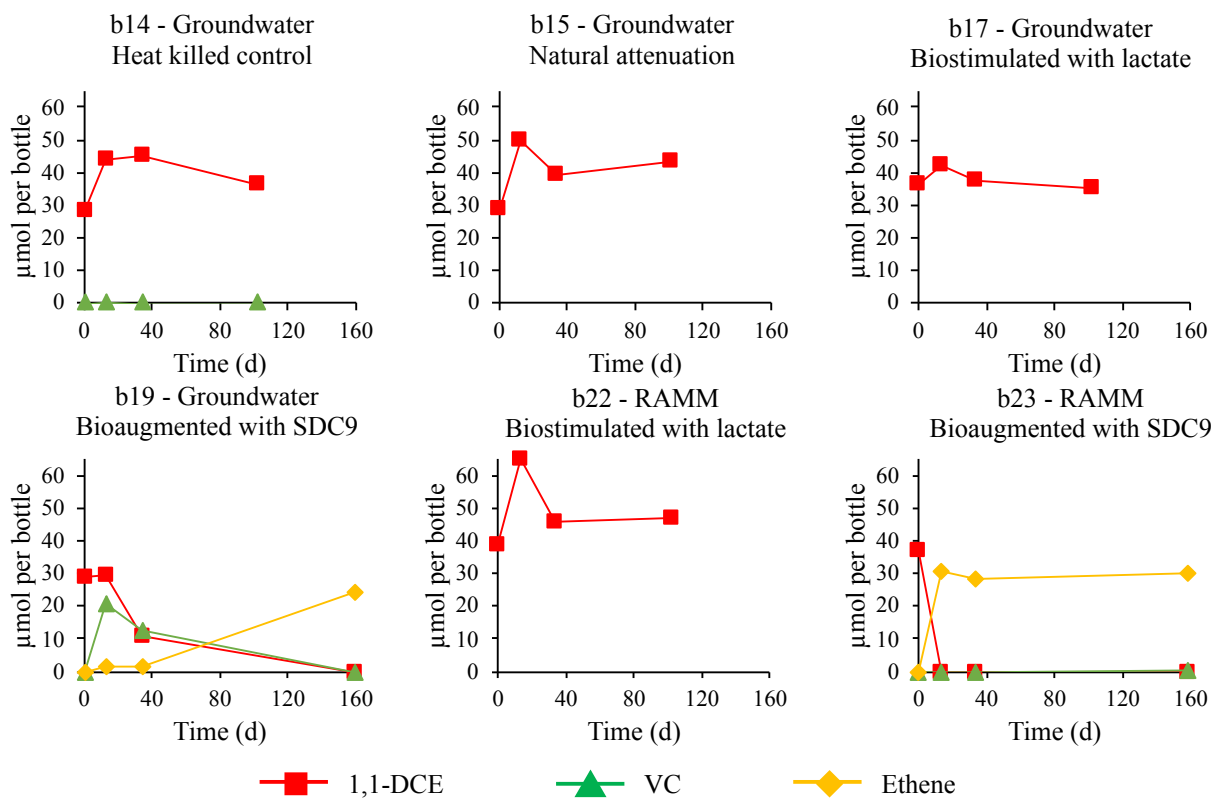


Figure 23. Borehole A3-7 m microcosm performance (1,1-DCE).

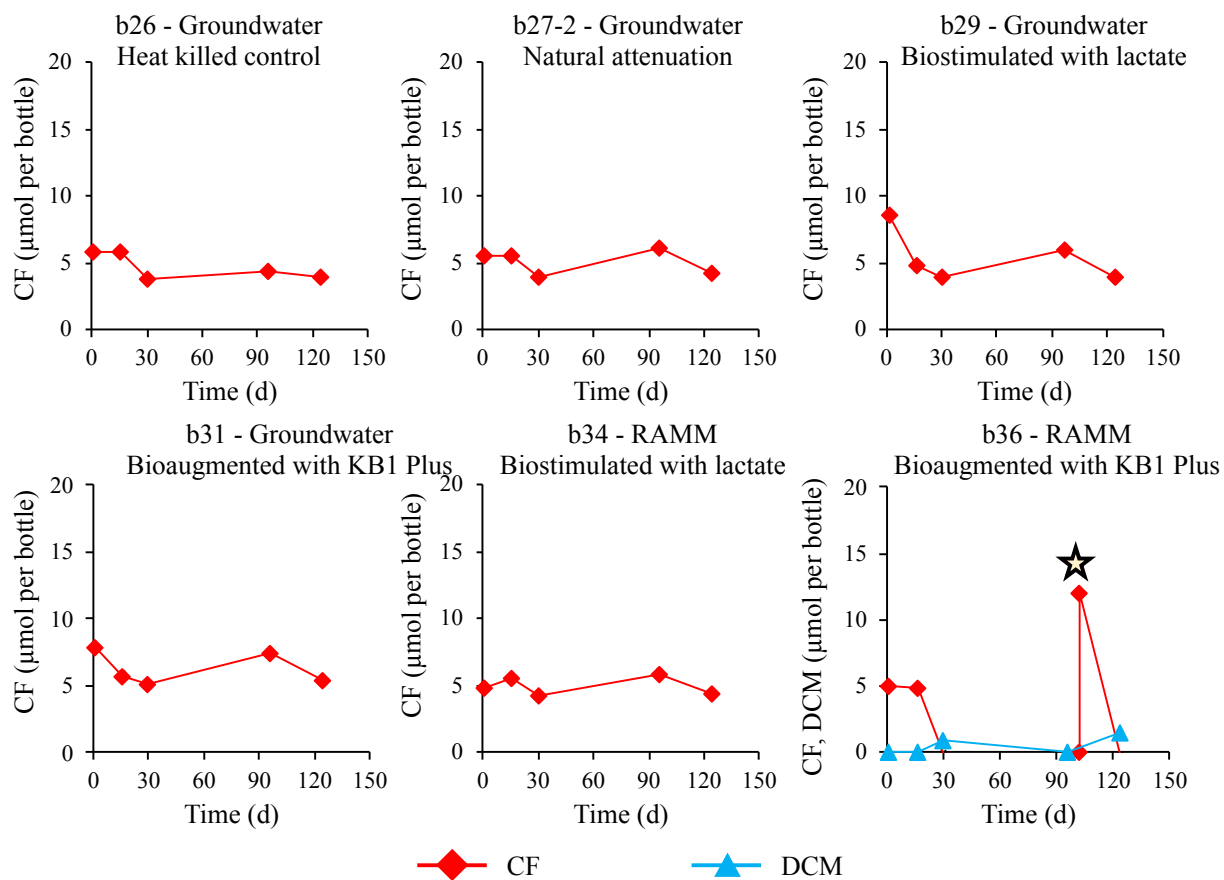


Figure 24. Borehole A3-7 m microcosm performance (CF).

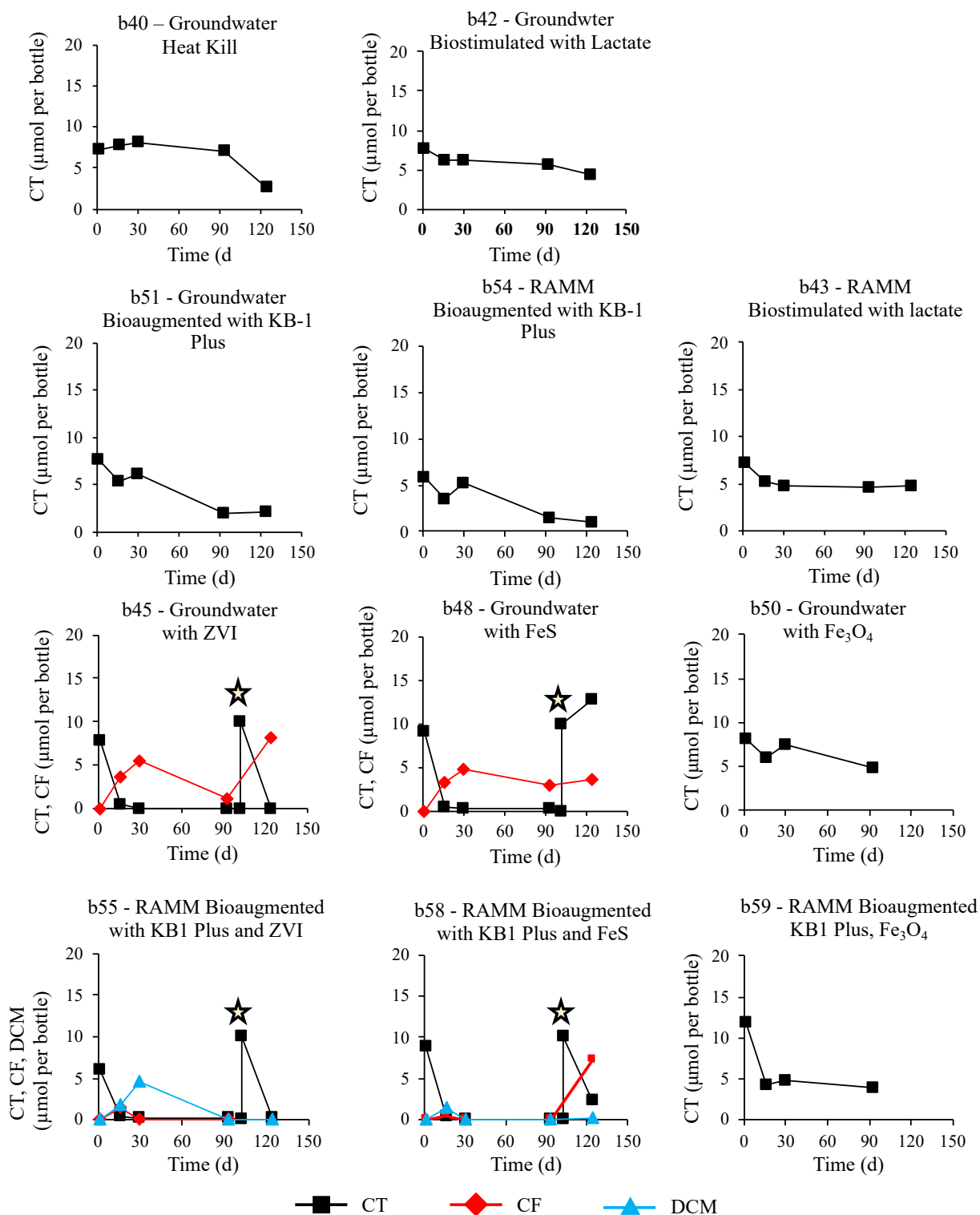


Figure 25. Borehole A3-7 m microcosm performance (CT).

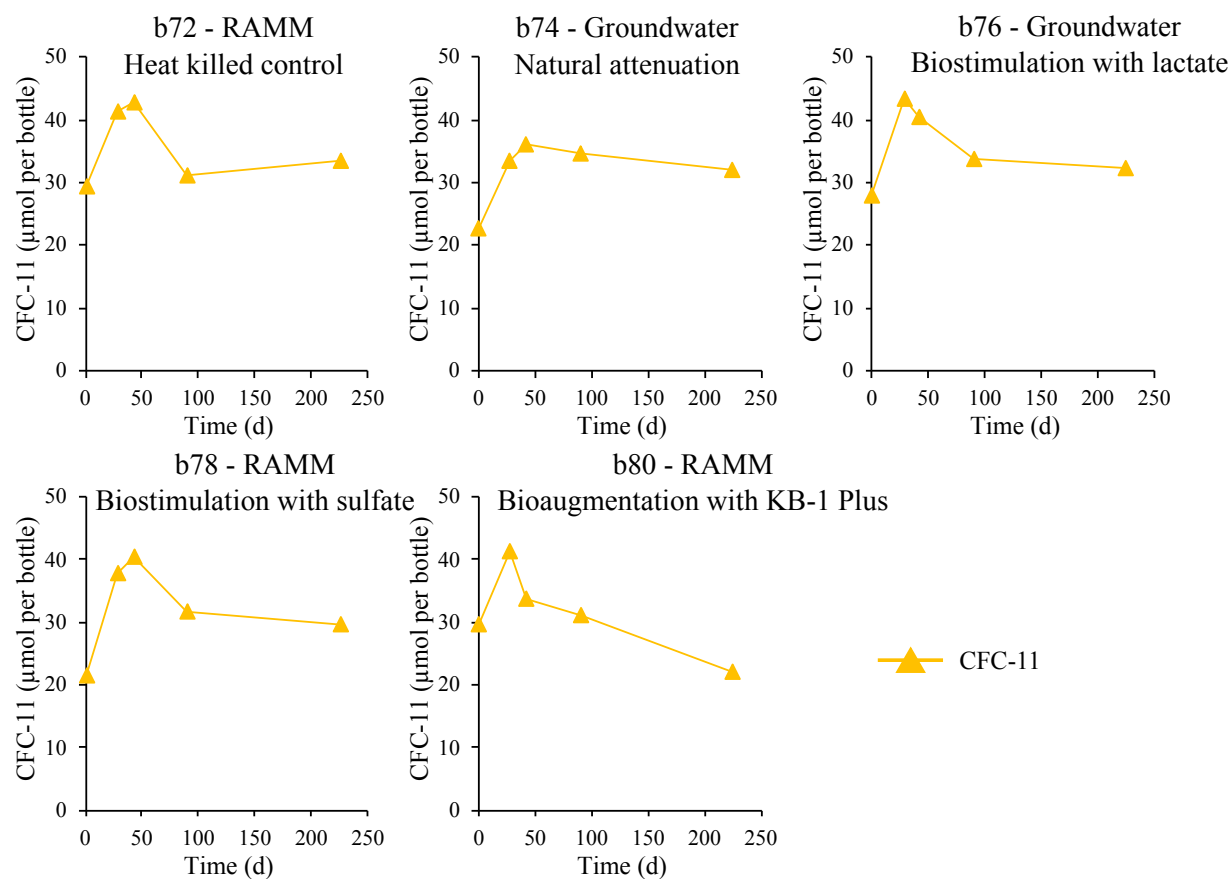


Figure 26. Borehole A3-7 m microcosm performance (CFC-11).

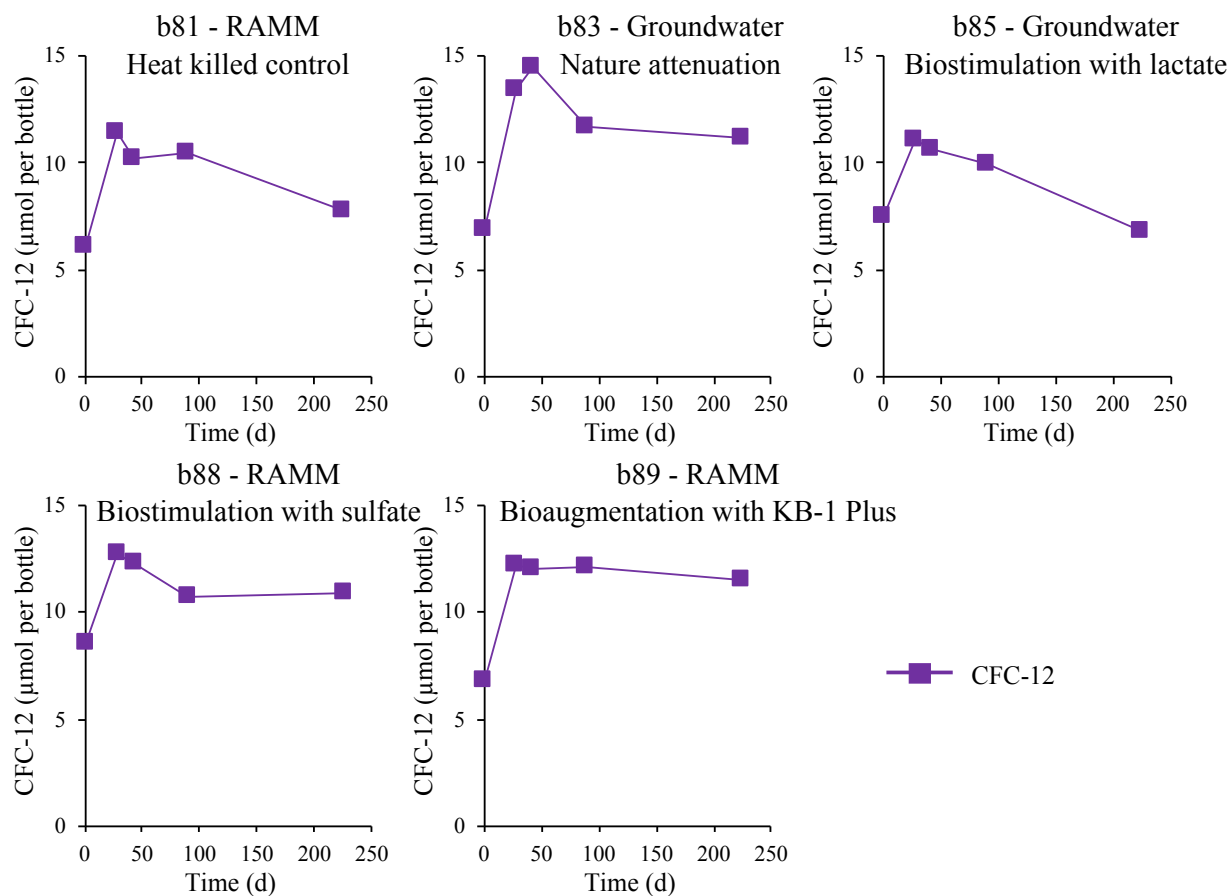


Figure 27. Borehole A3-7 m microcosm performance (CFC-12).

Table 15. Borehole A3-13.90 m lactate fermentation performance.

BHA3 - 13.90 m	PCE	1,1-DCE	CT	CF	CFC-11	CFC-12
Bioaugmentation	(4/4)	(4/4)	(4/4)	(4/4)	(4/4)	(4/4)
Biostimulation	(4/4)	(4/4)	(4/4)	(4/4)	(0/4)	(4/4)

The ratio of bottles in which lactate fermentation was observed in biostimulated and bioaugmented microcosms for each contaminant at borehole A3-13.90 m.

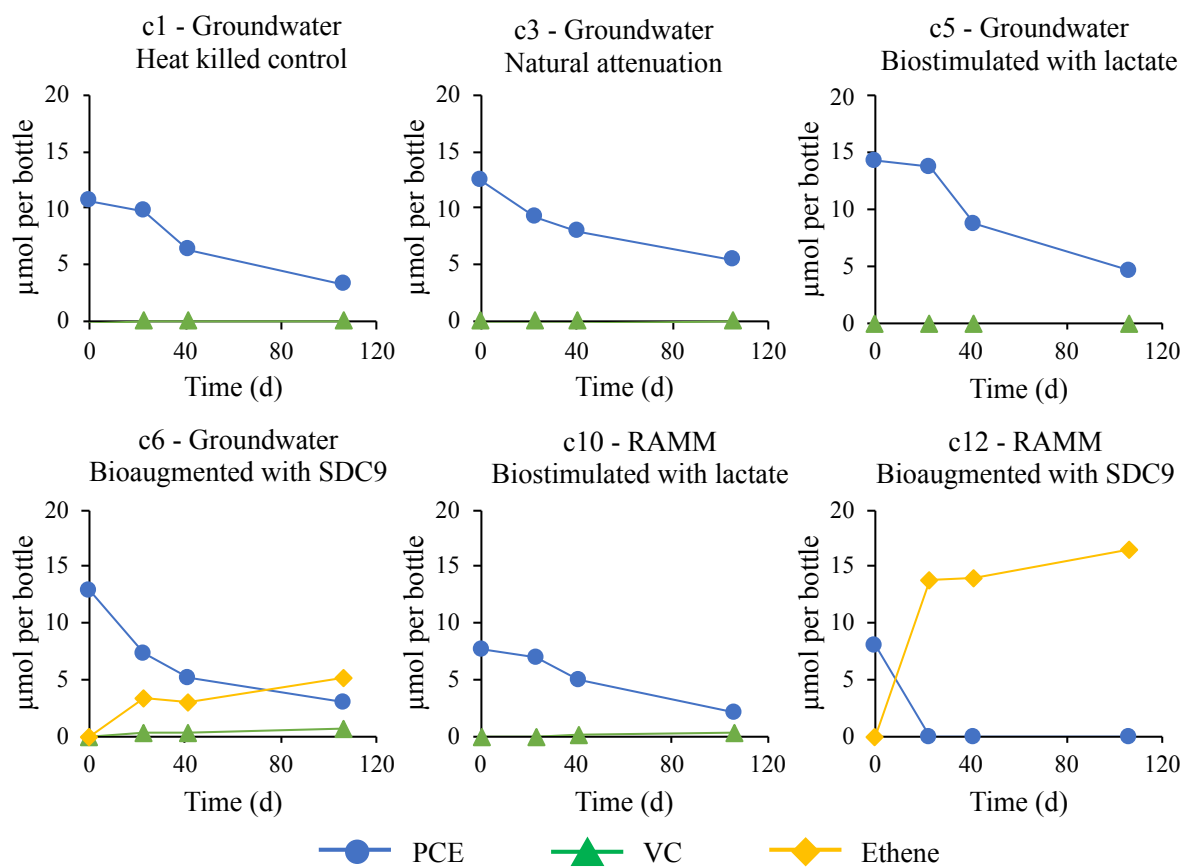


Figure 28. Borehole A3-15 m microcosm performance (PCE).

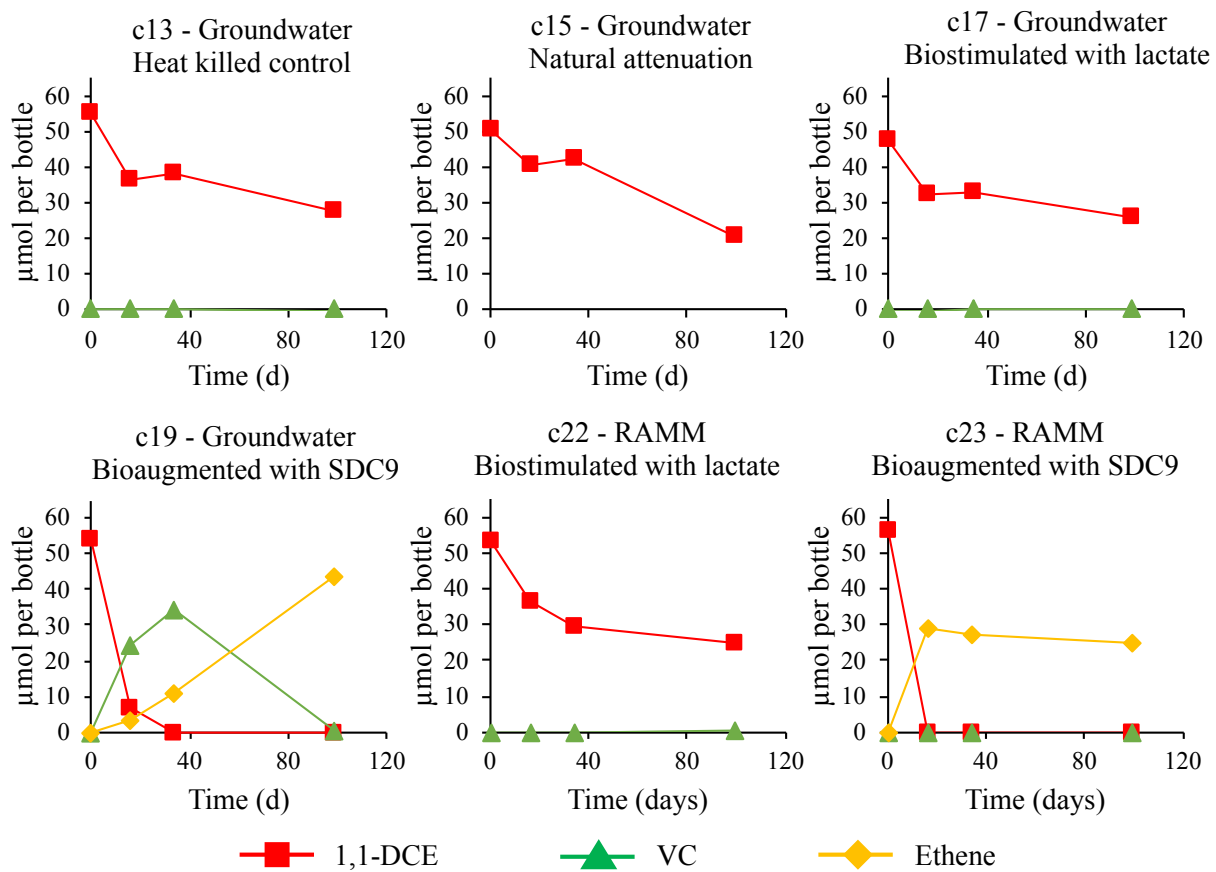


Figure 29. Borehole A3-15 m microcosm performance (1,1-DCE).

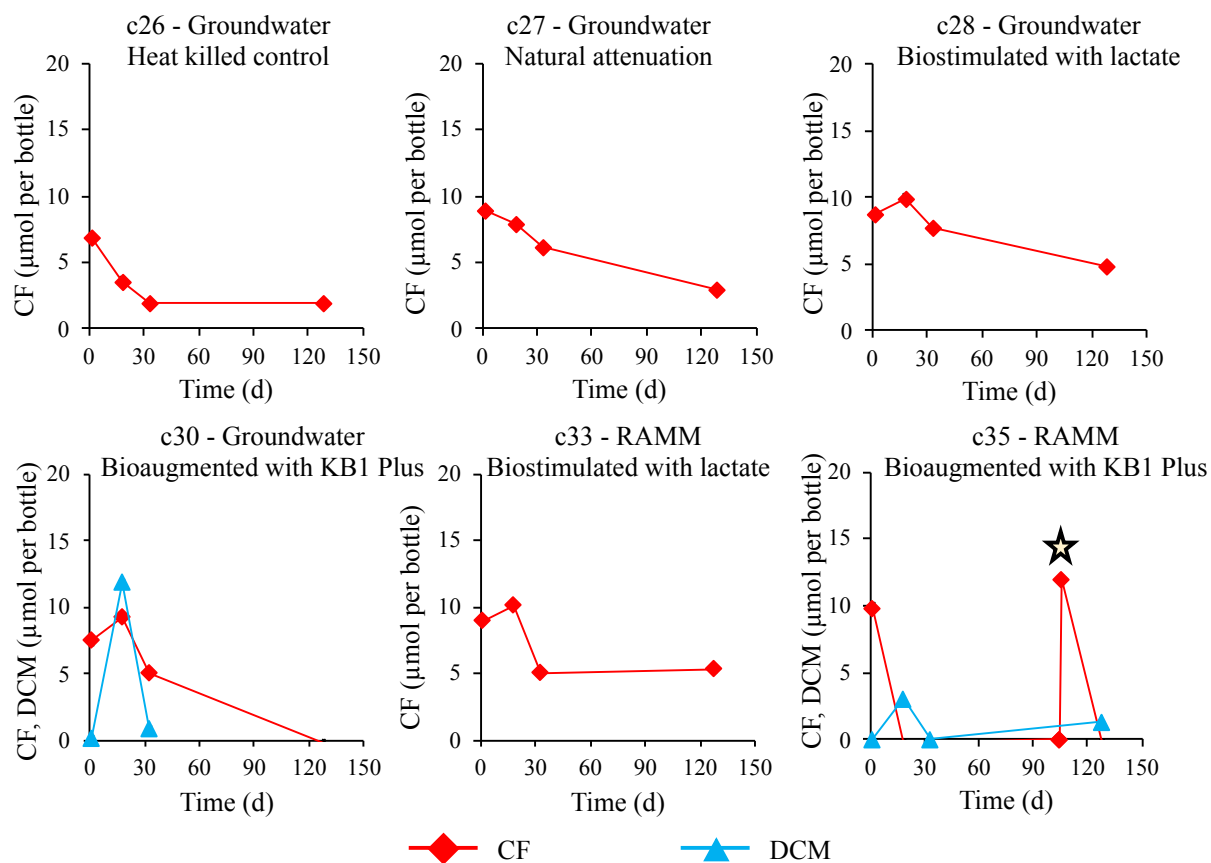


Figure 30. Borehole A3-15 m microcosm performance (CF).

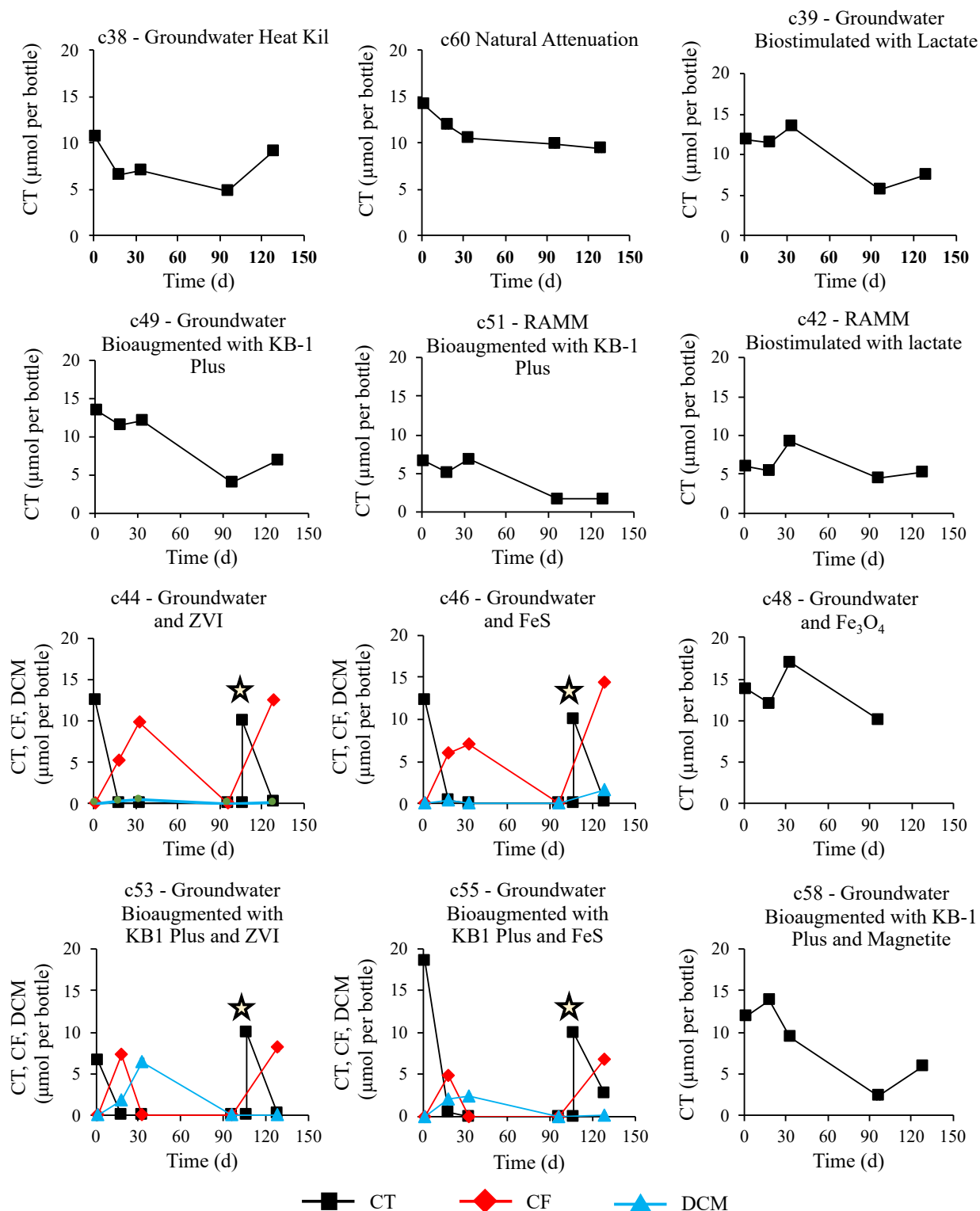


Figure 31. Borehole A3-15 m microcosm performance (CT).

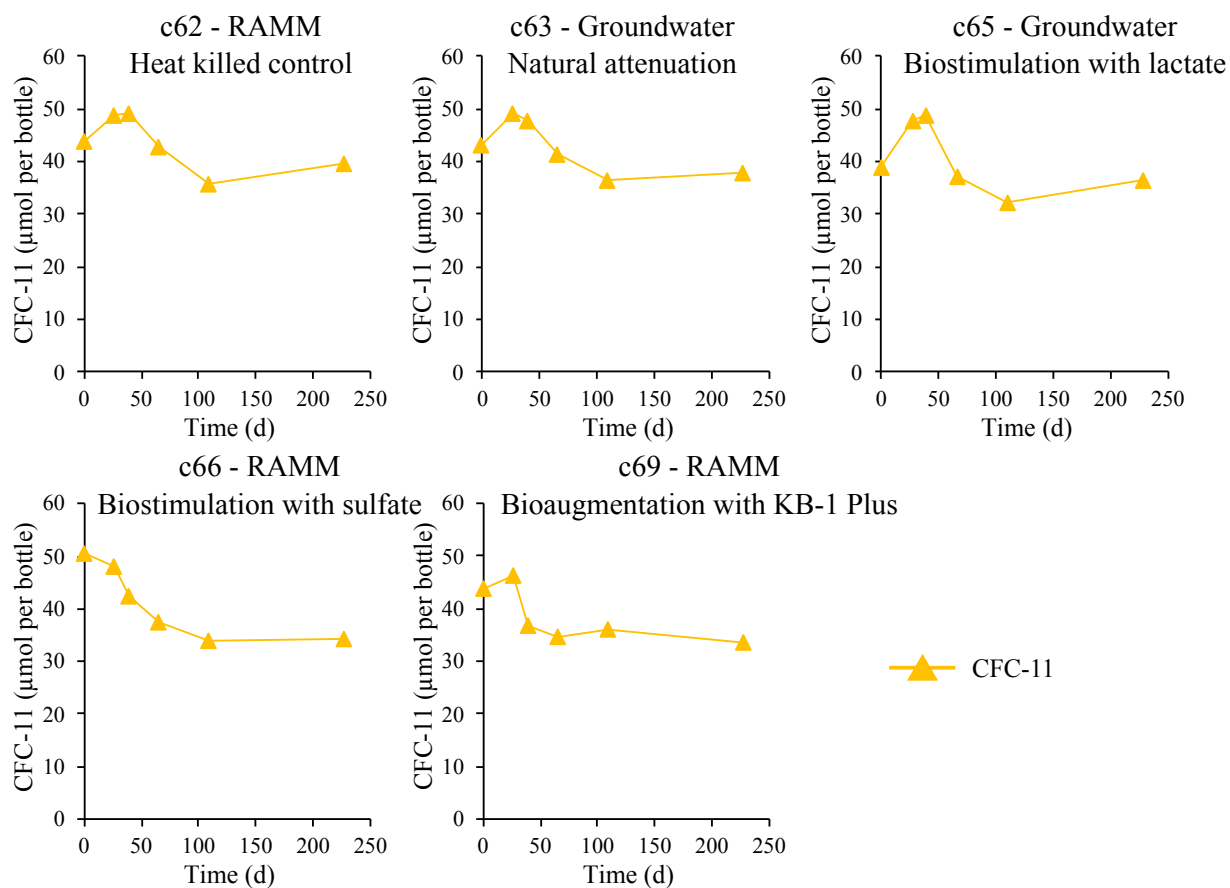


Figure 32. Borehole A3-15 m microcosm performance (CFC-11).

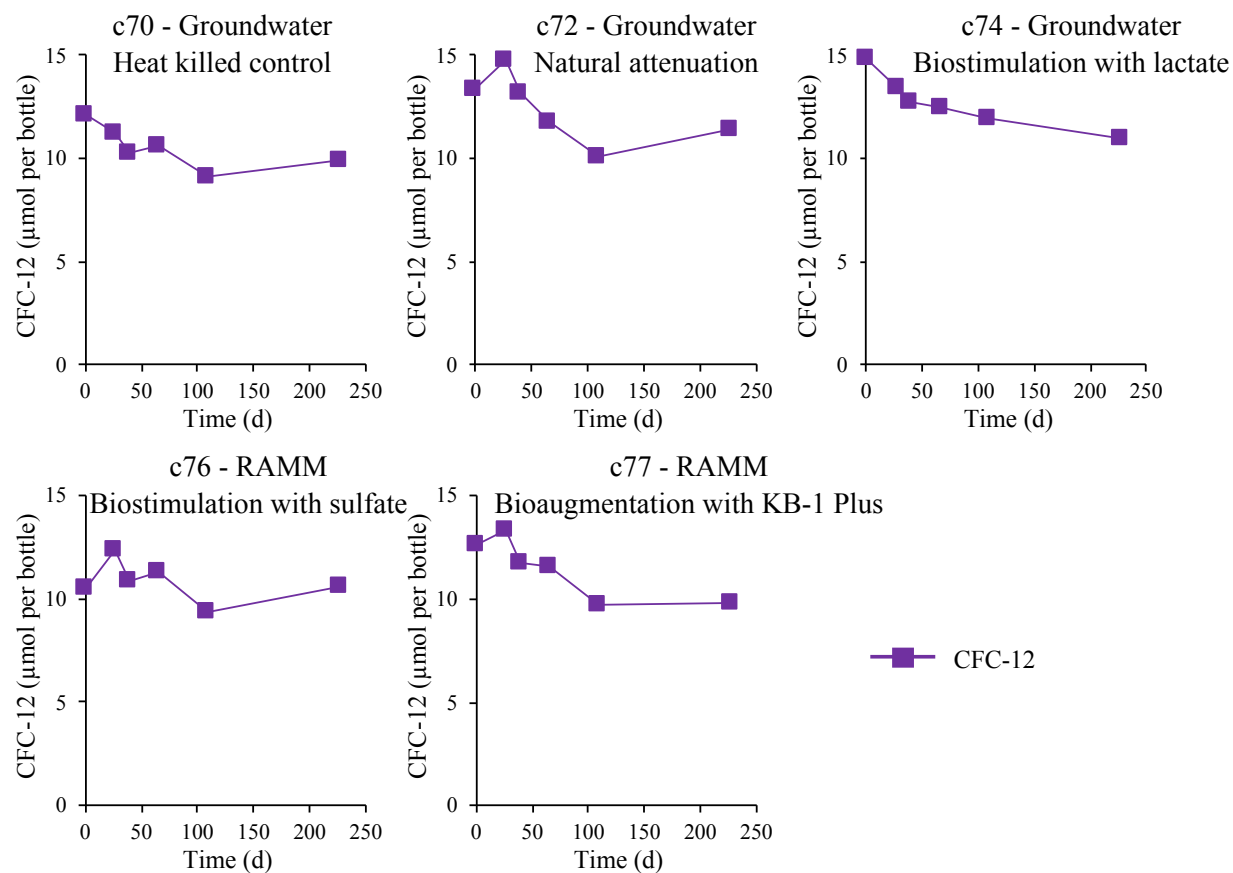


Figure 33. Borehole A3-15 m microcosm performance (CFC-12).

Table 16. Borehole A3-24.20 m lactate fermentation performance

BHA3 - 24.20 m	PCE	1,1-DCE	CT	CF	CFC-11	CFC-12
Bioaugmentation	(4/4)	(4/4)	(4/4)	(4/4)	(4/4)	(4/4)
Biostimulation	(4/4)	(2/4)	(4/4)	(4/4)	(0/4)	(2/4)

The ratio of bottles in which lactate fermentation was observed in biostimulated and bioaugmented microcosms for each contaminant at borehole A3-24.20 m.

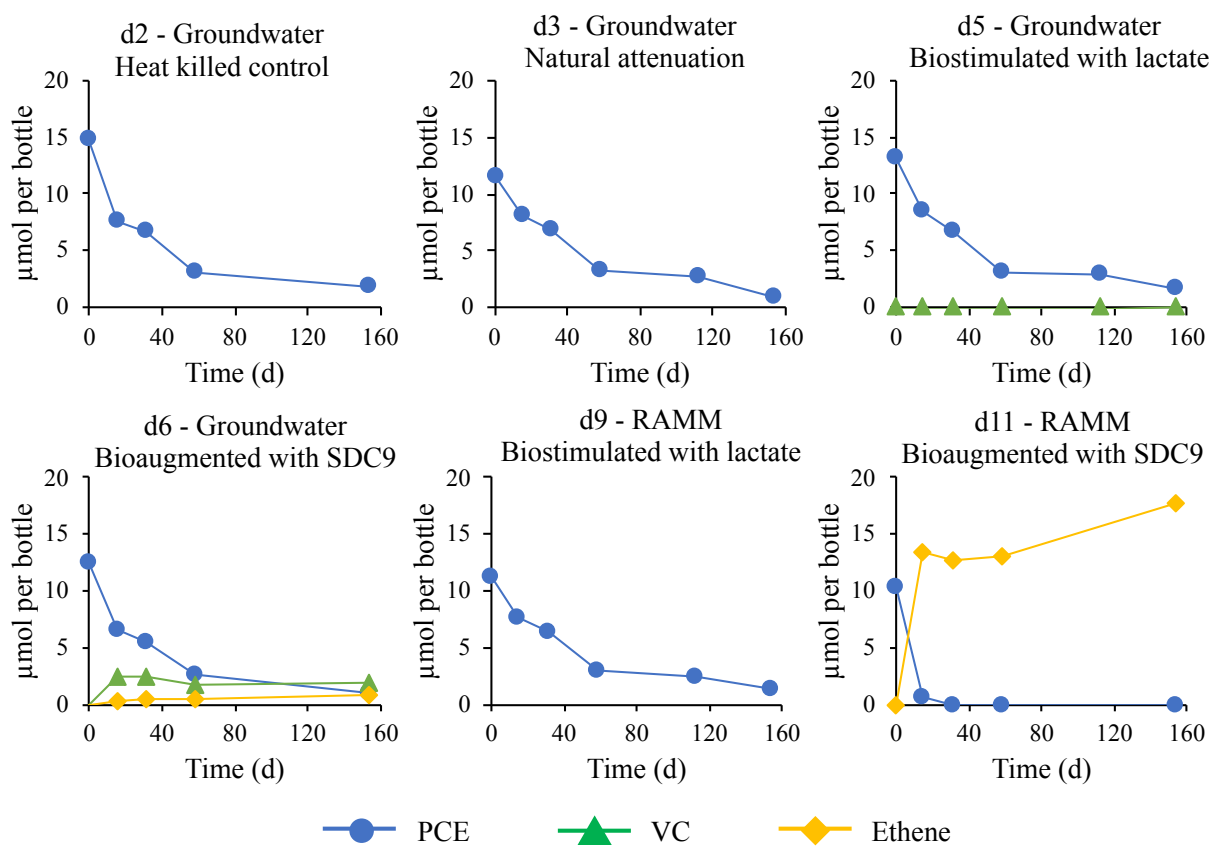


Figure 34. Borehole A3-24 m microcosm performance (PCE).

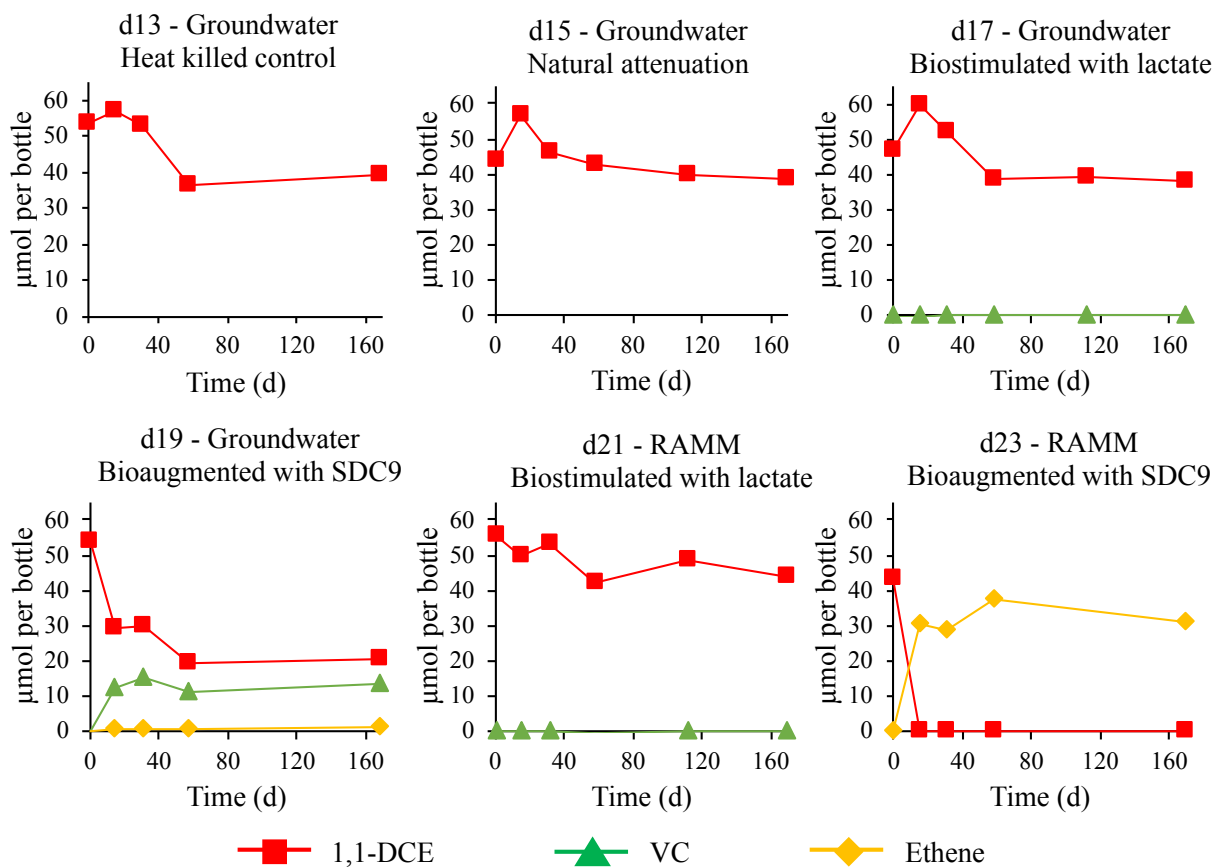


Figure 35. Borehole A3-24 m microcosm performance (1,1-DCE.)

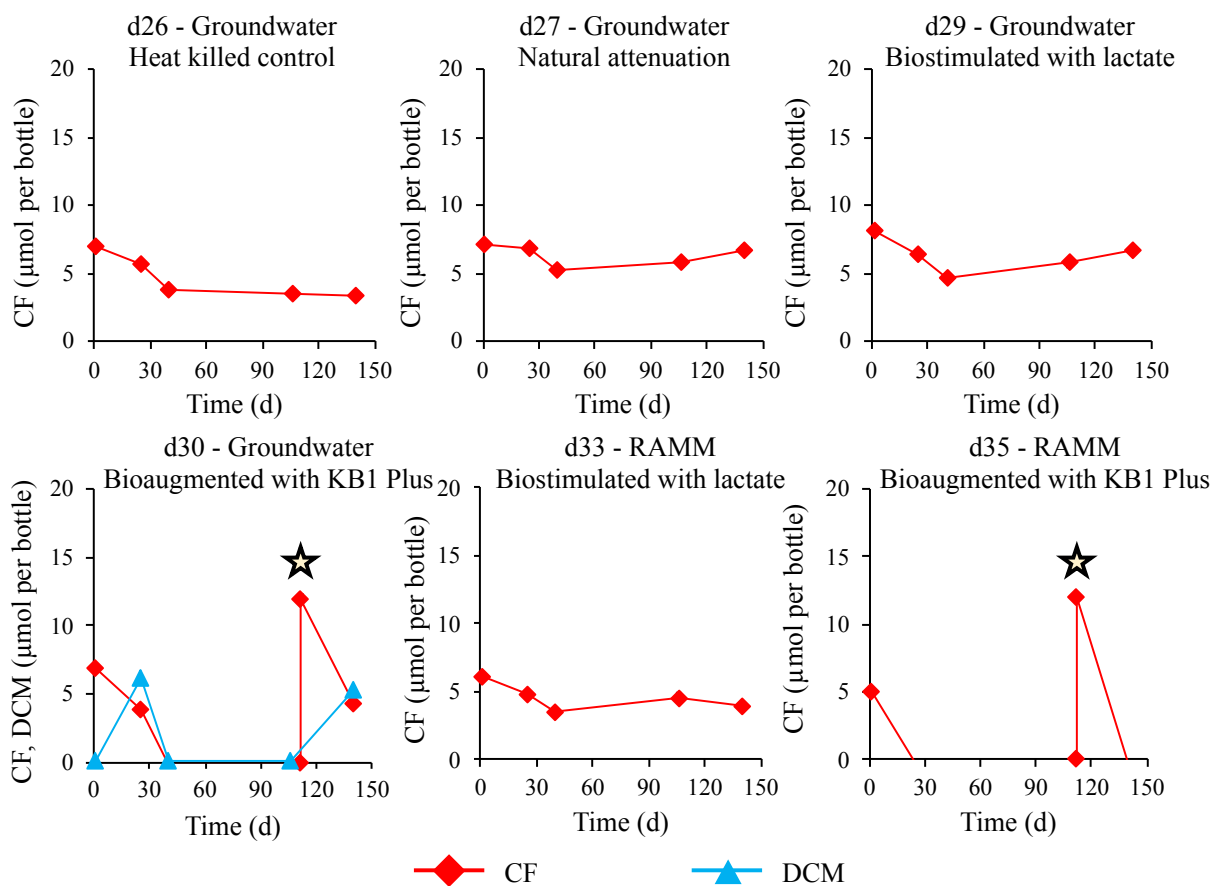


Figure 36. Borehole A3-24 m microcosm performance (CF).

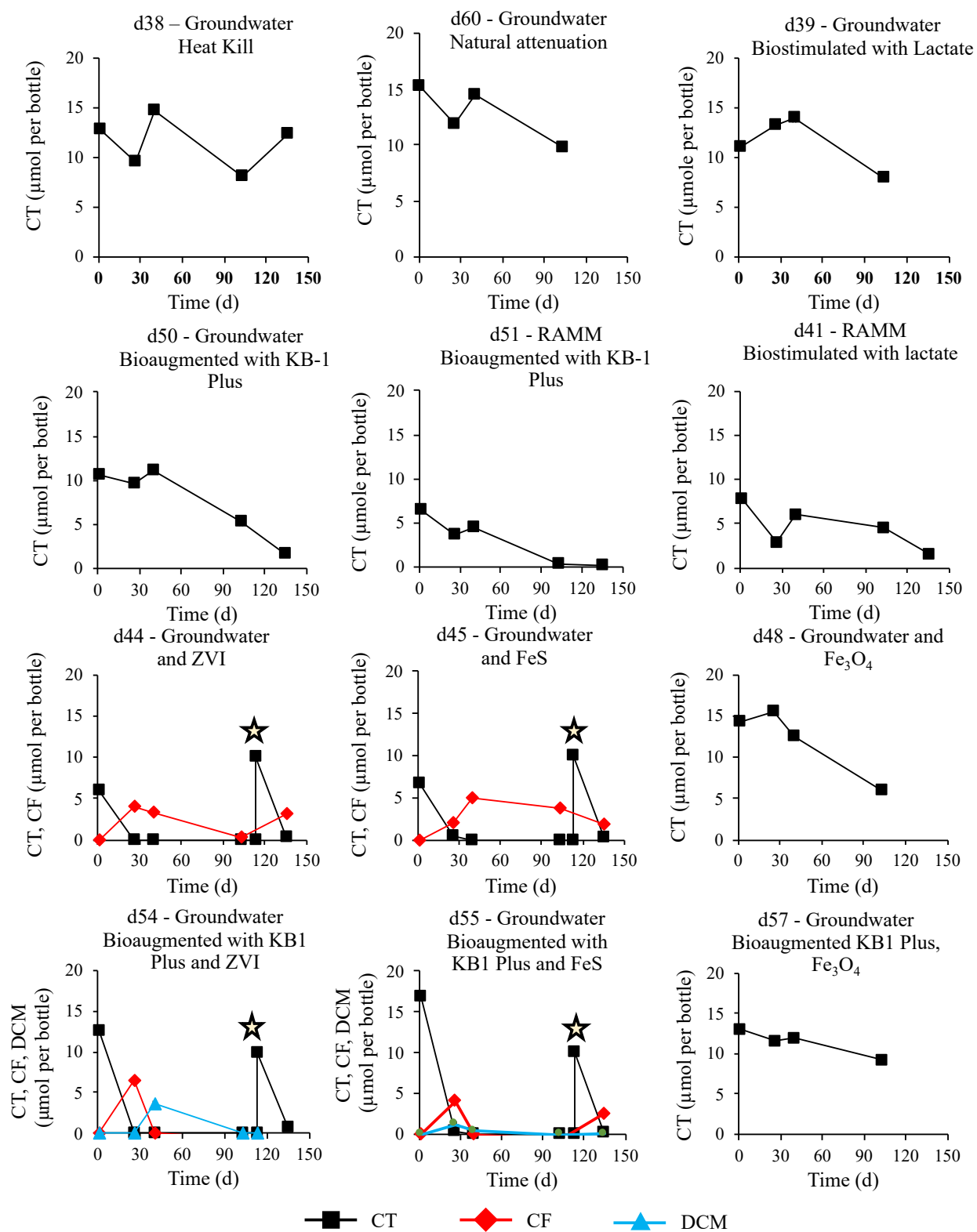


Figure 37. Borehole A3-24 m microcosm performance (CT).

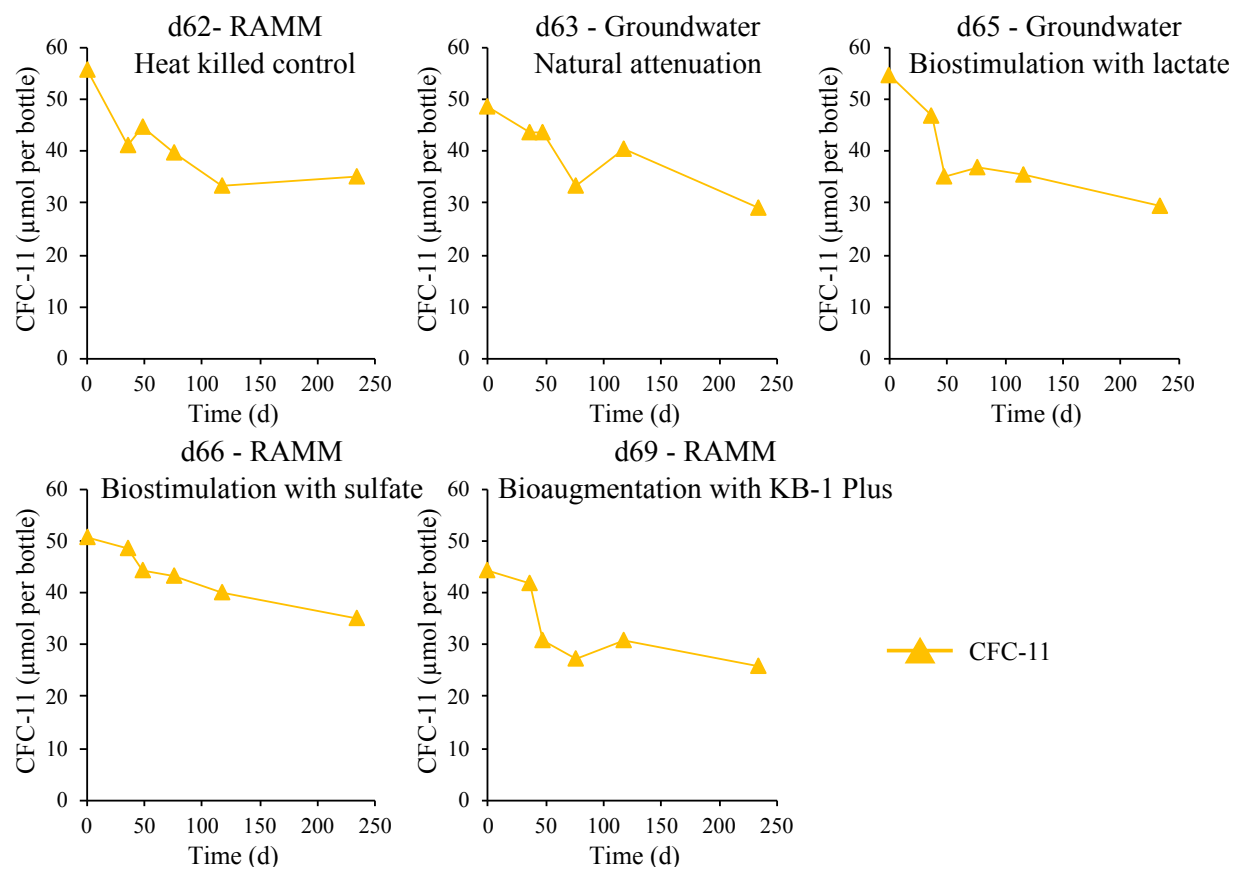


Figure 38. Borehole A3-24 m microcosm performance (CFC-11).

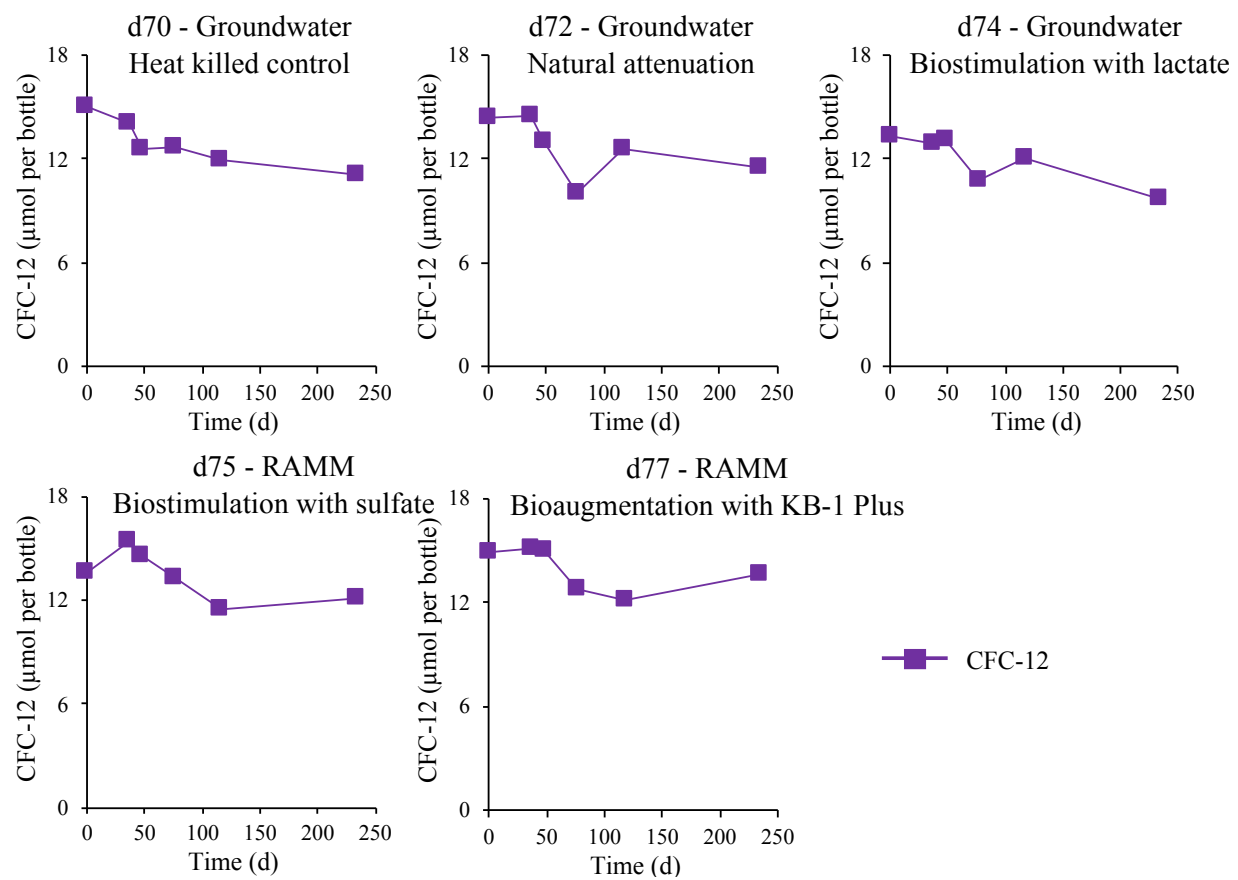


Figure 39. Borehole A3-24 m microcosm performance (CFC-12).

Table 17. Borehole A4-10.10 m lactate fermentation performance.

BHA4 - 10.10 m	PCE	1,1-DCE	CT	CF	CFC-11	CFC-12
Bioaugmentation	(4/4)	(4/4)	(4/4)	(4/4)	(4/4)	(4/4)
Biostimulation	(4/4)	(4/4)	(4/4)	(4/4)	(4/4)	(4/4)

The ratio of bottles in which lactate fermentation was observed in biostimulated and bioaugmented microcosms for each contaminant at borehole A4-10.10 m.

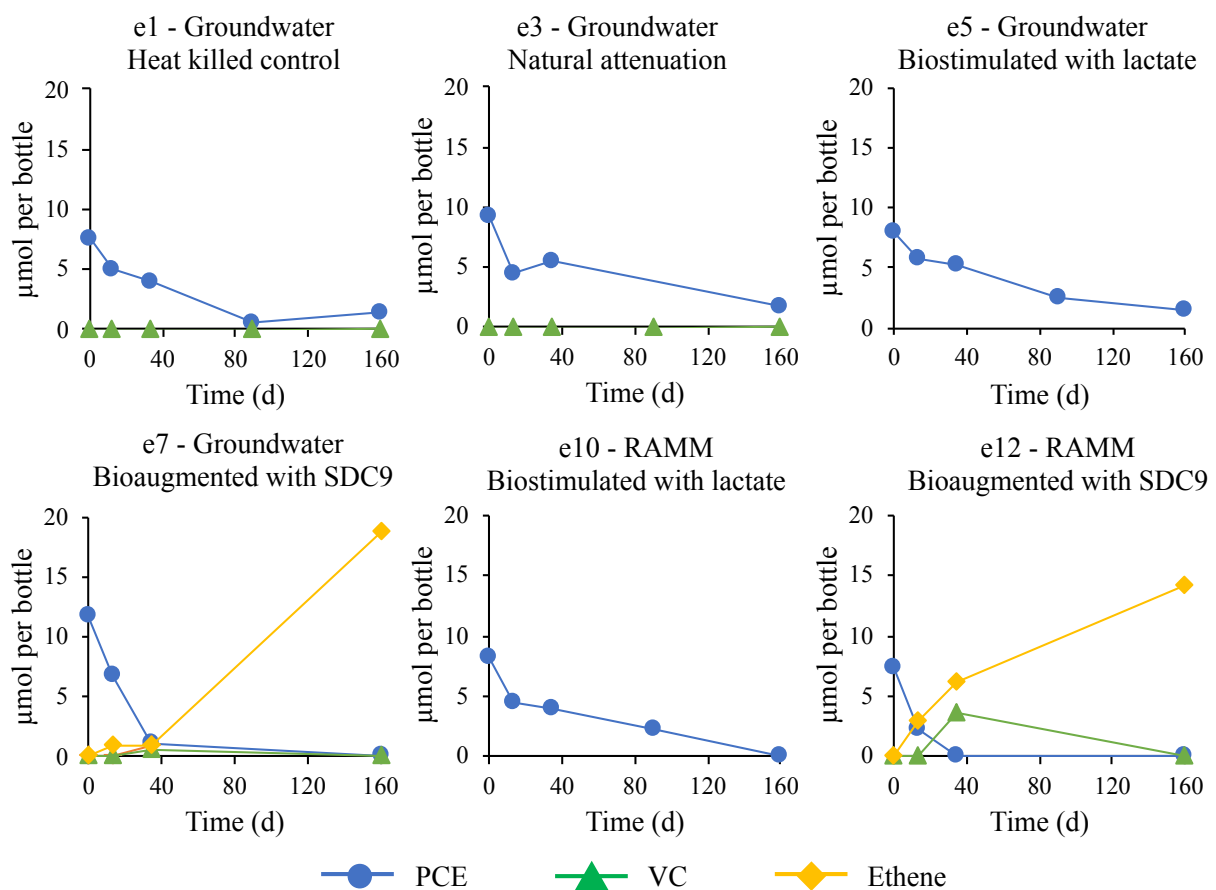


Figure 40. Borehole A4-10 m microcosm performance (PCE).

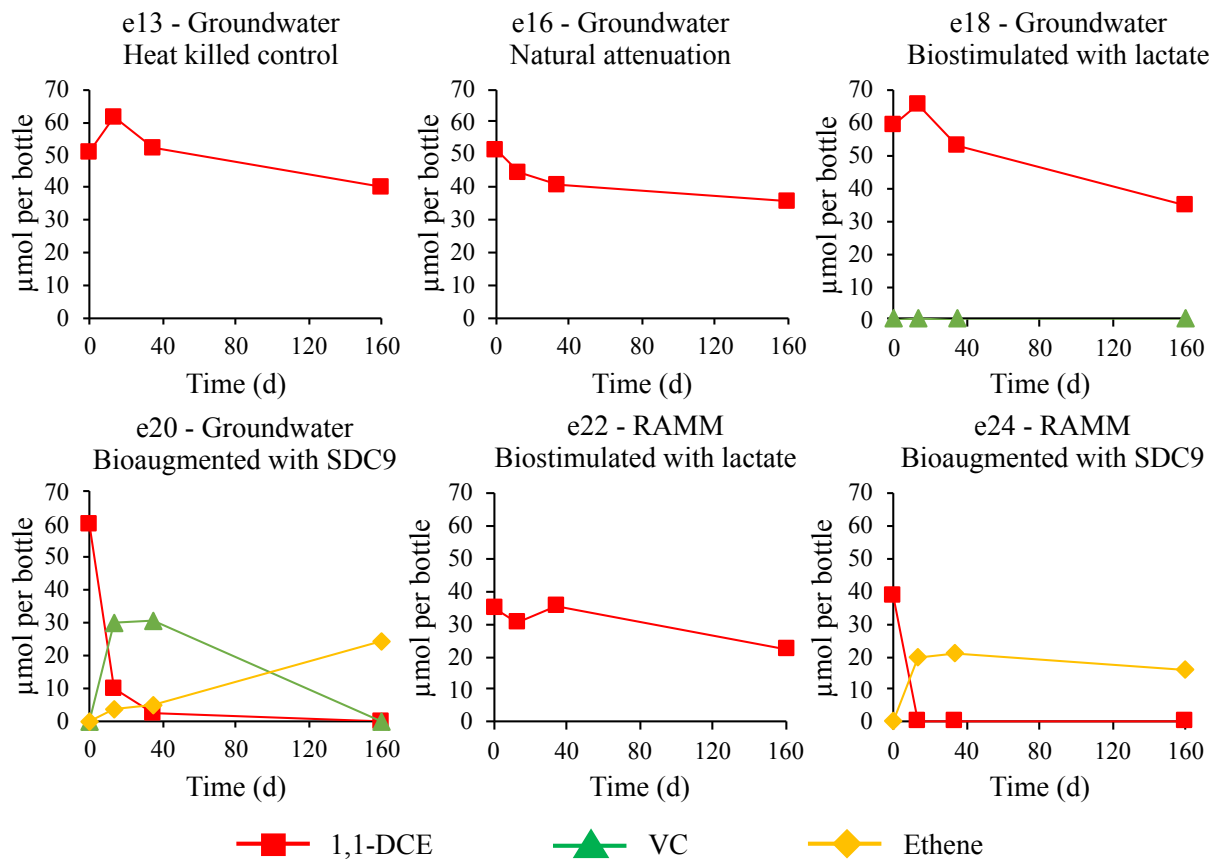


Figure 41. Borehole A4-10 m microcosm performance (1,1-DCE).

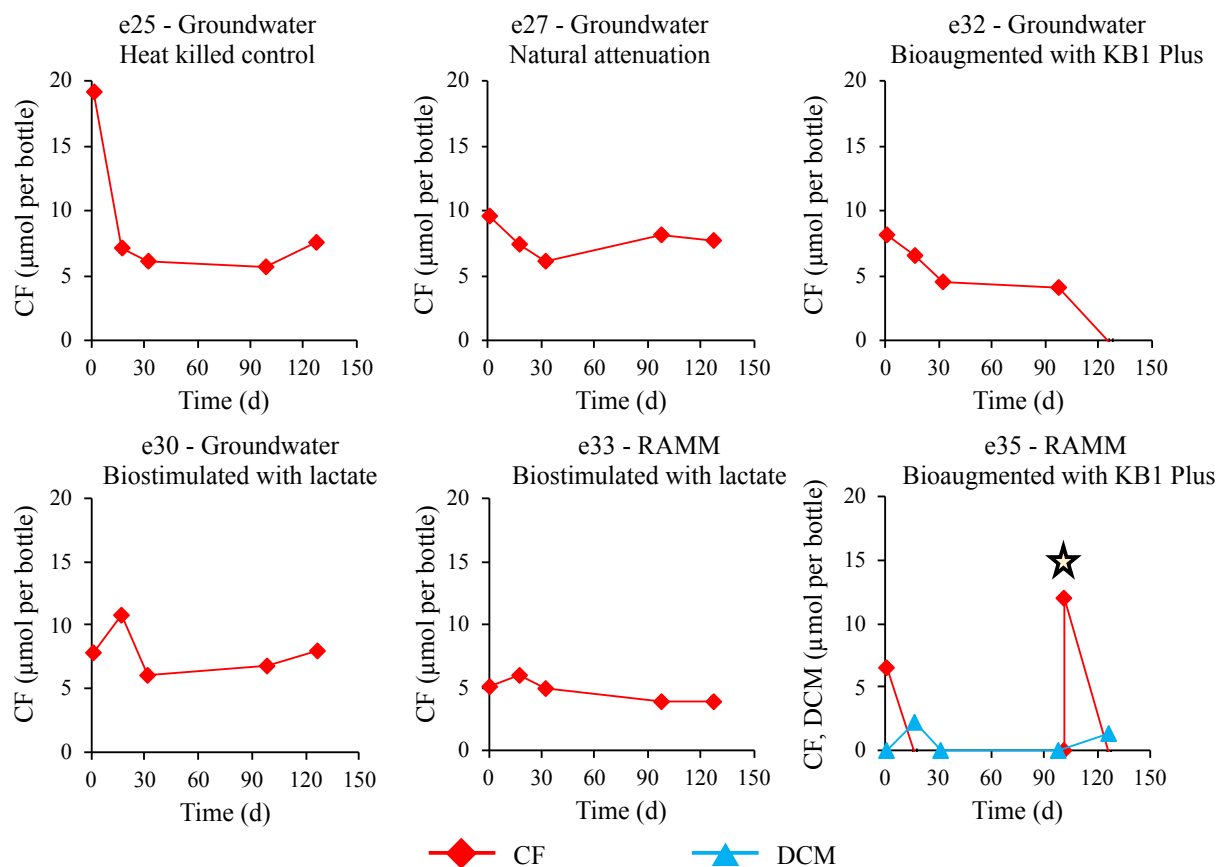


Figure 42. Borehole A4-10 m microcosm performance (CF).

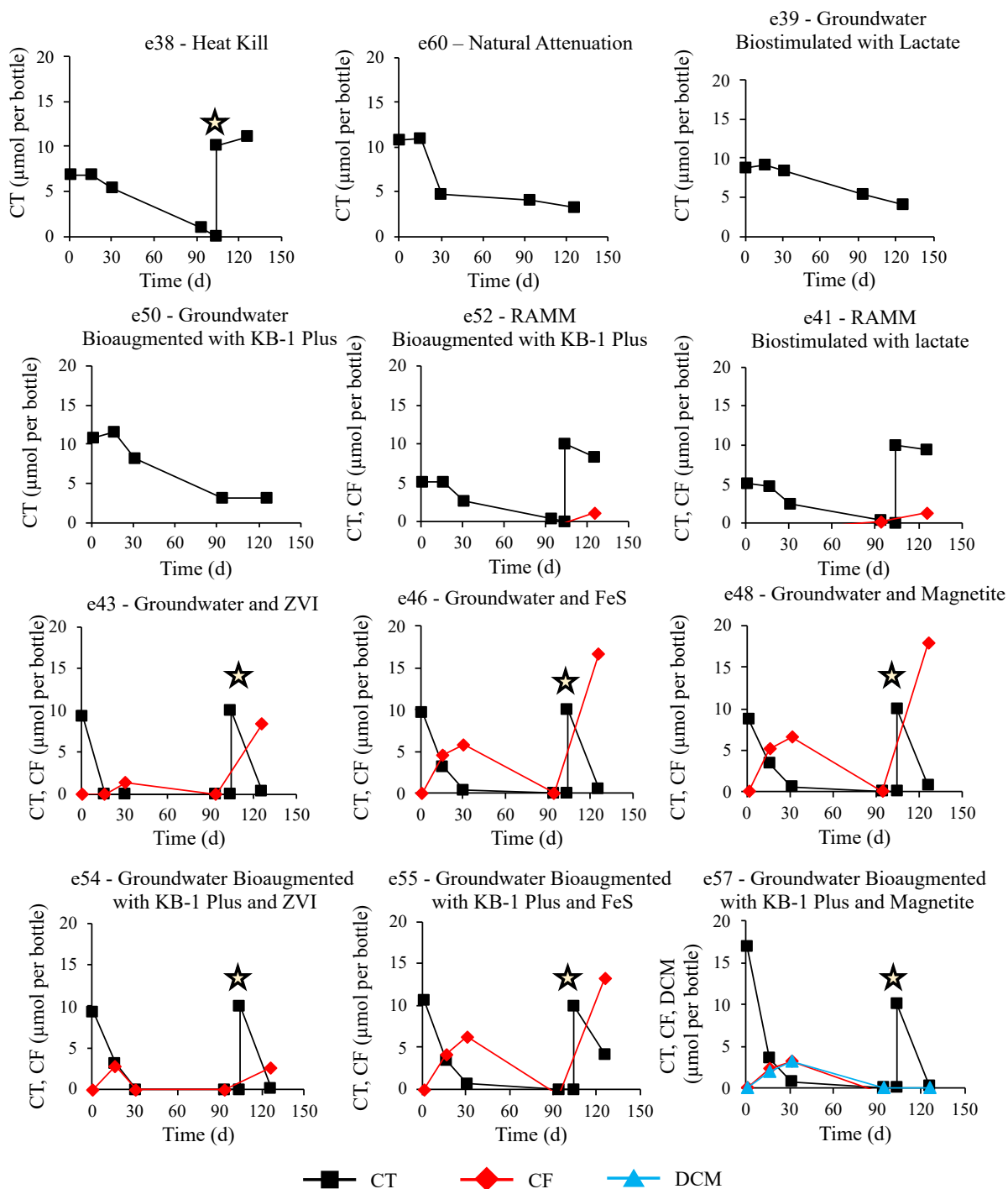


Figure 43. Borehole A4-10 m microcosm performance (CT).

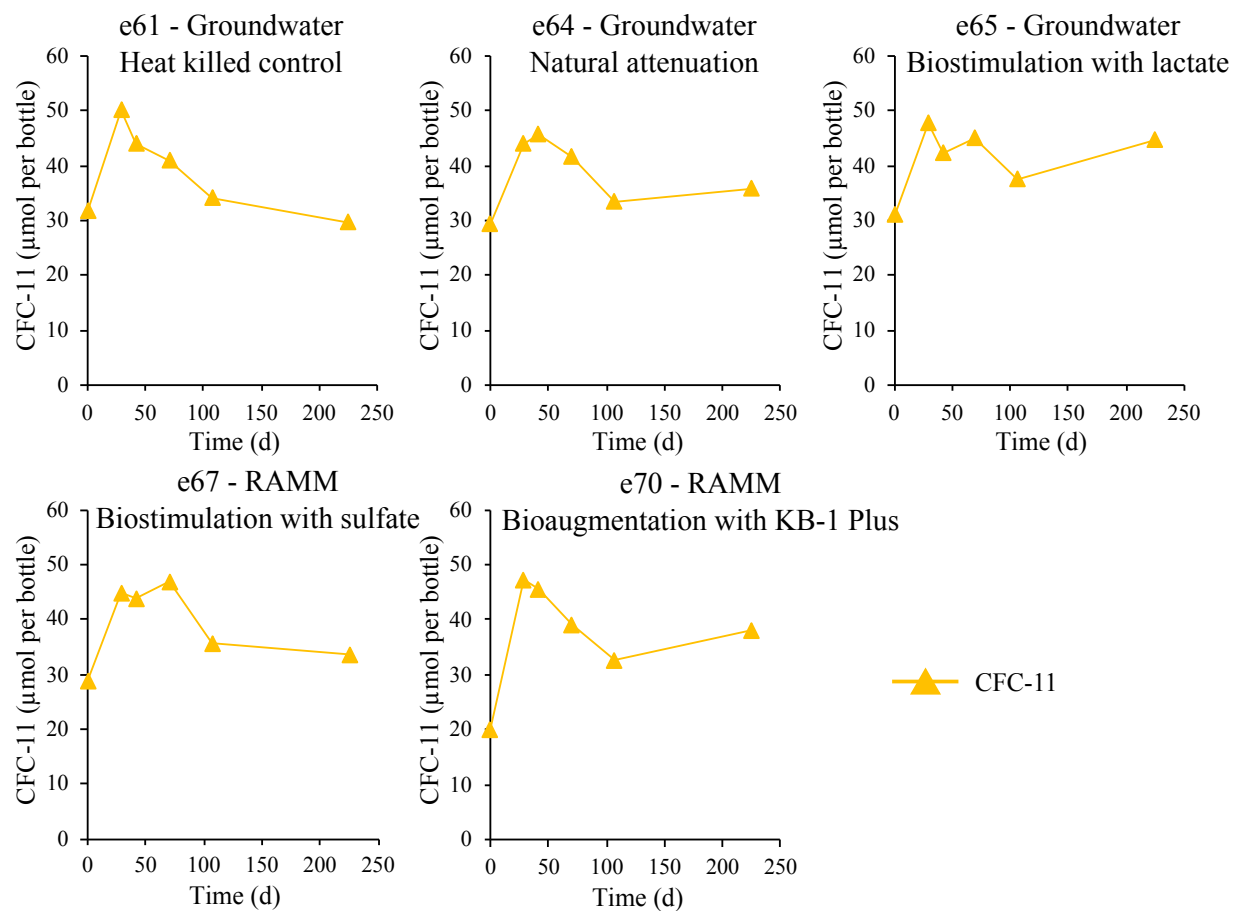


Figure 44. Borehole A4-10 m microcosm performance (CFC-11).

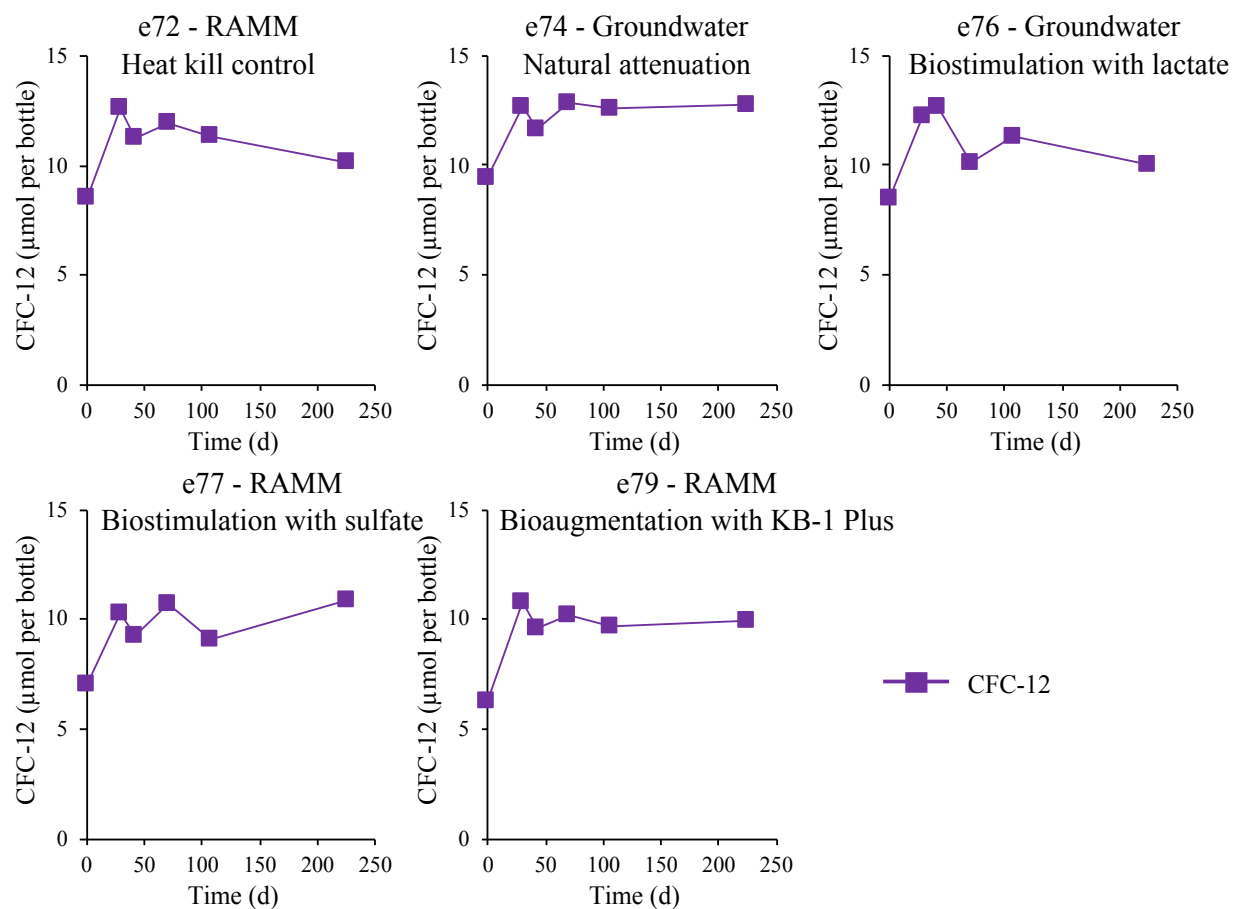


Figure 45. Borehole A4-10 m microcosm performance (CFC-12).

Table 18. qPCR data from Sterivex filters collected at site A.

Sample Name	Volume of Sample Filtered (mL)	DNA Conc. (ng/μl)	Volume of purified DNA (μl)	<i>Dehalococcoides</i> 16S rRNA		<i>Dehalobacter</i> 16S rRNA		<i>Dehalogenimonas</i> 16S rRNA	
				Ave Gene copies/mL	STD Error	Ave Gene copies/mL	STD Error	Ave Gene copies/mL	STD Error
BER-RT1-BHA3-SF-1 (ST-2A)	760	0.50	100	ND	ND	DNQ	DNQ	ND	ND
BER-RT1-BHA3-SF-2 (ST-3A)	760	0.60	100	ND	ND	DNQ	DNQ	ND	ND
BER-RT1-BHA3-SF4 (ST-1A)	760	0.36	100	ND	ND	DNQ	DNQ	ND	ND
BER-RT1-BHA3-SF3 (ST-4B)	790	0.37	100	ND	ND	DNQ	DNQ	ND	ND
BER-RT1-BHA4-SF1 (ST-5A)	790	0.38	100	ND	ND	170	6.10	ND	ND
BER-RT1-BHA4-SF2 (ST-8A)	310	too low	100	ND	ND	DNQ	DNQ	ND	ND
BER-RT1-BHA4-SF3 (ST-7B)	630	0.08	100	ND	ND	DNQ	DNQ	ND	ND
BER-RT1-BHA4-SF4 (ST-9A)	520	0.18	100	ND	ND	DNQ	DNQ	ND	ND
BER-RT1-BHA4-SF5 (ST-17A)	500	0.33	100	ND	ND	117	3.32	ND	ND
BER-RT1-BHA4-SF6 (ST-6B)	520	0.32	100	ND	ND	134	9.75	ND	ND

DNQ = Detected Not Quantifiable

ND = Not Detected

CHAPTER III

A LABORATORY-SCALE TREATABILITY STUDY OF BIOSTIMULATION, BIOAUGMENTATION, AND AMENDMENTS WITH REACTIVE IRON SPECIES FOR TETRACHLOROETHENE, TETRACHLOROMETHANE, AND TRICHLOROMETHANE DEGRADATION AT A CONTAMINATED SITE.

Abstract

A bench-scale treatability study was performed with aquifer material from a contaminated site in the US, which is predominantly impacted by carbon tetrachloride (CT), chloroform (CF), and tetrachloroethene (PCE). Microcosms were constructed in 160-mL glass vessels containing about 18 g (wet weight) of aquifer material and 80 mL of site groundwater or entirely synthetic, reduced anoxic mineral salt medium (RAMM). Chlorinated solvents were monitored by GC-FID, and the organic acid concentration was monitored by HPLC. Four remedial treatments were evaluated: monitored natural attenuation (MNA), biostimulation, biostimulation combined with bioaugmentation, and the amending of microcosms with zero-valent iron (ZVI). Groundwater was filtered through Sterivex filters to identify organohalide-respiring bacteria (OHRB) by qPCR. The lack of degradation of PCE, CT, or CF in microcosms set up to simulate MNA and biostimulation conditions, along with the limited detection of OHRB in qPCR assays, suggests that a community of OHRB are not active at this contaminated site. OHRB were detected at one of the four sampling locations in low abundance. In samples from location B-2-S, the qPCR assay for *Dehalococcoides* detected 5.5 gene copies per mL, and the Dehalogenimonas assay detected 6.5 gene copies per mL. Furthermore, bioaugmentation combined with biostimulation effectively degraded PCE and CF in microcosms that contained RAMM, but no activity was observed in microcosms that contained groundwater. This observation suggests that a constituent of the groundwater inhibits the growth of native and amended OHRB. Transformation of CT to CF was observed in microcosms amended with ZVI.

Introduction

The samples used in this study were from a chlorofluorocarbon manufacturing facility. The aquifer at this contaminated site is impacted by various chlorinated volatile organic compounds (cVOCs), including PCE, CT, and CF **Table 19**, Appendix C. The presence of trichloroethene (TCE; 0.02 mg/L), *cis*-1,2-dichloroethene (cDCE; 0.02 mg/L), and vinyl chloride (VC; 0.01 mg/L), which are typical PCE transformation products, was observed in groundwater monitoring wells. In addition, dichloromethane (methylene chloride; DCM; 0.05 mg/L) and chloromethane (methyl chloride; CM; 0.01 mg/L), both potential transformation products of CT and CF, were detected in groundwater samples. These observations suggest that indigenous processes transform PCE, CT, and CF to products with a lower degree of chlorination; however, the extent of *in situ* dechlorination is limited, and the transformation products are also regulated compounds (*i.e.*, detoxification is not achieved). In addition, fluorinated volatile organic compounds were observed in groundwater monitoring wells, 1,1,2-trichloro-1,2,2-trifluoroethane (CFC-113) was observed at a concentration of 1.10 mg/L, and dichlorodifluoromethane (CFC-12; 0.01 mg/L) and trichlorofluoromethane (CFC-11; 0.01 mg/L) were observed.

CT, CF, and PCE are recalcitrant cVOCs listed on the EPA's priority pollutants list.¹⁶⁵ These VOCs have densities of 1.49 g/cm³ (CF), 1.59 g/cm³ (CT), and 1.62 g/cm³ (PCE) and tend to form dense non-aqueous phase liquids in the subsurface upon release.¹⁶⁶ Such dense non-aqueous phase liquid source zones can feed extensive groundwater contaminant plumes for many decades.¹⁴⁴

A variety of processes, including biotic and abiotic degradation, convert cVOCs into non-hazardous products.^{68–70,167} Degradation of cVOCs by zero-valent iron (ZVI) or reactive mineral phases, such as iron sulfide (FeS) and magnetite (Fe₃O₄), has been documented in the literature, and installation of permeable reactive barriers can be a strategy to intercept plumes and degrade cVOCs.⁷⁰ Reactions between mineral phases and cVOCs are influenced by various biogeochemical parameters, including pH, sulfate/sulfide concentrations, natural organic matter content, iron availability, and the resident microbial community (*e.g.*, sulfate-reducing and ferric iron-reducing bacteria).

In addition to abiotic processes, microbial degradation is a significant attenuation process for cVOCs. A diversity of bacteria has been discovered that use cVOCs as respiratory electron acceptors in a process known as organohalide respiration.^{143,167} In the context of cVOCs, the focus has been on chlorinated ethenes such as PCE, TCE, cDCE, and VC. Microbes capable of reductively dechlorinating cVOCs have been reported in the literature.^{143,167} If these microbes are present and the environmental conditions support their activity, *in situ* contaminant degradation can lead to contaminant mass reduction and detoxification. If in such a scenario, the indigenous degradation rates meet remedial goals, the implementation of monitored natural attenuation (MNA) can be considered.

At many sites, bacteria capable of degrading cVOCs are present, but site-specific conditions are not favorable to support efficient contaminant degradation. In such cases, altering the site conditions can stimulate biodegradation activity. For instance, adding a fermentable substrate (*i.e.*, biostimulation) to consume oxygen can establish favorable redox conditions and simultaneously increase the flux of hydrogen, which is the preferred electron donor for many organohalide-respiring bacteria. To further enhance contaminant degradation rates, or to initiate the microbial reductive dechlorination process at sites where the key dechlorinators are absent or present in low abundances, the injection of a microbial consortium (*i.e.*, bioaugmentation) can be considered.^{168–171} Two commercial microbial consortia were tested in this study, including APTIM's SDC-9 culture, which was used to stimulate the degradation of PCE in microcosms, and SiREM's KB-1 Plus culture, which was used to stimulate the degradation of CT and CF in microcosms.

The overarching goal of this bench-scale treatability study was to assess the potential for *in situ* degradation of the target contaminants by MNA, enhanced *in situ* bioremediation (*i.e.*, biostimulation alone and combined with bioaugmentation), and abiotic degradation of CT by ZVI in aquifer material from the site. Degradation activity was measured by following the concentrations of target contaminants (*i.e.*, PCE, CT, and CF) and the formation of potential transformation products (*i.e.*, cDCE, VC, DCM, CM, ethene, and methane). Additional measures included the consumption of the electron donor (*i.e.*, lactate), the formation of lactate fermentation products (*i.e.*, acetate and propionate), and the presence of known dechlorinating bacteria in biomass associated with the groundwater.

Methodology

Sample Collection.

Aquifer material and groundwater samples were collected near a former miscellaneous landfill area at the contaminated site. Four temporary wells were established to a depth of 20 ft and

placed 5–8 ft north, south, east, and west of the monitoring well **Figure 46**, Appendix C. Each aquifer core was 23 in long and encased in a cellulose acetate butyrate (CAB) liner. Groundwater was collected from each of the four temporary wells in 1-L plastic containers closed with gas-tight screw caps. Aquifer material and groundwater samples were classified with the label “B” (for borehole = temporary well) 1 through 4, with the numerals indicating the order in which the samples were obtained and with “N,” “S,” “E,” and “W” for the location of the boreholes relative to the monitoring well **Figure 46**, Appendix C. These identifiers (*e.g.*, B-1-E, B-2-S, B-3-W, and B-4-N) were used to identify aquifer and groundwater origins.

The field crew measured basic groundwater parameters at the time of sampling, and the data are summarized in **Table 20**, Appendix C. In addition, groundwater from each of the four temporary wells was filtered on site through Sterivex cartridges to collect biomass associated with the groundwater. The volume of the groundwater that passed through each cartridge was recorded and noted on the respective cartridge housing **Table 21**, Appendix C. From each temporary well location, two Sterivex cartridges were obtained (eight cartridges total). Aquifer material cores, groundwater, and Sterivex cartridges were shipped in a cooler with blue ice packs via an overnight carrier to the University of Tennessee, and the shipment arrived on June 19, 2019.

Sample processing and microcosm setup.

Two liters of composite groundwater were prepared and used as the liquid phase for microcosm setup. To prepare composite groundwater, equal volumes (250 mL) of groundwater collected from temporary well locations B-1-E, B-2-S, B-3-W, and B-4-N were mixed in 2-L vessels under a constant flow of nitrogen (N₂). To remove oxygen and VOCs, sterile, oxygen-free N₂ gas was bubbled through the composite groundwater for 30 min. Prior to the vessels being capped, 20 mL was transferred from each vessel to a clean beaker, and pH was measured. The pH of the composite groundwater in both vessels was 7.7 ± 0.05 pH units. The 2-L vessels with N₂ headspace were closed with butyl rubber stoppers secured with aluminum crimps and were stored at 4°C until used for microcosm setup.

A total of four ~23-inch-long aquifer material cores were obtained with the designations B-1-E, B-2-S, B-3-W, and B-4-N. The aquifer cores were cut into two segments and immediately closed with a plastic cap, and the seal was secured with electrical tape. The bottom 33 cm portion of the core was considered the lower core, and the top ~10-inch portion of the core was considered the upper core **Figure 47**, Appendix C.

Following the separation of cores, the term “lower” refers to the bottom portions, and the term “upper” refers to the top portions. The cut cores were transferred inside an anoxic glove box filled with N₂/H₂ (97:3, vol/vol) to transfer aquifer material from the CAB core liners into sterile glass Mason jars. The aquifer material had a firm consistency that was very difficult to remove from the CAB liners. The upper portion of the core material contained more of a fine material, with some visible sand and white, visibly porous pebbles. The lower portion of the core contained a higher proportion of clay. Homogenization of the aquifer materials by physical mixing was impossible due to the material’s firm texture, and it was impossible to distribute the material into glass serum bottles for microcosm setup. Pipettable slurries were made in order to distribute the homogenized material into glass serum bottles for microcosm setup. To make the pipettable slurries groundwater from the corresponding sampling locations was added to the

Mason jars containing the solids in an attempt to prepare pipettable slurries **Table 22**, Appendix C. Then, manual homogenization of the slurries was performed outside of the glove box under a constant stream of anoxic, sterile N₂. This process was time-consuming due to the firm texture of the aquifer material, and it took several hours before this process yielded pipettable slurries. The Mason jars containing the slurries with N₂ headspace were closed with gas-tight lids, and the contents were mixed with a magnetic stirrer at 130 rpm at room temperature overnight.

The following day, composite slurries were prepared to represent the upper and lower portions of the cores **Table 22**, Appendix C. The composite slurry representing the upper segments was prepared by mixing equal volumes (~200 mL) of the four slurries made from the top portions of the B-1-E, B-2-S, B-3-W, and B-4-N aquifer material cores **Table 22**, Appendix C. The composite slurry representing the lower portions was prepared by mixing ~200 mL of each of the slurries established with the lower portions of the cores. To prepare pipettable slurries, an additional volume of 100 mL of composite groundwater was added to the upper and lower composite slurries. The Mason jars containing the pipettable composite slurries were then capped and manually shaken for homogenization. **Table 22**, Appendix C provides information about the amount of aquifer material and groundwater used to prepare the individual slurries. **Table 22**, Appendix C summarizes the amounts of aquifer material and volumes of groundwater that were used to establish the different slurries. The individual upper and lower core slurries were then mixed to form the upper and lower composite slurries identified in the boxes on the right-hand side of **Table 22**, Appendix C.

Microcosms were prepared at the gassing (Hungate) station under a constant flow of an anoxic, sterile mixture of N₂ and carbon dioxide (CO₂; 80/20, vol/vol; **Figure 48**, Appendix C and **Figure 49**, Appendix C). All vessels were flushed with the 80/20 (vol/vol) N₂/CO₂ gas mixture to replace air. First, 18 mL of the designated composite slurry was transferred into sterile 160-mL glass serum bottles with a sterile 25-mL plastic pipette **Figure 48**, Appendix C. Then, 80 mL of either RAMM-containing resazurin as a redox indicator or anoxic composite groundwater was added **Figure 50**, Appendix C. The pH of the groundwater decreased from 7.7 to 7.2 ± 0.2 pH units. The microcosms were sealed with blue butyl rubber stoppers held in place with aluminum crimps. All microcosms received 0.2 mM sulfide from a 100-mM sterilized stock solution of sodium sulfide. Lactate (~5 mM) was added to the 2-L bottles of RAMM when the medium was prepared.¹⁷² For microcosms containing groundwater, lactate was added by injecting 1 mL from a 1-M stock solution with a 1-mL plastic syringe equipped with a sterile 0.2- μ m membrane filter **Table 24**, Appendix C. One replicate microcosm per treatment was autoclaved (40 min at 121°C at 15 psi) immediately after microcosm setup, and a second time the following morning, to serve as heat-killed control. Heat-killed controls received PCE, CT, and CF after autoclaving. All cVOCs were added with designated Hamilton glass syringes equipped with reproducibility (Chaney) adaptors. The concentrations of PCE, CT, and CF added to the microcosms are shown in **Table 23**, Appendix C.

Figure 49, Appendix C shows the system used to dispense groundwater or RAMM into 160-mL glass serum bottles containing the composite aquifer material slurries. The 2-L bottle containing anoxic groundwater or sterilized RAMM was inverted and placed on a three-legged stand (depicted on the left in **Figure 49**, Appendix C). The 2-L bottle was pressurized with anoxic, sterile N₂ gas. A sterile PVC line with a needle penetrating the bottle's rubber stopper delivered

RAMM or groundwater to the serum bottles with the slurries. This RAMM transfer line was equipped with a valve to regulate flow. Each 160-mL serum bottle received 80 mL of RAMM or groundwater. A 160-mL serum bottle containing 100 mL of RAMM was bubbled continuously with the same N₂/CO₂ gas mixture to serve as a proxy for monitoring RAMM pH. A RAMM pH of 7.2 ± 0.2 pH units was maintained by adjusting the percentage of CO₂ in the gas mixture. All serum bottles were flushed with this N₂/CO₂ (~80/20, vol/vol) gas mixture and capped with blue rubber butyl stoppers, which were secured with aluminum crimps **Figure 50**, Appendix C.

All microcosms were equilibrated overnight at room temperature with the stoppers down. The next morning, the redox indicator in microcosms established with RAMM was pink, indicating the redox potential was above -110 mV, potentially unfavorable for reductive dechlorination. To lower the redox potential in the microcosms, an additional 0.2 mM sodium sulfide was added to all microcosms, including those that had been established with groundwater (note that the groundwater microcosms did not contain the redox indicator resazurin and received the same treatment as did the microcosms established with RAMM.) After an additional day of incubation, the redox indicator remained pink. At this time, cVOCs were measured in headspace samples using a gas chromatograph (GC) equipped with a flame ionization detector (FID). Headspace samples were also injected into a GC equipped with a micro electron capture detector to detect low concentrations of oxygen. These measurements did not provide evidence for elevated concentrations of oxygen in any of the microcosms. Aliquots (1 mL) of the liquid phase were withdrawn from all microcosms to measure organic acids (*i.e.*, lactate, propionate, and acetate) by using high-performance liquid chromatography (HPLC).

Bioaugmentation.

Microcosms containing PCE were amended with culture SDC-9, a commercial bioaugmentation consortium capable of degrading a variety of halogenated contaminants.¹⁷³ The SDC-9 culture was received from APTIM on June 21, 2019 and was stored at 4°C until it was injected into the designated microcosms on July 1, 2019 **Table 24**, Appendix C.

Microcosms containing CT and CF were augmented with consortium KB-1 Plus provided by SiREM. The KB-1 Plus culture was received on June 20, 2019 and stored at 4°C until it was injected into the designated microcosms on July 1, 2019 **Table 24**, Appendix C. Microcosms received 3 mL of the respective bioaugmentation cultures using 3-mL, N₂-flushed plastic syringes with 25G needles. Although the microcosms containing the redox indicator resazurin still had a pink sheen, the microcosms were incubated without further manipulations with the stoppers down in the dark and without agitation at room temperature (~21°C). For all treatments, negative control microcosms were established to document potential contaminant loss (*e.g.*, sorption) and clearly demonstrate the benefits of the respective treatments.

Abiotic degradation.

Experiments were set up at the gassing (Hungate) station under a constant flow of N₂ and CO₂ (80/20, vol/vol). Composite aquifer material slurry (18 mL) was transferred to 160-mL glass serum bottles containing 80 mL of anoxic MilliQ water. The bottles were closed with butyl rubber stoppers and crimped prior to CT being added with a designated Hamilton syringe equipped with Chaney adaptors **Table 23**, Appendix C. The bottles were equilibrated overnight with the stoppers down at room temperature. The following morning, time zero measurements of contaminant concentrations in the headspace were acquired by manually injecting 100-μL

headspace samples into a GC-FID. Immediately after sampling, bottles 37a, 38a, and 39a were amended with 3 mL of a ZVI slurry containing 500 mg/mL ZVI by using a 3-mL plastic syringe equipped with an 18G needle. The ZVI slurry was prepared inside the anoxic glove box using anoxic deionized water and -200 mesh iron powder from Alfa Aesar (99+% metals-basis grade). Contaminant concentrations were monitored over the next 48 hr via headspace sampling and GC-FID analysis.

Microcosm monitoring.

cVOCs and organic acids were measured every 14 days over a 98-day period. One additional cVOC measurement occurred after 226 days. For cVOC measurements, plastic 1-mL syringes were used to withdraw 100 μ L of headspace sample, which was manually injected into a GC-FID. Organic acids were measured in the aqueous phase, and 1 mL of liquid was withdrawn with a 3-mL plastic syringe and transferred to a 1.5-mL plastic tube. Following centrifugation, 200 μ L of the supernatant was transferred to a 200- μ L plastic HPLC vial containing 2 μ L of 1 M sulfuric acid. The HPLC vials were stored at 4°C overnight and analyzed the following day by HPLC. Concentrations of analytes were determined by comparing the peak areas to externally generated standard curves.

Data analysis.

Peak areas from the GC-FID chromatograms were determined by integration performed with the Agilent software package OpenLab ChemStation (Rev. C.01.10). Peak areas from the HPLC chromatograms were determined by integration performed with Agilent software ChemStation for LC 3D systems (Rev. B.04.02). The peak areas for GC-FID and HPLC were recorded in an Excel spreadsheet and plotted. If the GC-FID measurements indicated that replicate microcosms performed similarly, then the cVOC data were averaged and plotted as a single graph. Separate graphs were prepared if replicate microcosms showed distinct dechlorination performance. For GC-FID analysis, the limit of detection (LOD) and limit of quantification (LOQ) for each chemical were calculated using the standard deviation of the response of the slope method.^{174,175}

Additional amendments.

On day 63 after microcosm setup, an additional dose of 0.5 mM sodium sulfide was added to all microcosms because the microcosms with RAMM, which contained the redox indicator resazurin, remained slightly pink. The bioaugmentation microcosms received an additional dose of 5 mM lactate and a second 3-mL inoculum of the respective bioaugmentation culture SDC-9 or KB-1 Plus on day 63 **Table 24**, Appendix C.

DNA isolation.

Biomass samples collected with Sterivex cartridges from temporary well locations B-1-E, B-2-S, B-3-W, and B-4-N were kept at -80°C upon delivery. To isolate DNA from Sterivex membrane filters, the filters were removed from the Sterivex cartridge housing, cut into ~1-cm-long pieces using a sterile scalpel, and placed into plastic tubes with beads provided with the Qiagen DNeasy PowerLyzer PowerSoil DNA Kit. DNA was isolated according to the manufacturer's recommendations except for the application of a bead-beating step at 5 m/s for 3 min to improve cell lysis (OMNI Bead Rupter Homogenizer, OMNI International, GA, USA). DNA was eluted into nuclease-free water, and the DNA concentration and purity were determined with a

NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA was stored at -80°C until analysis.

Quantitative polymerase chain reaction (qPCR).

qPCR assays using TaqMan detection chemistry were used to enumerate biomarker gene copy numbers associated with the biomass samples collected on the Sterivex membrane filters. The qPCR runs were performed with the specific assays targeting the 16S rRNA genes of *Dehalococcoides mccartyi*,¹⁷⁶ *Dehalobacter*,¹⁷⁷ and total bacteria.¹⁴⁶ Prior to qPCR analysis, the DNA samples were diluted with nuclease-free water to achieve 1:10, 1:100, and 1:1,000 dilutions of the initial DNA concentrations. This routine procedure assists in the identification of potential PCR inhibitors in the samples.¹⁷⁸ TaqMan qPCR assays (10 µL total assay volume) were prepared as follows: 5 µL of TaqMan Universal PCR Master Mix No AmpErase UNG (Applied Biosystems, Foster City, CA), 0.3 µM of each primer, 0.3 µM of the probe, and 2.0 µL of template DNA. The PCR cycle parameters were 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. TaqMan qPCR assays were run using QuantStudio 12K Flex Real-Time PCR System (Life Technologies, Grand Island, NY) in 384-well plates. A synthetic 1,500 bp linear DNA fragment covering primer/probe binding sites of target genes was used as a DNA standard for the preparation of standard curves for the target assays. A range of 10 to 10⁸ gene copies/µL was prepared for each target gene by a 10-fold serial dilution series of standard linear DNA fragments. Standards and samples were run in triplicate, and the results were analyzed with the QuantStudio 12K Flex System Software (Life Technologies).

Results

Detection and quantification of known dechlorinating bacteria with qPCR.

Bacterial 16S rRNA genes were detected in groundwater collected from all four sampling locations. Total bacterial abundances ranged from 362 gene copies per mL at sampling location B-1-E to 6.68 x 10⁴ gene copies per mL at sampling location B-2-S. Intermediate bacterial 16S rRNA gene copy numbers were observed in groundwater collected from sampling locations B-3-W and B-4-N **Table 25**, Appendix C. Bacterial 16S rRNA gene copy numbers varied significantly between the four groundwater sampling locations B-1-E, B-2-S, B-3-W, and B-4-N, suggesting that microorganisms in the aquifer surrounding the monitoring well are heterogeneously distributed. Dhc 16S rRNA genes were detected in a quantifiable amount in samples from B-2-S. Additionally, Dhc 16S rRNA genes were detected in samples from B-4-N, but were below the LOQ (10 gene copies/reaction) in groundwater sampling location B-4-N. At sampling locations B-1-E and B-3-W, Dhc 16S rRNA genes were below the LOD (5 gene copies/reaction; **Table 25**, Appendix C). Dehalobacter 16S rRNA genes were detected at all four groundwater sampling locations, but the abundances were generally too low to be quantified (*i.e.*, DNQ; **Table 25**, Appendix C). The qPCR analysis indicated that organohalide-respiring bacteria belonging to the genera *Dehalococcoides* and *Dehalobacter* are present in groundwater near well OW-09DB but in very low abundances. Active reductive dechlorination has been observed at Dhc cell abundance exceeding 1.0 x 10⁷ per mL, and this cell titer is recognized as a threshold for effective reductive dechlorination at contaminated sites.¹⁷⁹ Due to the low abundance of Dhc and Dhb 16S rRNA genes in the groundwater (<10 cells/mL), reductive dehalogenase genes were not quantified.

Microcosm performance.

No cVOC degradation was apparent in any of the microcosms after a 63-day incubation period. At that time, all of microcosms established with RAMM had a pink sheen, presumably due to the presence of some oxidized resazurin. The microcosms established with groundwater did not receive resazurin, and the pink sheen was not apparent, suggesting that the pink sheen was indeed caused by oxidized resazurin. Apparently, reducing conditions had not been established in the microcosms, and all microcosms received an additional dose of 0.5 mM sulfide. It is possible that oxygen was introduced during the extensive manipulation that was necessary to create a slurry of the aquifer material and set up microcosms, though anoxic techniques were used during the handling of aquifer material. Another explanation for reducing conditions having not been met is that the aquifer material may have been oxidizing. Data collected by Parsons on the day of sampling at the monitoring well reported a redox value of 102.2 mV and the dissolved oxygen concentrated to be 0.53 mg/L. Furthermore, historical measurements which were taken biannually since 2005 show a mean redox value of 44 and a mean dissolved oxygen concentration of 1.12 mg/L.

Lactate consumption had only occurred in the bioaugmented microcosms by day 63, which received an additional dose of 5 mM lactate. In addition, the bioaugmented microcosm received a second 3-mL inoculum with the respective bioaugmentation culture SDC-9 or KB-1 Plus. Lactate was not consumed in any of the biostimulation microcosms that contained groundwater as the liquid phase, which was an unexpected observation. Lactate is used by aerobic microorganisms and is readily fermented by ubiquitous microorganisms under anoxic conditions. Numerous studies have used lactate as an electron donor to stimulate reductive dechlorination processes, and lactate fermentation is a robust process and generally not inhibited by the presence of cVOCs that support organohalide-respiring bacteria. Possible explanations for this unexpected observation include the presence of low amounts of CT and/or CFC-113 in the microcosms. The initial CT and CFC-113 concentrations in the groundwater were 2.1 mg/L and 1.1 mg/L, respectively, which were substantially reduced by flushing the groundwater with N₂ gas for 30 min; however, it is possible that low levels of CT and CFC-113 remained in the groundwater or were introduced to the microcosms with the aquifer material. Neither compounds are utilized as electron acceptors by organohalide-respiring bacteria, and toxic effects have been documented.^{30,31,34,180,181} It is conceivable that the CT concentrations remained sufficiently high to prevent microbial lactate utilization in the microcosms. Little is known about the toxicity of CFC-113 for microorganism; however, a recent study demonstrated inhibitory effects on *Dhc*.¹⁸² Im et al. also showed that homoacetogen *Sporomusa ovata* is capable of reducing CFC-113 to cis-1,2-dichloroethene (cis-DCE). It is thought that the reduction of CFC-113 to cis-1,2-dichloroethene (cis-DCE) by *Sporomusa ovata* is due to its corrinoid-producing capabilities, as Im et al. also report the super nucleophilic corrinoids can reduce CFC-113 to chlorotrifluoroethene and trifluoroethene. Additionally, lactate consumption was observed in the bioaugmented microcosms, suggesting that increased biomass can circumvent potential toxicity caused by CT and/or CFC-113.

Microcosms were set up with i) upper aquifer material and groundwater, ii) upper aquifer material and RAMM, and iii) lower aquifer material and RAMM to explore the potential of MNA, biostimulation, and bioaugmentation as remediation strategies **Table 24, Appendix C**. Additionally, heat-killed control microcosms were set up and monitored. Microcosms were not

set up with lower aquifer material and groundwater due to limitations in laboratory resources. Analysis of the GC-FID data for microcosms containing i) upper aquifer material and groundwater, ii) upper aquifer material and RAMM, and iii) lower aquifer material and RAMM revealed that the microcosms behaved consistently within MNA, biostimulation, and bioaugmentation treatment groups, with the exception of PCE and CF bioaugmentation microcosms that contained RAMM.

Data from all microcosms that underwent the same treatment (*i.e.*, MNA, biostimulation, and bioaugmentation) and contained the same contaminant were averaged and plotted together due to the consistent behavior between microcosms that contained different combinations of aquifer material and groundwater or RAMM **Figure 51**, Appendix C, **Figure 53**, Appendix C, and **Figure 54**, Appendix C. Bioaugmentation microcosms that contained PCE and CF were plotted individually to highlight the differences between microcosms set up with i) upper aquifer material and groundwater, ii) upper aquifer material and RAMM, and iii) lower aquifer material and RAMM **Figure 52**, Appendix C and **Figure 55**, Appendix C.

PCE transformation.

PCE can be reductively dechlorinated to TCE, cDCE, VC, and ethene, and these transformation products can be captured in headspace samples analyzed by GC-FID. PCE concentrations decreased in all microcosms; however, equivalent amounts of transformation products were not observed, suggesting that this decrease in PCE concentration is due to sorption to the stopper and/or aquifer material solids rather than reductive dechlorination **Figure 51**, Appendix C. The formation of some cDCE (1.0–4.5 $\mu\text{mol/bottle}$) was observed in all microcosms that had received PCE following the addition of 0.5 mM of sodium sulfide on day 63. A possible explanation for the formation of cDCE following the addition of 0.5 mM sodium sulfide on day 63 is that the added sulfide scavenged any ferrous iron (Fe[II]) in the system, resulting in the formation of ferrous sulfide (FeS).¹³⁵ FeS has been demonstrated to dechlorinate chlorinated ethenes including PCE and TCE.¹⁵¹ Data for ferrous iron in the groundwater or associated with the aquifer material were not available, but ferrous iron (~ 0.1 g/bottle) was present in microcosms containing RAMM.

In PCE-containing heat-killed controls, an average decrease of 11.7 ± 0.5 μmol of PCE per bottle was observed on day 226 **Figure 51**, Appendix C. Plausible explanations for the loss of PCE include sorption to the stopper and/or the aquifer material solids. Some cDCE formation (1.5 $\mu\text{mol/bottle}$) was observed after the addition of sulfide. Prior to the addition of 0.5 mM sulfide on day 63, no cDCE was detected in heat-killed controls. In PCE-containing MNA microcosms, the measured starting amount of PCE in the MNA microcosms was 37.0 ± 0.5 μmol per bottle, and decreases of 21.9 ± 0.5 μmol of PCE per bottle were observed following a 226-day incubation period. No cDCE was formed prior to the addition of 0.5 mM sulfide on day 63. Following a 98-day incubation period, an average of 4.4 ± 0.5 μmol of cDCE was detected in PCE-containing MNA microcosms.

Biostimulation microcosms that contained PCE had a measured starting amount of PCE of 38.7 μmol per bottle. A decrease of 24.9 ± 0.5 μmol of PCE per bottle was observed after a 226-day incubation period. Additionally, 2.5 ± 0.5 μmol of cDCE was identified. Prior to the addition of 0.5 mM sulfide on day 63, no cDCE was detected in PCE-containing biostimulation microcosms.

An average decrease of 28.8 ± 0.5 μmol of PCE per bottle was observed in PCE-containing bioaugmentation microcosms. Variability in the performance of the bioaugmentation microcosms was observed; thus, they were plotted individually **Figure 52**, Appendix C. Microcosms #4a–b contained groundwater as the liquid phase, and an average decrease of 25.3 ± 0.5 μmol of PCE per bottle was observed on day 226. Microcosms #4a–b produced 1.3 ± 0.5 μmol of cDCE after the addition of 0.5 mM of sodium sulfide on day 63, similar to what was observed in the heat-killed control, MNA, and biostimulation microcosms. It is possible that reductive dechlorination was inhibited in the bioaugmentation microcosms established with groundwater due to the presence of CT or CFC-113.^{30,31,34,180–182} Evidence for PCE reductive dechlorination was observed in bioaugmentation microcosms containing RAMM as the liquid phase (#8a–b and 28a–b). In microcosms #8a–b, an average decline of 34.9 ± 0.5 μmol of PCE per bottle and the formation of 3.4 ± 0.5 μmol of cDCE and 12.4 ± 0.5 μmol of ethene were observed on day 226 **Figure 52**, Appendix C. In microcosms #28a–b, an average decline of 29.9 ± 0.5 μmol of PCE and the formation of 1.8 ± 0.5 μmol of cDCE and 9.2 ± 0.5 μmol of ethene were observed on day 226. Ethene was detected in all bioaugmentation microcosms (#4a–b, 8a–b, 28a–b), but was below the LOD in microcosms #4a–b, which had groundwater as the liquid phase. The LOD and LOQ for ethene were 0.3 ± 0.5 μmol and 1.0 ± 0.5 μmol , respectively. Quantifiable amounts of ethene were observed in bioaugmentation microcosms containing RAMM as the liquid phase (#8a–b and 28a–b) on day 226 **Figure 52**, Appendix C. VC was not detected in any of the microcosms. The LOD for VC is 1.0 ± 0.5 μmol per bottle. Methane was detected in bioaugmentation microcosms on day 226. An average of 1.2 ± 0.5 μmol of methane was detected in microcosms #4a–b, 29.7 ± 0.5 μmol methane was observed in microcosms #8a–b, and 18.6 ± 0.5 μmol methane was observed in microcosms #28a–b. The GC-FID is quite sensitive to methane. The LOD is 0.04 μmol of methane per bottle, and the LOQ is 0.1 μmol of methane per bottle. Methane formation was not observed in the heat-killed control, MNA, or biostimulation microcosms. The data generated from the bioaugmentation microcosms suggests that reductive dechlorination did not occur prior to the second addition of 0.5 mM sodium sulfide on day 63.

Lactate fermentation in PCE-containing microcosms.

Lactate is a fermentable substrate that generally leads to the formation of hydrogen, propionate, and acetate. Organic acid concentrations were monitored by HPLC every 14 days **Table 26**, Appendix C. The target concentration for microcosms containing RAMM as the aqueous phase was 5 mM, but the actual measured starting concentrations were 4.5 mM or 3.8 mM. Two 2-L bottles of RAMM were used to set up microcosms, and they had different concentrations of lactate. The variation in starting lactate concentrations between the two 2-L bottles of RAMM may have been due to the retention of some of the 60% (w/w) sodium DL-lactate syrup on the neck of the 2-L flask during medium preparation, causing incomplete dissolution of the lactate added to the medium. Microcosms (ID# 27, 28) were set up with RAMM that had 4.5 mM of lactate. Microcosms (ID# 7, 8) were set up with RAMM that had 3.8 mM of lactate. Microcosms (ID# 3, 4) contained groundwater as the aqueous phase, and lactate was added by injecting 0.5 mL from an 800-mM stock solution using a 3-mL syringe equipped with a 2- μm filter, resulting in a starting concentration of 4 mM for microcosms containing groundwater.

In biostimulated microcosms containing PCE, lactate fermentation occurred in microcosms that contained RAMM as the liquid phase (ID# 7, 27; **Table 26**, Appendix C). Lactate fermentation did not occur in biostimulated microcosms that contained groundwater as the liquid phase (ID# 3). These data suggest that the groundwater contains a chemical that is inhibitory to lactate-fermenting organisms. It is possible that residual amounts of CT or CFC-113 are responsible for the inhibition of lactate fermentation in the PCE-containing biostimulated microcosms.^{30,31,34,180–182} All microcosms set up to test the effectiveness of combined bioaugmentation and biostimulation as a remediation strategy fermented lactate regardless of the liquid phase. These data suggest that the bioaugmentation culture SDC-9 contains lactate fermenters that are resistant to the inhibitory effects of the compound in the groundwater.

CT transformation.

Some decline in the amounts of CT was observed in all microcosms, including the heat-killed controls, MNA, biostimulation, and bioaugmentation microcosms, but no transformation products were observed **Figure 53**, Appendix C. In heat-killed controls, CT decreased by 8.2 ± 0.5 μmol per bottle following a 226-day incubation period. CT decreases of 6.2 ± 0.5 μmol , 11.0 ± 0.5 μmol , and 9.5 ± 0.5 μmol were observed in MNA, biostimulation, and bioaugmentation microcosms, respectively, following a 226-day incubation period.

Bioaugmentation culture KB-1 Plus contains amorphous FeS, which has been shown to transform CT to CF.^{73,151} Based on information provided by SiREM, the amount of FeS present in the KB-1 Plus bioaugmentation culture is 0.02 g/L. Each bioaugmentation microcosm with CT was amended with 3 mL of the KB-1 Plus at the start, and an additional 3 mL of KB-1 Plus was added on day 63. Thus, the total amount of FeS added to each CT-containing bioaugmentation microcosm was 0.0012 g/L. This small amount of FeS added to the bioaugmentation microcosms did not result in observable increases in CT transformation compared to the other treatments.

Lactate fermentation and formation in CT-containing microcosms.

Organic acid concentrations were monitored by HPLC every 14 days **Table 27**, Appendix C. The target concentration for microcosms containing RAMM as the aqueous phase was 5 mM, but the actual measured starting concentrations were 4.5 mM or 3.8 mM. Two 2-L bottles of RAMM were used to set up microcosms, and they had different concentrations of lactate. The variation in starting lactate concentrations between the two 2-L bottles of RAMM may have been due to retention of some of the 60% (w/w) sodium DL-lactate syrup on the neck of the 2-L flask during medium preparation, causing incomplete dissolution of the lactate added to the medium. Microcosms (ID# 31, 32) were set up with RAMM that had 4.5 mM lactate. Microcosms (ID# 15, 16) were set up with RAMM that had 3.8 mM lactate. Microcosms (ID# 11, 12) contained groundwater as the aqueous phase, and lactate was added by injecting 0.5 mL from an 800-mM stock solution using a 3-mL syringe equipped with a 2- μm filter, resulting in a starting concentration of 4 mM for microcosms containing groundwater.

Lactate fermentation did not occur in any biostimulated microcosms that contained CT (ID# 11, 15, 31; **Table 27**, Appendix C). Lactate fermentation occurred in all CT-containing microcosms that were bioaugmented and biostimulated (ID# 12, 16, 32), though fermentation occurred more slowly here than it did in bioaugmented microcosms that contained PCE or CF. This suggests

that the lactate-fermenting organisms present in KB1 Plus were able to overcome the inhibitory effects of the CT that these microcosms were treated with.

CF transformation.

CF concentrations decreased in all microcosms, including the heat-killed control microcosms. Transformation products were not observed, suggesting that the decrease in CF concentration was due to sorption to the stopper or aquifer material, not dechlorination **Figure 54**, Appendix C. On average, CF amounts declined by 4.1 ± 0.5 μmol , 2.4 ± 0.5 μmol , and 1.9 ± 0.5 μmol per bottle in heat-killed control, MNA, and biostimulation microcosms, respectively. In bioaugmentation microcosms (ID# 20a–b, 24a–b, 36a–b), an average decline of 12.8 ± 0.5 μmol per bottle was observed. There was variability in the performance of the bioaugmentation microcosms; thus, they were plotted individually **Figure 55**, Appendix C. Microcosms #20a–b were established with groundwater as the liquid phase, and an average decrease of 2.5 ± 0.5 μmol of CF per bottle was observed on day 226. This decrease in CF is similar to the decrease observed in the heat-killed control, MNA, and biostimulation microcosms and is likely due to sorption to the stopper and/or the aquifer material. A possible explanation for the lack of dechlorination in the bioaugmentation microcosms containing groundwater is inhibition by residual CT and CFC-113.^{30,31,34,180,181} The bioaugmentation microcosms containing aquifer material and RAMM as the liquid phase (ID# 24a–b, 36a–b) provided evidence for CF dechlorination. In microcosms #24a–b, which contained upper aquifer material and RAMM, an average of 20.3 ± 0.5 μmol of CF per bottle was consumed by day 86. These microcosms produced 7.4 ± 0.5 μmol of DCM by day 86, which was completely consumed by day 98. Microcosms #36a–b, which were established with aquifer material from the lower section of the sediment cores and RAMM, consumed an average of 15.7 ± 0.5 μmol of CF. In these microcosms, an average of 3.6 ± 0.5 μmol of DCM per bottle had been produced by day 70 and consumed by day 86. Methane was detected in all bioaugmentation microcosms, though much less methane was formed in #20a–b (0.69 ± 0.5 μmol , which contained groundwater as the liquid phase). In microcosms #24a–b, 634.71 ± 0.5 μmol of methane were detected, and in microcosms #36a–b, 747.4 ± 0.5 μmol of methane were detected. Methane formation was not observed in heat-killed control, MNA, or biostimulation microcosms containing CF. The LOD for methane is 0.04 μmol , and the LOQ is 0.1 μmol of methane per bottle. Bioaugmentation microcosms amended with CF were inoculated with the KB-1 Plus culture. Bioaugmentation culture KB-1 Plus contains a *Dehalobacter* population capable of reductively dechlorinating CF to DCM.^{183,184} DCM can then be degraded by organisms such as *Dehalobacterium formicoaceticum* or the ‘*Candidatus Dichloromethanomonas elyunquensis*’ strain RM.^{170,185} Dechlorination of CF was observed in microcosms containing aquifer material and RAMM as the liquid phase after the addition of a second dose of 0.5 mM of sodium sulfide on day 63, suggesting that non-reducing conditions had prevented dechlorination prior to day 63.

Lactate fermentation in CF-containing microcosms.

Organic acid concentrations were monitored by HPLC every 14 days **Table 28**, Appendix C. The target concentration for microcosms containing RAMM as the aqueous phase was 5 mM, but the actual measured starting concentrations were 4.5 mM or 3.8 mM. Two 2-L bottles of RAMM were used to set up microcosms, and they had different concentrations of lactate. The variation in starting lactate concentrations between the two 2-L bottles of RAMM may have been due to retention of some of the 60% (w/w) sodium DL-lactate syrup on the neck of the 2-L flask during

medium preparation, causing incomplete dissolution of the lactate added to the medium. Microcosms (ID# 23, 24, 35, 36) were set up with RAMM that had 4.5 mM of lactate. Microcosms (ID# 19, 20) contained groundwater as the aqueous phase, and lactate was added by injecting 0.5 mL from an 800-mM stock solution using a 3-mL syringe equipped with a 2- μ m filter, resulting in a starting concentration of 4 mM for microcosms containing groundwater.

In biostimulated microcosms containing CF, lactate fermentation occurred in microcosms that contained RAMM as the liquid phase (ID# 23, 35; **Table 28**, Appendix C). Lactate fermentation did not occur in biostimulated microcosms that contained groundwater as the liquid phase (ID# 19). These data suggest that the groundwater contains a chemical that is inhibitory to lactate-fermenting organisms. It is possible that residual amounts of CT or CFC-113 are responsible for the inhibition of lactate fermentation in the CF-containing biostimulated microcosms.^{30,31,34,180–182} All microcosms set up to test the effectiveness of combined bioaugmentation and biostimulation as a remediation strategy fermented lactate regardless of the liquid phase. These data suggest that the bioaugmentation culture KB1 Plus contains lactate fermenters that are resistant to the inhibitory effects of the compound in the groundwater.

Microcosms testing abiotic degradation.

Microcosms were established to explore the capacity of ZVI to transform CT **Figure 56**, Appendix C. Contaminant concentrations were monitored for 96 hr using headspace samples. Complete degradation of CT and formation of CF were observed in all abiotic microcosms containing ZVI (ID# 37a, 38a, 39a) within 48 hr. The use of ZVI to remove CT may alleviate the inhibiting effects of CT and CFC-113 on microbial reduction of PCE and CF in the aquifer material.^{186,187}

Discussion

In this treatability study, four remediation strategies were tested for their effectiveness at degrading PCE, CT, and CF in the aquifer material from the site. Anoxic conditions were used because known OHRB required anaerobic conditions. Degradation was not observed under biostimulation or MNA conditions. Biostimulation and MNA as remediation strategies rely on the presence of native microorganisms (*e.g.*, OHRB) in the groundwater aquifer capable of degrading the target contaminants. These observations combined with the low detection of known OHRB via qPCR assays suggest that there is not an active community of OHRB present at this site and that indigenous processes are unlikely to have a major impact on remediating the target contaminants.

Degradation of PCE and CF was observed in microcosms set up to test bioaugmentation combined with biostimulation that contained groundwater as the liquid phase, but degradation was unsuccessful in microcosms set up with groundwater as the liquid phase. These observations suggest that a compound that is inhibitory to OHRB is present in the groundwater. It is possible that CT or another contaminant is responsible for this inhibition. Degradation of CT was not observed in any microcosms set up to test bioaugmentation combined with biostimulation as a remediation strategy. This observation is not surprising, as CT is not respired by any known OHRB, and is known to be toxic to bacteria.^{30,31,34,180,181} If bioaugmentation combined with biostimulation was to be implemented as a remediation strategy, removal or transformation of the inhibitory compounds would be necessary at the site. Additionally, the aquifer would need to

be reduced for bioaugmentation combined with biostimulation to be an effective remediation strategy.

Amendments of ZVI to microcosms containing CT facilitated the transformation of CT to CF. In addition to transforming CT to CF, ZVI is a reductant, and addition of ZVI to the aquifer may help to establish reducing conditions.^{186,187}

Appendix C

Table 19. Contaminant concentrations measured in the groundwater at the contaminated site.

Compound	Concentration (mg/L)
Carbon tetrachloride (CT)	2.10
Chloroform (CF)	0.65
Tetrachloroethene (PCE)	1.10
1,1,2-Trichloro-1,2,2-trifluoroethane (CFC-113)	1.10

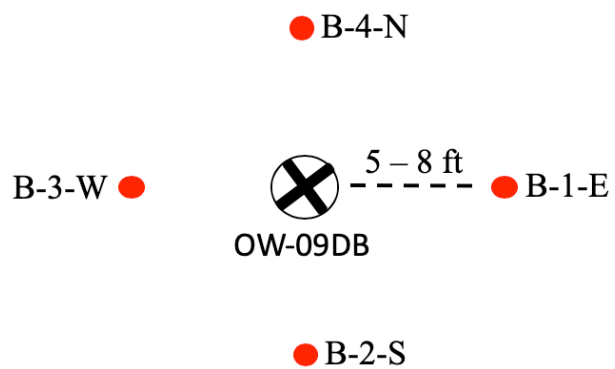


Figure 46. Schematic showing the temporary well locations surrounding the monitoring well at the contaminated site.

Table 20. Groundwater parameters determined in the monitoring well located in the center of the four sampling locations (data provided by Parsons).

Parameter	Value/Observation	Units
Color	Clear	-
Water Table Depth ^a	9.01	ft
Dissolved Oxygen ^b	0.53	mg/L
Odor	None	-
pH	7.28	-log[H ⁺]
Redox	102.2	mV
Specific Conductance ^b	33,954	µmho/cm
Temperature	25.77	°C
Turbidity ^c	3.9	NTU
Total Depth	19.15	ft

^a The depth to water from the top of the well casing is reported in feet.

^b The unit of specific conductance used is micromhos per centimeter (µmho/cm).

^c Turbidity is reported in nephelometric turbidity units (NTU).

Table 21. Volumes of groundwater filtered through each Sterivex cartridge.

Sampling location	Volume of groundwater filtered (mL)	
	Sterivex cartridge 1	Sterivex cartridge 2
B-1-E	1,200	1,000
B-2-S	2,000	1,300
B-3-W	1,250	2,000
B-4-N	1,500	1,750



Figure 47. Aquifer material cores that were received prior to separating the cores into lower (bottom) and upper (top) cores.

Table 22. Preparation of pipettable composite slurries for microcosm setup.

Core	Groundwater	Aquifer material (g)	Groundwater volume (mL)	
B-1-E upper	B-1-E	350	70	 The upper composite slurry was made from equal volumes of the upper slurries
B-2-S upper	B-2-S	374	100	
B-3-W upper	B-3-W	348	100	
B-4-N upper	B-4-N	393	100	
B-1-E lower	B-1-E	288	200	 The lower composite slurry was made from equal volumes of the lower slurries
B-2-S lower	B-2-S	522	150	
B-3-W lower	B-3-W	513	100	
B-4-N lower	B-4-N	487	150	

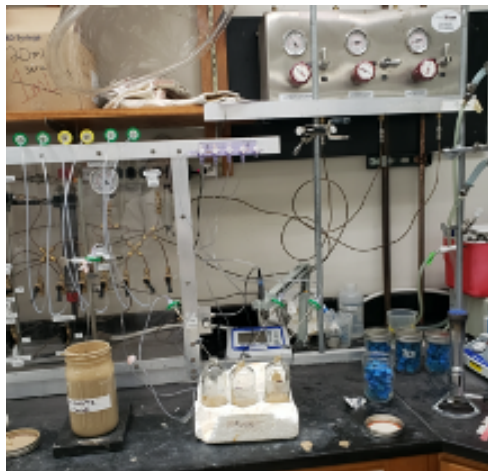


Figure 48. Transfer of aquifer material slurry to sterile and N_2/CO_2 -flushed 160-mL glass serum bottles.

Table 23. cVOCs added to microcosms.

Contaminant	μL added	μmol per bottle	mM (aq)
PCE	3	29	0.205
CT	2	20	0.118
CF	2	25	0.227



Figure 49. Dispensing of synthetic, defined reduced anoxic mineral salt medium (RAMM) to the glass serum bottles containing the composite slurry.



Figure 50. Microcosms established with aquifer slurries and composite groundwater or RAMM.

Table 24. Microcosms established to assess target contaminant degradation.

Identifier (ID#)	Source Aquifer Material	Electron Donor ^a	Treatment	Liquid Phase ^b	cVOC (μmol/bottle)
1a	Upper	-	Killed	GW	PCE (29)
2a	Upper	-	Native	GW	PCE (29)
3a	Upper	Yes	Stimulated	GW	PCE (29)
3b	Upper Aquifer Material	Yes	Stimulated	GW	PCE (29)
4a	Upper Aquifer Material	Yes	SDC-9	GW	PCE (29)
4b	Upper Aquifer Material	Yes	SDC-9	GW	PCE (29)
5a	Upper Aquifer Material	-	Killed	RAMM	PCE (29)
6a	Upper Aquifer Material	-	Native	RAMM	PCE (29)
7a	Upper Aquifer Material	Yes	Stimulated	RAMM	PCE (29)
7b	Upper Aquifer Material	Yes	Stimulated	RAMM	PCE (29)
8a	Upper Aquifer Material	Yes	SDC-9	RAMM	PCE (29)
8b	Upper Aquifer Material	Yes	SDC-9	RAMM	PCE (29)
9a	Upper Aquifer Material	-	Killed	GW	CT (20)
10a	Upper Aquifer Material	-	Native	GW	CT (20)
11a	Upper Aquifer Material	Yes	Stimulated	GW	CT (20)
11b	Upper Aquifer Material	Yes	Stimulated	GW	CT (20)
12a	Upper Aquifer Material	Yes	KB-1® Plus	GW	CT (20)
12b	Upper Aquifer Material	Yes	KB-1® Plus	GW	CT (20)
13a	Upper Aquifer Material	-	Killed	RAMM	CT (20)
14a	Upper Aquifer Material	-	Native	RAMM	CT (20)
15a	Upper Aquifer Material	Yes	Stimulated	RAMM	CT (20)
15b	Upper Aquifer Material	Yes	Stimulated	RAMM	CT (20)
16a	Upper Aquifer Material	Yes	KB-1® Plus	RAMM	CT (20)
16b	Upper Aquifer Material	Yes	KB-1® Plus	RAMM	CT (20)
17a	Upper Aquifer Material	-	Killed	GW	CF (25)
18a	Upper Aquifer Material	-	Native	GW	CF (25)
19a	Upper Aquifer Material	Yes	Stimulated	GW	CF (25)
19b	Upper Aquifer Material	Yes	Stimulated	GW	CF (25)
20a	Upper Aquifer Material	Yes	KB-1® Plus	GW	CF (25)
20b	Upper Aquifer Material	Yes	KB-1® Plus	GW	CF (25)
21a	Upper Aquifer Material	-	Killed	RAMM	CF (25)
22a	Upper Aquifer Material	-	Native	RAMM	CF (25)
23a	Upper Aquifer Material	Yes	Stimulated	RAMM	CF (25)
23b	Upper Aquifer Material	Yes	Stimulated	RAMM	CF (25)
24a	Upper Aquifer Material	Yes	KB-1® Plus	RAMM	CF (25)

Table 24. Continued.

Identifier (ID#)	Source Aquifer Material	Electron Donor ^a	Treatment	Liquid Phase ^b	cVOC (μmol/bottle)
24b	Upper Aquifer Material	Yes	KB-1® Plus	RAMM	CF (25)
25a	Lower Aquifer Material	-	Killed	RAMM	PCE (29)
26b	Lower Aquifer Material	-	Native	RAMM	PCE (29)
27a	Lower Aquifer Material	Yes	Stimulated	RAMM	PCE (29)
27b	Lower Aquifer Material	Yes	Stimulated	RAMM	PCE (29)
28a	Lower Aquifer Material	Yes	SDC-9	RAMM	PCE (29)
28b	Lower Aquifer Material	Yes	SDC-9	RAMM	PCE (29)
29a	Lower Aquifer Material	-	Killed	RAMM	CT (20)
30a	Lower Aquifer Material	-	Native	RAMM	CT (20)
31a	Lower Aquifer Material	Yes	Stimulated	RAMM	CT (20)
31b	Lower Aquifer Material	Yes	Stimulated	RAMM	CT (20)
32a	Lower Aquifer Material	Yes	KB-1® Plus	RAMM	CT (20)
32b	Lower Aquifer Material	Yes	KB-1® Plus	RAMM	CT (20)
33a	Lower Aquifer Material	-	Killed	RAMM	CF (25)
34a	Lower Aquifer Material	-	Native	RAMM	CF (25)
35a	Lower Aquifer Material	Yes	Stimulated	RAMM	CF (25)
35b	Lower Aquifer Material	Yes	Stimulated	RAMM	CF (25)
36a	Lower Aquifer Material	Yes	KB-1® Plus	RAMM	CF (25)
36b	Lower Aquifer Material	Yes	KB-1® Plus	RAMM	CF (25)
37a	Upper Aquifer Material	-	ZVI	Anoxic H ₂ O	CT (10)
38a	Lower Aquifer Material	-	ZVI	Anoxic H ₂ O	CT (10)
39a	-	-	ZVI	Anoxic H ₂ O	CT (10)

^a Lactate (5 mM) was provided as an electron donor.

^b RAMM: reduced anoxic mineral salt medium.

Table 25. Detection of total bacterial, *Dhc*, and *Dhb* 16S rRNA genes with specific qPCR assays in groundwater samples collected from the contaminated site.

Sample location	Total bacterial 16S rRNA gene copies/mL^a	<i>Dhc</i> 16S rRNA gene copies/mL^b	<i>Dhb</i> 16S rRNA gene copies/mL^b
B-1-E	$3.62 \times 10^2 \pm 1.11 \times 10^2$	Not detected	DNQ ^c
B-2-S	$6.68 \times 10^2 \pm 1.36 \times 10^4$	$5.52 \pm .96$	$6.45 \pm .54$
B-3-W	$1.23 \times 10^3 \pm 3.02 \times 10^2$	Not detected	DNQ ^c
B-4-N	$1.04 \times 10^2 \pm 2.79 \times 10^3$	DNQ ^b	DNQ ^c

^a Practical detection limit: 1 gene copy/mL, ^b Practical detection limit: 0.1 gene copies/mL, ^c detectable but not quantifiable

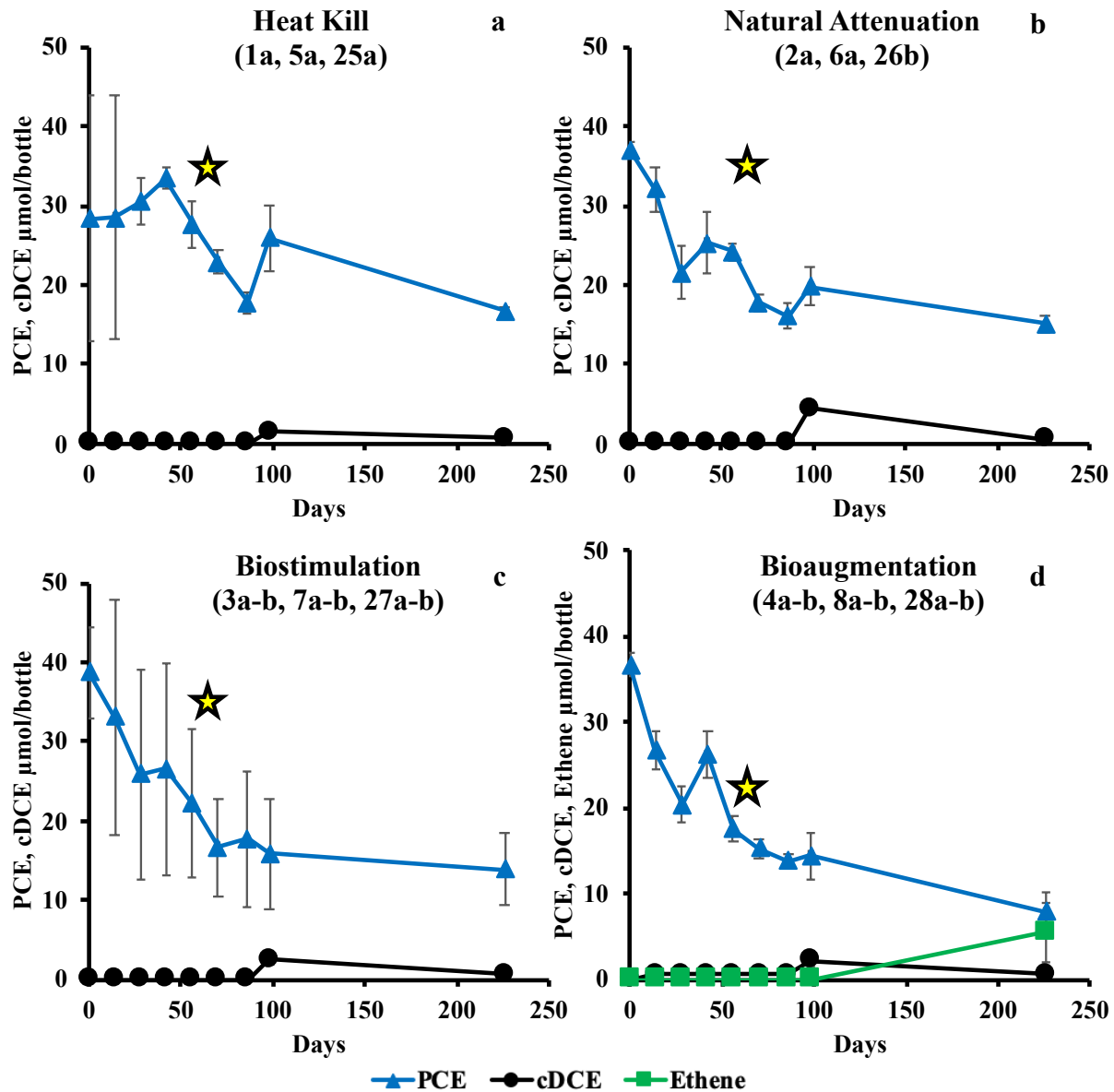


Figure 51. Amounts of PCE and transformation products over time.

Panel (a) shows cVOC data from heat-killed controls (ID# 1a, 5a, 25a), panel (b) contains cVOC data from MNA microcosms (ID# 2a, 6a, 26b), panel (c) contains cVOC data from biostimulation microcosms (ID# 3a-b, 7a-b, 27a-b), and panel (d) contains cVOC data from bioaugmentation microcosms (ID# 4a-b, 8a-b, 28a-b). The star indicates the addition of a second dose of 0.5 mM of sodium sulfide on day 63.

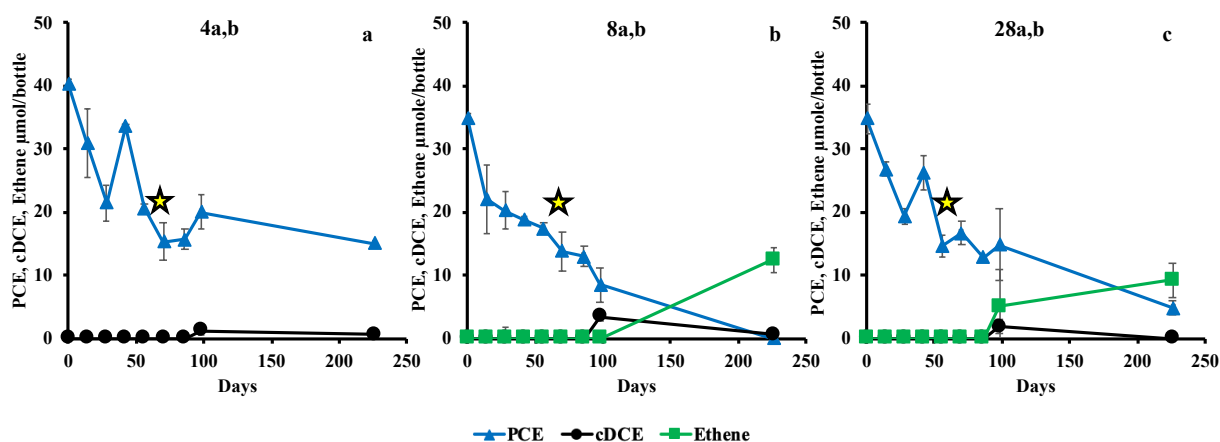


Figure 52. Changes in the amount of PCE, cDCE, and ethene in μmol per bottle over time.

Panel (a) shows cVOC data from microcosms #4a–b, which contain upper aquifer material and groundwater. Panel (b) shows cVOC data from microcosms #8a–b, which contain upper aquifer material and RAMM. Panel (c) shows cVOC data from microcosms #28a–b, which contain lower aquifer material and RAMM. All microcosms were bioaugmented with consortium SDC-9. The addition of a second dose of 0.5 mM sodium sulfide on day 63 is indicated by the star.

Table 26. Consumption of lactate and formation of acetate and propionate in lactate-amended microcosms containing PCE.

Day Sample ID	Lactate (mM)						mM
	3	4	7	8	27	28	
0	4.0	4.0	3.8	3.8	4.5	4.5	5.0
7	4.0	4.3	3.9	3.8	4.2	4.1	
14	4.1	1.8	3.5	0.0	3.8	0.0	
28	4.0	0.9	3.4	0.0	4.0	0.0	
42	3.6	0.5	3.3	0.0	3.9	0.0	
70	3.1	0.9	2.8	0.6	1.9	0.8	
86	3.4	0.8	1.9	0.5	1.2	1.0	
98	3.5	1.0	2.1	0.5	1.6	0.1	
Day Sample ID	Propionate (mM)						2.5
	3	4	7	8	27	28	
0	0.0	0.0	0.0	0.0	0.0	0.0	
7	0.0	0.0	0.0	0.1	0.0	0.0	
14	0.0	1.2	0.0	0.1	0.0	0.2	
28	0.0	1.1	0.0	0.1	0.1	0.2	
42	0.0	1.1	0.0	0.0	0.1	0.2	
70	0.0	0.0	0.1	0.4	0.2	0.5	
86	0.0	2.1	0.1	1.1	0.3	1.1	
98	0.0	1.9	0.1	1.3	0.3	1.4	
Day Sample ID	Acetate (mM)						0.0
	3	4	7	8	27	28	
0	0.0	0.0	0.0	0.0	0.0	0.0	
7	0.0	0.3	0.0	2.9	0.0	3.7	
14	0.0	1.8	0.0	3.0	0.0	3.5	
28	0.0	1.5	0.0	2.7	0.0	3.2	
42	0.0	1.5	0.0	2.4	0.0	2.8	
70	0.0	2.7	0.2	4.4	1.1	4.7	
86	0.0	2.9	0.6	4.0	0.9	4.1	
98	0.0	2.2	0.7	4.2	0.9	4.4	

The numbers at the top of each column refer to the sample ID#, as described in the text and **Table 24** the “Day” column refers to the days after microcosm initiation. The reported value is the average of two replicates. A heat map function is used to indicate the consumption of lactate and the formation of propionate and acetate. All microcosms were amended with an additional 0.5 mM of sodium sulfide on day 63. Microcosms (ID# 8a–b, 28a–b) were also amended with an additional 5 mM of lactate on day 63.

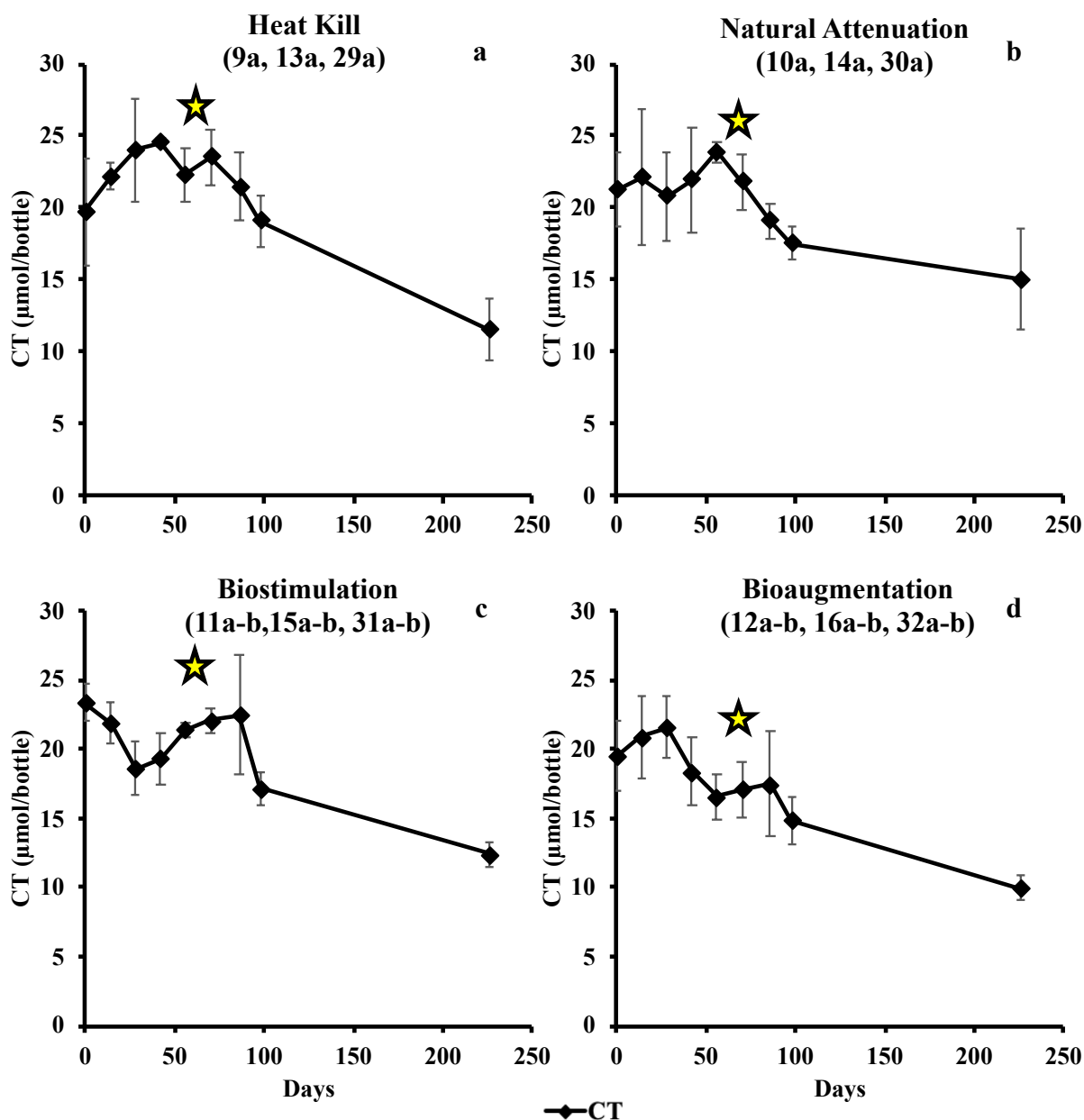


Figure 53. Amounts of CT in the microcosms over time.

Panel (a) contains cVOC data from heat-killed control microcosms (ID# 9a, 13a, 29a). Panel (b) contains cVOC data from MNA microcosms (ID# 10a, 14a, 30a). Panel (c) contains cVOC data from biostimulation microcosms (ID# 11a-b, 15a-b, 31a-b). Panel (d) contains cVOC data from bioaugmentation microcosms (ID# 12a-b, 16a-b, 32a-b). The star indicates the addition of a second dose of 0.5 mM of sodium sulfide on day 63.

Table 27. Consumption of lactate and formation of acetate and propionate in lactate-amended microcosms containing CT.

Day Sample ID	Lactate (mM)						mM		
	11	12	15	16	31	32			
0	4.0	4.0	3.8	3.8	4.5	4.5			
7	4.1	4.0	3.5	3.6	4.8	4.7			
14	4.3	4.3	3.9	3.7	4.9	4.8			
28	4.0	4.2	3.7	3.0	4.8	4.9			
42	3.9	4.2	3.3	3.1	4.6	4.5			
70	3.9	3.9	3.6	3.4	4.4	4.4			
86	3.5	3.4	3.1	2.9	4.3	2.4			
98	3.7	3.4	3.4	3.1	4.4	2.1			
Day Sample ID	Propionate (mM)								
	11	12	15	16	31	32			
0	0.0	0.0	0.0	0.0	0.0	0.0			
7	0.0	0.0	0.0	0.0	0.0	0.1			
14	0.0	0.0	0.0	0.0	0.0	0.1			
28	0.0	0.0	0.0	0.1	0.0	0.1			
42	0.0	0.0	0.0	0.1	0.0	0.1			
70	0.0	0.0	0.0	0.1	0.0	0.0			
86	0.0	0.0	0.0	0.0	0.0	0.0			
98	0.0	0.0	0.0	0.1	0.0	0.2			
Day Sample ID	Acetate (mM)								
	11	12	15	16	31	32			
0	0.0	0.0	0.0	0.0	0.0	0.0			
7	0.0	0.0	0.0	0.0	0.0	0.3			
14	0.0	0.0	0.0	0.0	0.0	0.3			
28	0.0	0.0	0.0	1.0	0.0	0.3			
42	0.0	0.0	0.0	1.0	0.0	0.7			
70	0.0	0.8	0.0	1.3	0.0	1.4			
86	0.0	0.9	0.0	1.1	0.0	1.2			
98	0.0	0.9	0.0	1.3	0.0	1.0			

The numbers at the top of each column refer to the sample ID#, as described in the text and **Table 24**. The “Day” column refers to the days after microcosm initiation. The reported value is the average of two replicates. A heat map function is used to indicate the consumption of lactate and the formation of propionate and acetate. All microcosms were amended with an additional 0.5 mM of sodium sulfide on day 63.

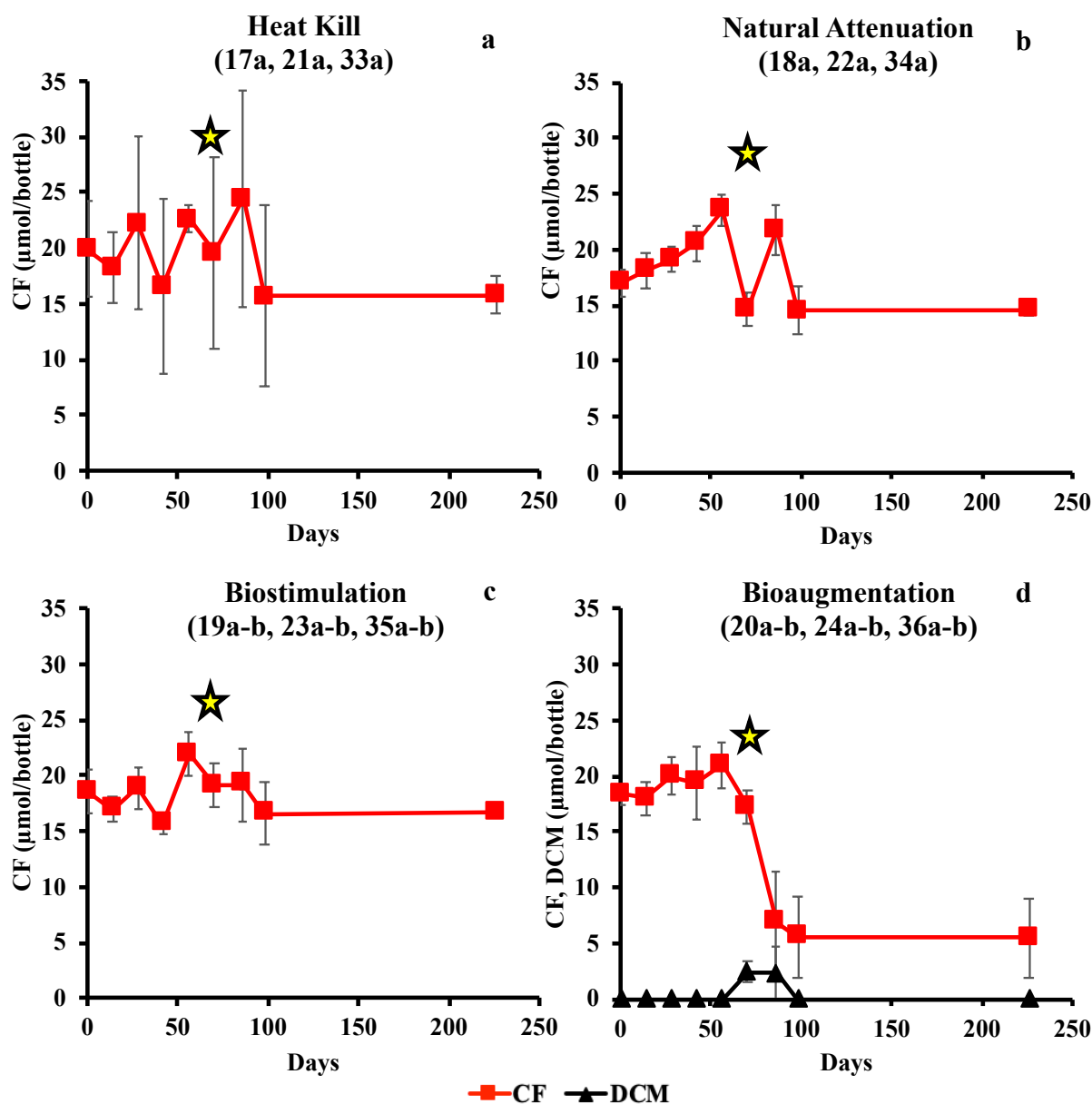


Figure 54. Amounts of CF and transformation products over time.

Panel (a) shows cVOC data from heat-killed control microcosms (ID# 17a, 21a, 33a). Panel (b) shows cVOC data from MNA microcosms (ID# 18a, 22a, 34a). Panel (c) depicts cVOC data from biostimulation microcosms (ID# 19a-b, 23a-b, 35a-b). Panel (d) shows cVOC data from bioaugmentation microcosms (ID# 20a-b, 24a-b, 36a-b). The star indicates the addition of a second dose of 0.5 mM of sodium sulfide on day 63.

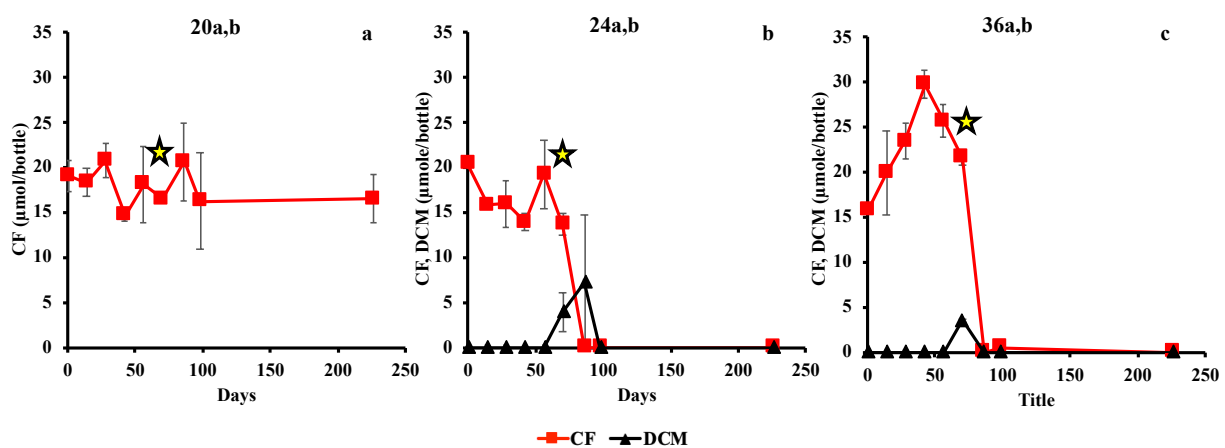


Figure 55. Amounts of CF and transformation products measured in bioaugmentation microcosms #20a–b, 24 a–b, and 26 a–b over time.

Table 28. Consumption of lactate and formation of acetate and propionate in lactate-amended microcosms containing CF.

Day Sample ID	Lactate (mM)						mM 5.0 2.5 0.0
	19	20	23	24	35	36	
0	4.0	4.0	4.5	4.5	4.5	4.5	
7	4.5	4.1	3.8	3.5	4.9	2.0	
14	4.5	3.9	3.7	0.2	4.3	0.0	
28	4.2	3.7	4.0	0.0	4.8	0.0	
42	4.0	3.4	3.0	0.0	4.4	0.0	
70	3.9	2.5	1.3	0.4	3.6	0.8	
86	3.3	0.9	0.9	0.3	3.0	0.6	
98	4.0	0.8	1.0	0.3	2.5	1.2	
Day Sample ID	Propionate (mM)						
	19	20	23	24	35	36	
0	0.0	0.0	0.0	0.0	0.0	0.0	
7	0.0	0.0	0.1	0.0	0.0	0.0	
14	0.0	0.0	0.0	0.1	0.0	0.2	
28	0.0	0.0	0.1	0.1	0.0	0.2	
42	0.0	0.0	0.0	0.1	0.0	0.1	
70	0.0	0.3	0.2	0.8	0.0	0.3	
86	0.0	0.6	0.3	1.2	0.0	1.2	
98	0.0	0.6	0.4	1.2	0.0	0.4	
Day Sample ID	Acetate (mM)						
	19	20	23	24	35	36	
0	0.0	0.0	0.0	0.0	0.0	0.0	
7	0.0	0.0	0.0	2.5	0.0	1.0	
14	0.0	0.0	0.0	2.9	0.0	3.7	
28	0.0	0.0	0.0	2.4	0.0	3.3	
42	0.0	0.6	0.3	2.8	0.0	2.0	
70	0.0	1.3	1.0	4.9	0.0	5.1	
86	0.0	1.9	1.3	3.9	0.0	4.8	
98	0.0	2.0	1.0	4.4	0.0	2.5	

The numbers at the top of each column refer to the sample ID#, as described in the text and **Table 24**. The “Day” column refers to the days after microcosm initiation. The reported value is the average of two replicates. A heat map function is used to indicate the consumption of lactate and the formation of propionate and acetate. All microcosms were amended with an additional 0.5 mM of sodium sulfide on day 63. Microcosms (ID# 24a–b, 36a–b) were also amended with an additional 5 mM of lactate on day 63.

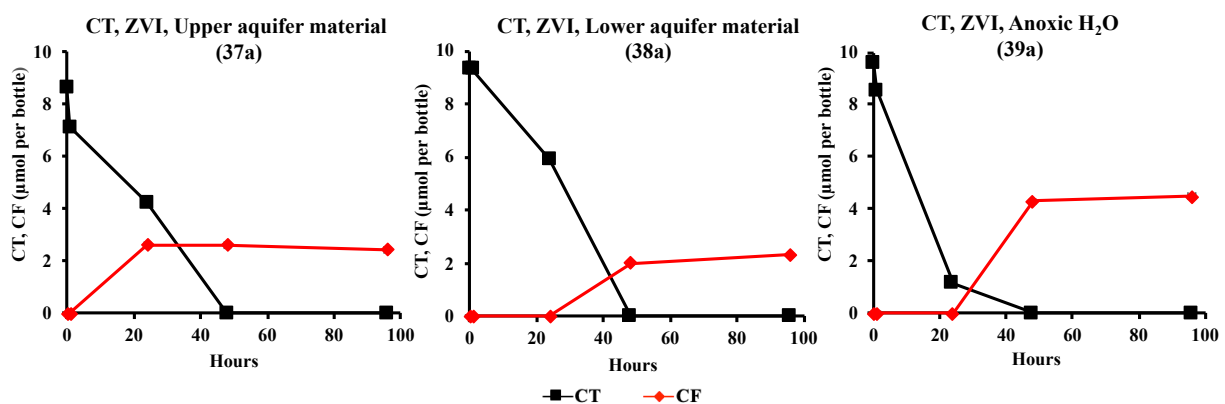


Figure 56. Abiotic transformation of CT by ZVI in microcosms established with upper aquifer material, lower aquifer material, and a control containing no aquifer material.

CHAPTER IV

EFFECT OF THE POTENTIAL OF HYDROGEN ON THE BIOMIMETIC DEHALOGENATION OF TRICHLOROFLUOROMETHANE, DICHLORODIFLUOROMETHANE, AND CARBON TETRACHLORIDE BY SUPER-NUCLEOPHILIC VITAMIN B12.

Abstract

Chlorofluorocarbons are recalcitrant contaminants found in the atmosphere and subsurface. This study examined the dehalogenation of trichlorofluoromethane (CFC-11), dichlorodifluoromethane (CFC-12), and tetrachloromethane (CT) by super-nucleophilic vitamin B₁₂ at pH 5, 7, and 9. Additionally, the dehalogenation by super-nucleophilic vitamin B₁₂ of possible transformation products of CFC-11 and CFC-12 was studied at pH 7. The potential transformation products studied were chlorofluoromethane, chlorodifluoromethane, difluoromethane, and fluoromethane. All reactions in this study occurred in closed anaerobic batch reactors. Changes in contaminant concentrations were monitored by gas chromatography with a flame ionization detector. Super-nucleophilic vitamin B₁₂ is capable of dehalogenating CFC-11, CFC-12, and carbon tetrachloride at pH 5, 7, and 9. Changes in pH affected which transformation products formed in reactions that contained CFC-11 and carbon tetrachloride. Chlorodifluoromethane was the only transformation product identified in the reactions with CFC-12 at all tested pHs. No dehalogenation was observed in the reactions with super-nucleophilic vitamin B₁₂ and chlorofluoromethane, chlorodifluoromethane, difluoromethane, or fluoromethane.

Introduction

Chlorofluorocarbons (CFCs) and tetrachloromethane (CT) are anthropogenic chemicals that were first synthesized as refrigerants.^{10,188} Their use was later expanded to many other industrial applications. Due to their extensive use and improper handling, CT and CFCs such as trichlorofluoromethane (CFC-11) and dichlorodifluoromethane (CFC-12) are common groundwater contaminants.^{37,84,188–190} Additionally, due to the high vapor pressure of CFC-11, CFC-12, and CT at ambient temperatures, these compounds collect in the atmosphere, where they facilitate ozone depletion.^{11,26,44,189,191,192} Implementation of the 1987 Montreal Protocol led to decreased production of CFC-11, CFC-12, and CT, but they are still produced in small quantities for the synthesis of other chemicals. Environmental cleanup of CFC-11, CFC-12, and CT still poses a problem as they are recalcitrant.³⁶

The environmental fate of CFC-11 and CFC-12 has been studied as they are used as groundwater tracers to calculate the age and recharge rate of young (~100 years old) groundwater.¹⁹³ They are considered useful groundwater tracers as they are thought to react conservatively, were released globally, and their production and release rates are known and measured. Though the transformation of CFCs in groundwater is considered conservative, degradation of CFC-11 and CFC-12 has been observed in pyrite-oxidizing aquifers and sulfate-reducing aquifers.^{85,86,194} Laboratory studies have reported degradation of CFC-11, CFC-12, and CT under sulfate-reducing and methanogenic conditions.^{77,78,90,158,195–197} This suggests that methanogenic archaea and sulfate-reducing bacteria may contribute to the degradation of CFC-11, CFC-12, and CT, possibly through their production of cobamides that can react with CFCs.^{198–202}

Cobamides are cofactors that contain a corrin ring with a cobalt center, an upper ligand coordinated with the cobalt center, and an exchangeable lower base which is connected to the corrin ring by a nucleotide loop.⁹⁹ The oxidation state of cobalt can vary from -1 to +4, though the +2 and +3 oxidation states are most frequently observed.²⁰³ The oxidation state of a coordinated cobalt at the center of a corrin ring can be reduced from the +2 or +3 oxidation states to a +1 oxidation state in the presence of a strong reductant like Ti(III)citrate or ZVI.^{93,204} When

the oxidation state of the centrally coordinated cobalt is +1, the cobamide is considered super-nucleophilic.^{182,204} Studies have shown that vitamin B₁₂ and other cobamides can dehalogenate CFC-11, CFC-12, and CT.^{93,147,205} Dehalogenation by vitamin B₁₂ occurs via electron donation from the cobalt center of the corrin ring.^{182,204,206,207} Vitamin B₁₂ acts as a catalytic electron shuttle when it reduces CFC-11, CFC-12 or CT, as such a single vitamin B₁₂ molecule can be regenerated and facilitate numerous reactions.^{94,207}

Krone and colleagues reported that carbon monoxide and a mixture of halogenated transformation products formed via the reduction of CFC-11, CFC-12, and CT by super-nucleophilic vitamin B₁₂.⁹³ They suggested that carbon monoxide was formed via a carbene intermediate that hydrolyzed to carbon monoxide. Understanding the factors that affect transformation product formation is crucial, as some transformation products are less favorable than others. For example, the formation of dichlorofluoromethane or chlorofluoromethane from CFC-11 is not favorable, as dichlorofluoromethane is more toxic than CFC-11 and chlorofluoromethane is carcinogenic and flammable.⁵⁸ It is conceivable that pH would affect the distribution of transformation products formed during a reaction between super-nucleophilic vitamin B₁₂ and CFC-11, CFC-12, or CT, as the amount of protons available in a solution is likely to affect, the ionic interactions between a di-halogen carbene and the cobalt center of a super-nucleophilic vitamin B₁₂ molecule.^{94,136} This work examined the effect of pH (5, 7, 9) on the distribution of transformation product formation from the dehalogenation of CFC-11, CFC-12, and CT by super-nucleophilic vitamin B₁₂. Experiments were also performed that examined the ability of super-nucleophilic vitamin B₁₂ to dehalogenate possible transformation products of CFC-11 and CFC-12; the transformation products tested were chlorofluoromethane, chlorodifluoromethane, difluoromethane, and fluoromethane. Hypothesis explored in this work are i) that changes in pH will effect which transformation products form during the dehalogenation of CFC-11, CFC-12, or CT by super-nucleophilic vitamin B₁₂, ii) that super-nucleophilic vitamin B₁₂ can dehalogenate chlorofluoromethane, chlorodifluoromethane, difluoromethane, and fluoromethane.

Materials and Methods

Chemicals.

CFC-11 (99%), CFC-12 (99%), fluoromethane (99%), difluoromethane (99%), chlorofluoromethane (99%), and chlorodifluoromethane (99 %), vinyl chloride (99%) were purchased from SynQuest Laboratories. CT (99%) and Vitamin B₁₂ were purchased from Fisher Scientific. Titanium(III)chloride (15%) in 10 % HCl, methane (99%), tetrachloroethene (99%), trichloroethene (99.5%), chloroform (99.5%), dichloromethane (99.8%), chloromethane (99.5%), cis-1,2-dichloroethene (97%), ethene (99.5%), dichlorofluoromethane (98%) were purchased from Millipore Sigma.

Cyanocobalamin-catalyzed dehalogenation.

Dehalogenation of CFC-11, CFC-12, and CT by super-nucleophilic vitamin B₁₂ was assessed in 30 mL anoxic water at pH 5, 7, or 9 and 30 mL of N₂ headspace in 60 mL glass serum bottles. CFC-11 (108 µmol per bottle) and CT (103 µmol per bottle) were added by designated Hamilton syringes, and CFC-12 (122 µmol per bottle) was added with 3 mL plastic syringes fitted with 25 G needles. Reaction vessels containing CFC-11, CFC-12, or CT were stored stopper down for 24

hours to allow the chemicals to equilibrate. Once equilibrated, headspace samples were taken to determine the starting concentration of the chemical in each bottle. Stock solutions of vitamin B₁₂ (1 mM) and Titanium(III)-citrate (200 mM) were prepared fresh for each experiment. Titanium(III)-citrate was added to a final concentration of 5.3 mM in each reaction vessel. The concentration of Titanium(III)-citrate was approximately 100 times that of the vitamin B₁₂ added to each reaction; this was done to ensure that the vitamin B₁₂ was reduced to the super-nucleophilic Co(I) state and that the super-nucleophilic vitamin B₁₂ could be regenerated. Solutions of NaOH (10 M and 1 M) and HCl (6 M and 1 M) prepared in anoxic MilliQ water were used to adjust the solution's pH before the addition of vitamin B₁₂. Samples were taken from each reaction vessel, and the pH was measured. Finally, vitamin B₁₂ (49.5 μM) was added to the reaction vessels, and the reactions were monitored by gas chromatography with a flame ionization detector. Deionized, degasses Milli-Q water (Milli-Q Corporation, Bedford, MA) was used to make the stock solutions. The Hungate method was used to remove oxygen from the stock solutions. Control bottles containing only vitamin B₁₂ or titanium(III)-citrate were run in parallel with each experiment.

Degradation of chlorofluoromethane, chlorodifluoromethane, difluoromethane, and fluoromethane by super-nucleophilic vitamin B₁₂ was explored in 60 mL glass bottles containing 30 mL anoxic water pH 7. Each reaction vessel received 122 μmol of chlorodifluoromethane, difluoromethane, fluoromethane, or chlorofluoromethane. 3 mL plastic syringes fitted with 25 G needles were used to inject the halogenated gasses. Titanium(III)-citrate (5.3 mM) and vitamin B₁₂ (49.5 μM) were added to the reaction vessels, and the reactions were monitored by gas chromatography with a flame ionization detector for 24 hours.

Analytical methods.

The methods used in this study were adapted from Im et al., 2019.¹⁸² Experiments in which CFC-11, CFC-12, chlorodifluoromethane, difluoromethane, fluoromethane, chlorofluoromethane were the starting chemical, were monitored by injecting headspace samples (100 μL) into an Agilent 7890 gas chromatograph equipped with a PLOTQ column (30 m × 535 μm × 40 μm), and a flame ionization detector. Experiments with CT as the starting chemical were monitored by injecting headspace samples (100 μL) into an Agilent 7890 gas chromatograph equipped with a DB-624 capillary column (60 m × 0.32 mm × 1.8 μm), and a flame ionization detector. Quantification was attained by applying external standard curves. Known amounts of each chemical were added to 60 mL bottles with the same liquid-to-headspace ratio as the experimental vessels to prepare standards. Microliter Hamilton syringes were used to add the liquid chemicals, and the gaseous chemicals were added with 3 mL plastic syringes.

Standard curves for CFC- 11, CFC-12, chlorofluoromethane, chlorodifluoromethane, dichlorofluoromethane, fluoromethane, difluoromethane, CT, CF, DCM, CM, CH₄, PCE, TCE, cis-DCE, VC, and ethene were analyzed using the Agilent 7890 gas chromatograph equipped a PLOTQ column (30 m × 535 μm × 40 μm), and a flame ionization detector **Figure 57**, Appendix D. Additionally, standard curves for CT, CF, DCM, CM, CH₄, PCE, TCE, cis-DCE, VC, and ethene were also analyzed on an Agilent 7890 gas chromatograph equipped with a DB-624 capillary column (60 m × 0.32 mm × 1.8 μm) **Figure 58**, Appendix D. Transformation products were determined by aligning the retention times on the chromatograms of peaks generated during an experiment with the known retention times of chemicals for which standard curves were

established. Analytical conditions of the gas chromatography methods and the established standard curves are available in the appendix **Table 29**, Appendix D and **Table 30**, Appendix D. Dimension-less Henry's constants were used to calculate the amount of halogenated compound in each phase (*i.e.*, liquid or headspace) **Table 31**, Appendix D.

Results

Experiments to determine if super-nucleophilic vitamin B₁₂ could dehalogenate CFC-11, CFC-12, or CT were performed at pH 5, 7, and 9. Complete transformation of CFC-11, CFC-12, and CT was observed at pH 5, 7, and 9 in the presence of super-nucleophilic vitamin B₁₂ **Figure 59**, Appendix D. Declines in the CFC-11, CFC-12, and CT concentrations were observed in control vessels that contained vitamin B₁₂ without a reductant and in control vessels that had only Ti(III)citrate. However, the declines in CFC-11, CFC-12, and CT concentration were less than those observed in the vessels containing both vitamin B₁₂ and Ti(III)citrate. Transformation products were not observed in control vessels that contained CFC-11 or CFC-12 **Table 32**, Appendix D and **Table 33**, Appendix D. CF and PCE were observed in control vessels that contained CT and Ti(III)citrate at pH 7.

The percentages of carbon accounted for as identified transformation products were less than 25% for CFC-12 and CT and greater than 50% for CFC-11 **Table 32**, Appendix D and **Table 33**, Appendix D. A greater percentage of carbon was accounted for as transformation products as the pH increased for vessels that contained CFC-11 and CFC-12. In contrast, the percentage of carbon accounted for as transformation products did not vary with pH for CT. Four transformation products were identified in vessels in which CFC-11 was degraded: chlorofluoromethane, fluoromethane, methane, and ethene **Table 32**, Appendix D. Chlorodifluoromethane was the only transformation product identified in vessels where CFC-12 was degraded **Table 33**, Appendix D. Chloroform, dichloromethane, chloromethane, methane, tetrachloroethene, and ethene were identified as transformation products in vessels where CT was degraded **Table 34**, Appendix D.

Transformation product experiment.

The ability of super-nucleophilic vitamin B₁₂ to dehalogenate possible transformation products of CFC-11 and CFC-12 was explored by monitoring vessels that contained vitamin B₁₂, Ti(III)citrate, and either chlorodifluoromethane, difluoromethane, fluoromethane, or chlorofluoromethane. Dehalogenation or a decline in the starting concentration of the target chemical was not observed in any vessels **Table 35**, Appendix D.

Discussion

Strategies to degrade CFCs are needed in order to remove them from the groundwater and atmosphere. This study explored the dehalogenation capacity of biomimetic degradation by super-nucleophilic vitamin B₁₂. Degradation of CFC-11, CFC-12, and CT was observed in the presence of super-nucleophilic vitamin B₁₂. Unfortunately, no dehalogenation was observed in reactions with super-nucleophilic vitamin B₁₂ and chlorodifluoromethane, difluoromethane, fluoromethane, chlorofluoromethane. The lower degree of halogenation of these chemicals may make them less likely to be reduced, as their reduction potential has decreased.⁶⁹

In vessels that contained CFC-11 and super-nucleophilic vitamin B₁₂, shifts in the formation of transformation products occurred as pH increased. The dominant transformation product at pH 5 was methane, with small amounts of ethene and chlorofluoromethane identified. At pH 9, chlorofluoromethane was the predominant transformation product. The formation of chlorofluoromethane is an unfavorable outcome, as it is carcinogenic and flammable.⁵⁸ Additionally, in reactions that occurred at pH 9 all of the carbon measured as CFC-11 at time zero was accounted for as transformation products at the 24 hour time point. In reactions that occurred at pH 5 and 7 roughly 40% of the carbon from the time zero measurement of CFC-11 was accounted for as transformation products at the 24 hour time point, this suggests that transformation products formed which were not identifiable by the method used in this study. It is possible that carbon monoxide formed via a carbene intermediate at pH 5 and 7, as reported by Krone and colleagues,⁹³ and that reaction pathways that form radical intermediates and halogenated transformation products are more favorable at pH 9, than reaction pathways that favor carbene intermediates and non-halogenated end products (**Figure 2**, Appendix A, Chapter 1). The ionic interaction between a carbene intermediate and the cobalt center of vitamin B₁₂ may be destabilized in the less protonated environment of a pH 9 solution.

The influence of pH on reactions between super-nucleophilic vitamin B₁₂ and CFC-12 was observable in the amounts of transformation products identified, but not in the composition of the transformation product, as chlorodifluoromethane was the sole transformation product observed. However, more chlorodifluoromethane was observed at the 24 hour time point in reactions that occurred at pH 9 than in reactions that occurred at pH 5 or 7. The formation of chlorodifluoromethane as a transformation product is favorable as it is not toxic, carcinogenic, or flammable.⁵⁸ It is unclear if defluorination occurred in reactions with super-nucleophilic vitamin B₁₂ and CFC-12 as less than 15% of the carbon measured as CFC-12 at time zero was accounted for as transformation products at the 24 hour time point. It appears that transformation products formed that were not identifiable with the given methodology.

In reactions between CT and super-nucleophilic vitamin B₁₂ at pH 9 only non-halogenated transformation products were observed. While in reactions between CT and super-nucleophilic vitamin B₁₂ at pH 5 and 7 a mixture of halogenated end products and methane formed. Twenty-five percent or less of the carbon measured as CT at time zero was accounted for as transformation products at the 24 hour time point, suggesting that transformation products formed that were not identifiable with the given methodology.

There are significant limitations to the methodologies used in this study. For example, the flame ionization detector is not the ideal detector for these compounds, as some halogenated methanes are considered non-combustible. High starting concentrations were used in this study to overcome the non-combustible nature CFC-11, CFC-12, and of possible transformation products. Additionally, though a PLOTQ column is useful in separating the volatile methanes likely to form via dehalogenation of CFC-11, CFC-12, or CT, a PLOTQ column is not ideal if halogenated ethanes or ethenes form via radical-radical coupling reactions, as they are retained on the column. For example, the method used in this study is 17.5 minutes long and reaches a temperature of 240°C, but a sample of tetrachloroethene when injected will not elute from the column during the runtime. Given the limitations of the methodologies used in this study, more

research should be done to confirm the identity of the transformation products produced. Gas chromatography-mass spectrometry would be a useful method to verify the identity of the transformation products. This methodology was unavailable for this study due to limitations in laboratory resources.

Conclusions

The hypothesis that super-nucleophilic vitamin B₁₂ can dehalogenate chlorofluoromethane, chlorodifluoromethane, difluoromethane, and fluoromethane was not supported. No chlorofluoromethane, chlorodifluoromethane, difluoromethane, and fluoromethane was dehalogenated in reactions containing super-nucleophilic vitamin B₁₂. The hypothesis that changes in pH will effect which transformation products form during the dehalogenation of CFC-11, CFC-12, or CT by super-nucleophilic vitamin B₁₂ was supported for. Changes in the formation of transformation products were observed in reactions between CFC-11, CFC-12, or CT and super-nucleophilic vitamin B₁₂ at pH 5, 7, 9. Unfortunately, the majority of the carbon measured as starting chemicals was not accounted for as transformation products at the 24 hour time point. This suggests that transformation products formed that were not detectable with this methodology. The development of tools that utilize super-nucleophilic vitamin B₁₂ could be useful in the degradation of these chemicals. Though, more research should confirm the identity of the transformation products formed, as it would be unwise to develop a tool for bioremediation that forms transformation products that are more toxic, carcinogenic, flammable, or recalcitrant. Additionally, in order for a super-nucleophilic vitamin B₁₂ to be a cost effective bioremediation tool, a method would need to be developed to stabilize it in the subsurface.

Appendix D

Table 29. Detailed instrument settings used for the analysis of headspace samples with GC – FID set up with the DB-624 column.

Chlorinated compounds	Front Inlet	Inlet/Detector Gases	Detector (FID) or (μECD)	Program
CT, CF, DCM, CM, CH ₄ , PCE, TCE, cis-DCE, VC, methane, and ethene	Heater = 200 °C Pressure = 23.193 psi Septum Purge flow = 1.5 mL/min Split = Split ratio of 50:1 w/Split flow of 150 mL/min	He = inlet N ₂ = detector	Heater = 200 °C Air Flow = 400 mL/min H ₂ Fuel flow = 30 mL/min	Initial Rate °C/min = 0 Initial Value °C = 60 Initial Hold Time min = 2 Initial Run Time min = 2 Ramp 1 Rate °C/min = 25 Ramp 1 Value °C = 200 Ramp 1 Hold Time min = 1 Ramp 1 Run Time min = 8.6

Table 30. Detailed instrument settings used for the analysis of headspace samples with GC – FID PLOTQ column.

Chlorinated compounds	Back Inlet	Inlet/Detector Gases	Detector (FID)	Program
CFC- 11, CFC-12, chlorofluoromethane, chlorodifluoromethane, dichlorofluoromethane, fluoromethane, difluoromethane, CT, CF, DCM, CM, CH ₄ , PCE, TCE, cis-DCE, VC, methane, and ethene	Heater = 250 °C Pressure = 4.0198 psi Septum Purge flow = 4 mL/min Split = Split ratio of 10:1 w/Split flow of 30 mL/min	He = inlet N ₂ = detector	Heater = 300 °C Air Flow = 400 mL/min H ₂ Fuel flow = 30 mL/min	Initial Rate °C/min = 0 Initial Value °C = 40 Initial Hold Time min = 5 Initial Run Time min = 5 Ramp 1 Rate °C/min = 80 Ramp 1 Value °C = 80 Ramp 1 Hold Time min = 2 Ramp 1 Run Time min = 7.5 Ramp 2 Rate °C/min = 80 Ramp 2 Value °C = 120 Ramp 2 Hold Time min = 2 Ramp 2 Run Time min = 10 Ramp 3 Rate °C/min = 80 Ramp 3 Value °C = 160 Ramp 3 Hold Time min = 2 Ramp 3 Run Time min = 12.5 Ramp 4 Rate °C/min = 80 Ramp 4 Value °C = 200 Ramp 4 Hold Time min = 2 Ramp 4 Run Time min = 15 Ramp 5 Rate °C/min = 80 Ramp 5 Value °C = 240 Ramp 5 Hold Time min = 2 Ramp 5 Run Time min = 17.5

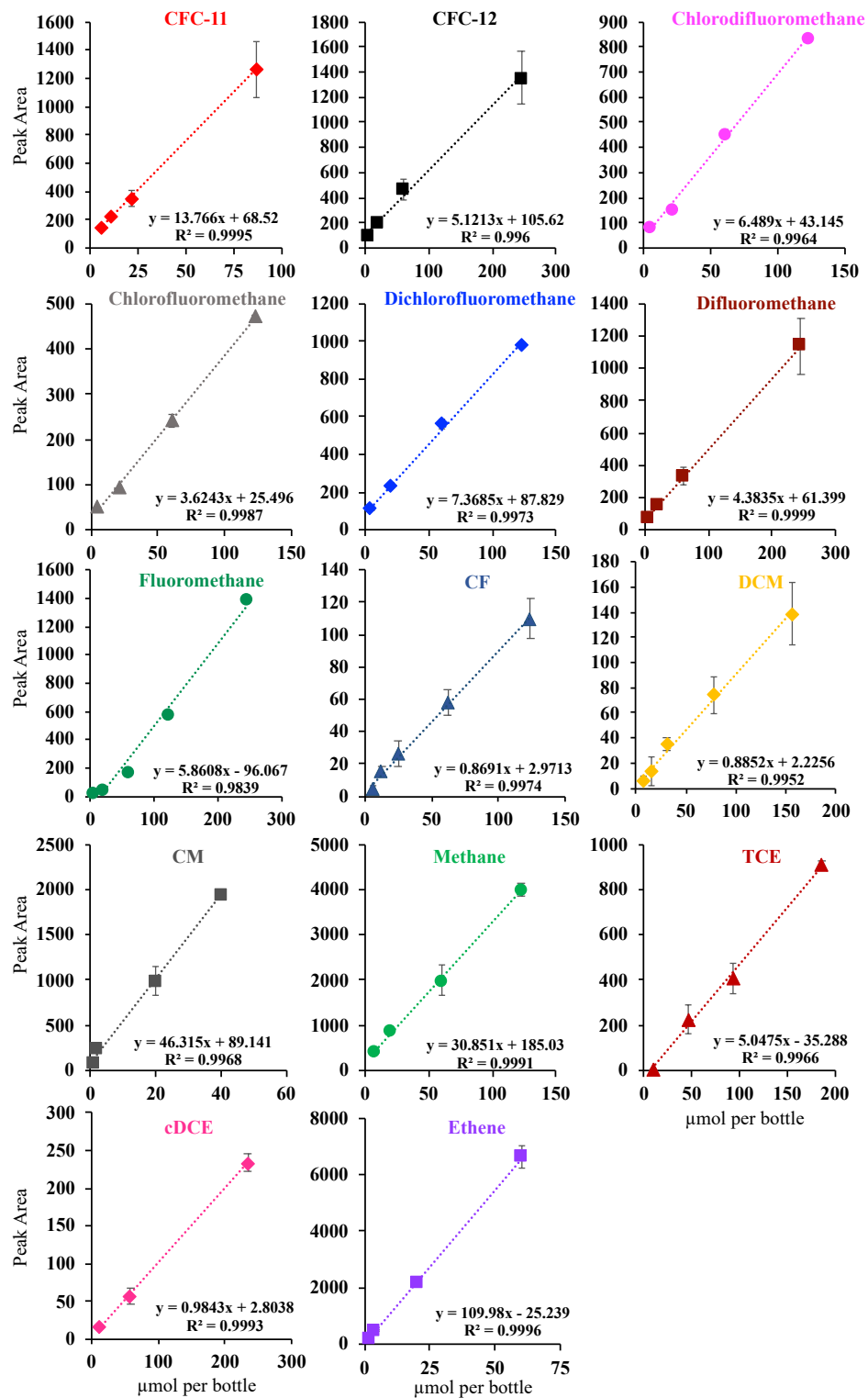


Figure 57. Standards run on GC-FID with a PLOTQ column.

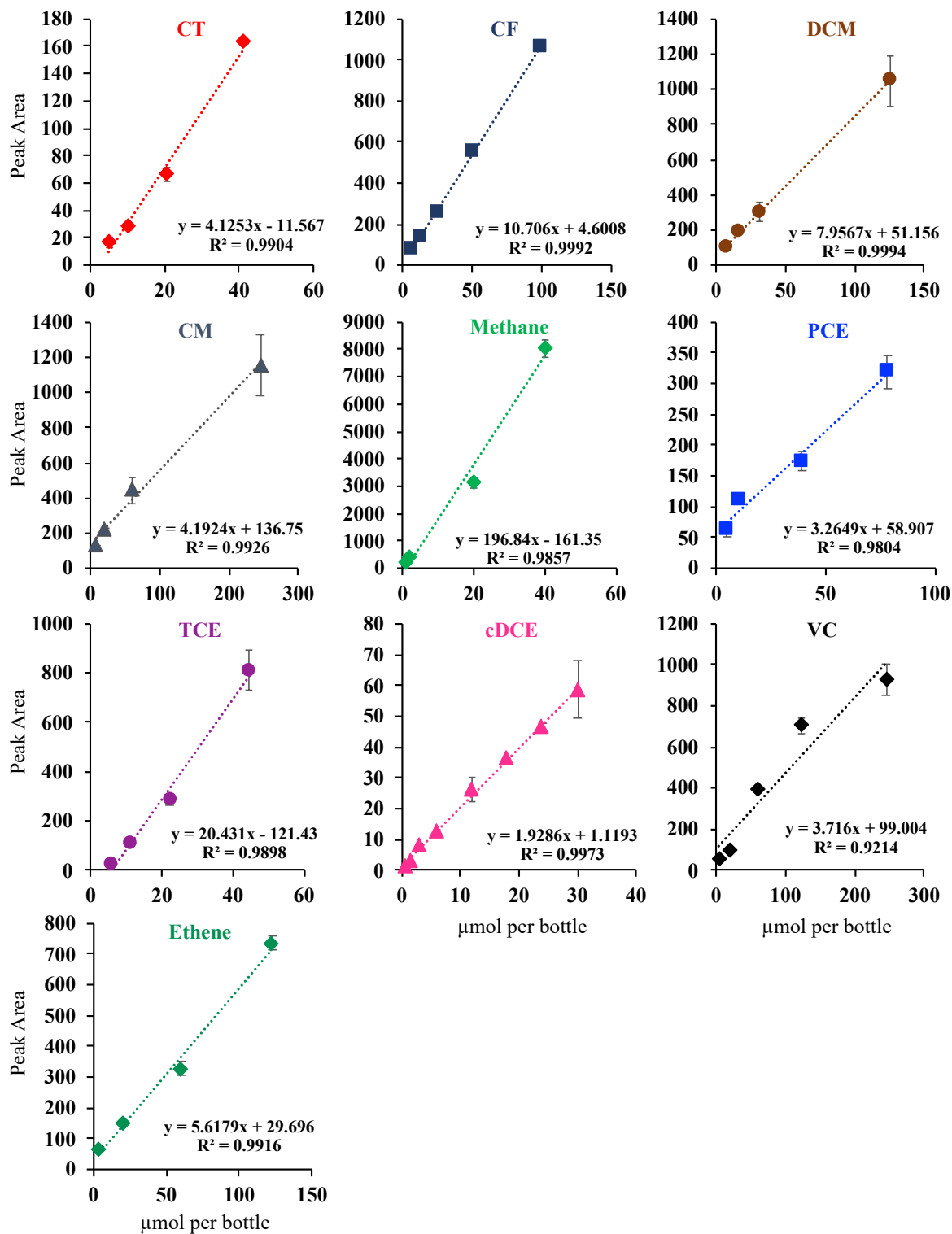


Figure 58. Standards run on GC-FID with a DB-624 column.

Table 31. Physical and Chemical Properties

Compound	Molecular Weight (g/mol)	Density (g/mL)	Aqueous Solubility (mg/L)	K^o_H
Tetrachloroethene	165.833	1.62	206	0.057
Trichloroethene	131.388	1.4642	1,280	0.11
<i>cis</i> -Dichloroethene	96.943	1.2837	3500	0.27
Vinyl Chloride	62.498	0.9106	2700	0.038
Ethene	28.0532	– g	131	0.0047
Carbon Tetrachloride	153.823	1.59	793	0.033
Chloroform	119.378	1.4788	7950	0.28
Dichloromethane	84.933	1.325	13,200	0.47
Chloromethane	50.488	0.911	5040	0.12
Methane	16.0425	– g	22	0.0013
CFC-11	37.368	1.49	1,100	0.0012
CFC-12	120.914	1.35	280	0.0025
Chlorodifluoromethane	86.468	1.194	2770	0.034
Chlorofluoromethane	68.478	– g	1190	0.15
Dichlorofluoromethane	102.923	1.48	18800	0.19
Difluoromethane	52.0234	– g	12850	0.086
Fluoromethane	34.0329	– g	1787	0.059

NIST – for Molecular weight and K^o_H

PubChem – for Density and aqueous solubility

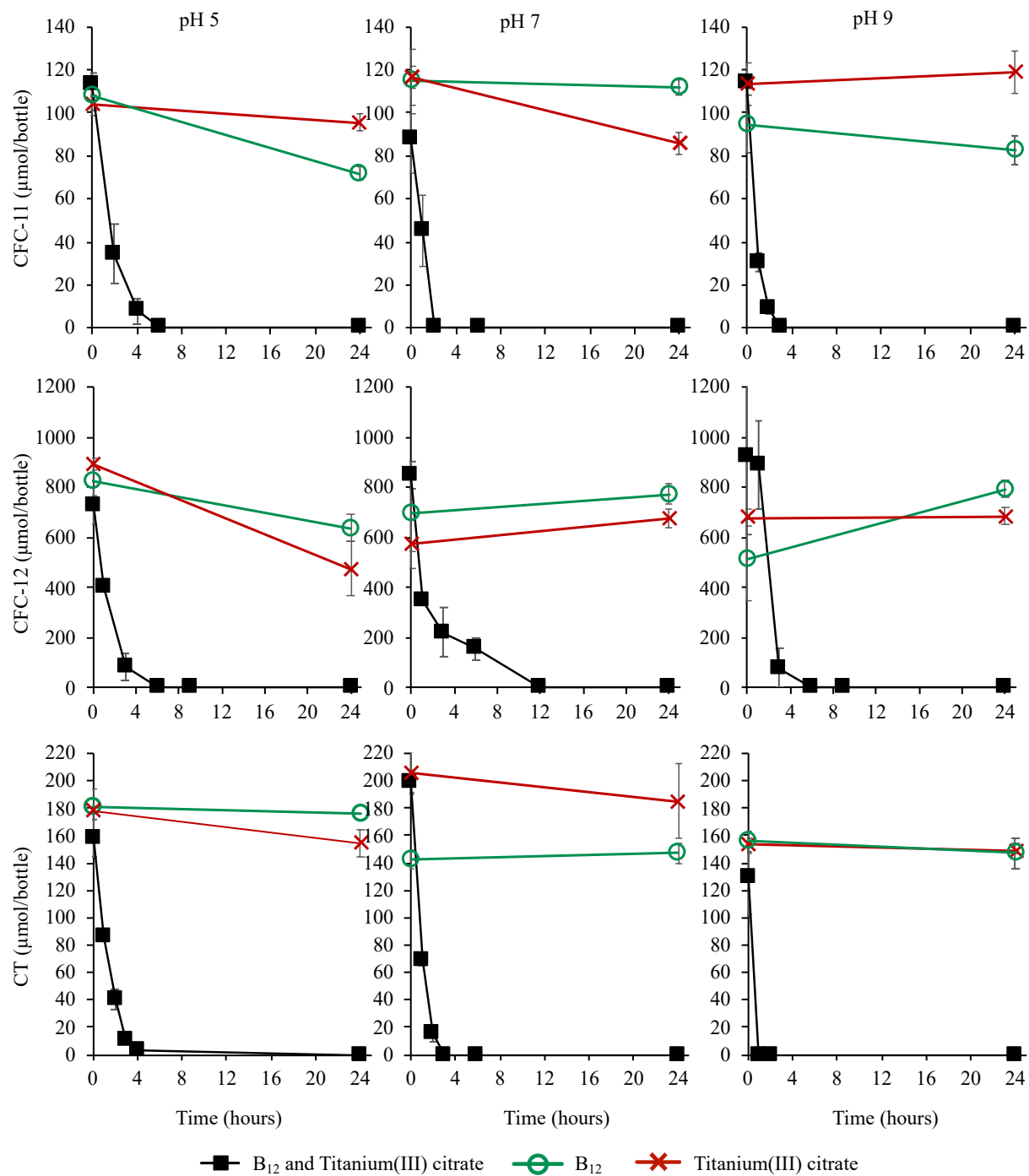


Figure 59. Reactions with super-nucleophilic vitamin B₁₂ and Ti(III) citrate.

Table 32. CFC-11 pH variation experiment performance at 24 hours.

Chemical	Experimental conditions	Decline in CFC-11 concentration (%)	Carbon identified as transformation products (%)	Methane (μmol)	Ethene (μmol)	Fluoromethane (μmol)	Chlorofluoromethane (μmol)
CFC-11	Vitamin B ₁₂ , Ti(III)citrate at pH 5	100	43	34	5	0	5
CFC-11	Vitamin B ₁₂ , Ti(III)citrate at pH 7	100	39	7	2	11	11
CFC-11	Vitamin B ₁₂ , Ti(III)citrate at pH 9	100	102	7	2	0	106
CFC-11	Vitamin B ₁₂ pH 5	34	0	0	0	0	0
CFC-11	Vitamin B ₁₂ pH 7	3	0	0	0	0	0
CFC-11	Vitamin B ₁₂ pH 9	0	0	0	0	0	0
CFC-11	Ti(III)citrate pH 5	8	0	0	0	0	0
CFC-11	Ti(III)citrate pH 7	11	0	0	0	0	0
CFC-11	Ti(III)citrate pH 9	13	0	0	0	0	0

Table 33. CFC-12 pH variation experiment performance at 24 hours.

Chemical	Experimental conditions	Decline in CFC-12 concentration (%)	Carbon identified as transformation products (%)	Chlorodifluoromethane (μmol)
CFC-12	Vitamin B ₁₂ and Ti(III)citrate at pH 5	100	8	59
CFC-12	Vitamin B ₁₂ and Ti(III)citrate at pH 7	100	6	47
CFC-12	Vitamin B ₁₂ and Ti(III)citrate at pH 9	100	14	131
CFC-12	Vitamin B ₁₂ pH 5	23	0	0
CFC-12	Vitamin B ₁₂ pH 7	0	0	0
CFC-12	Vitamin B ₁₂ pH 9	0	0	0
CFC-12	Ti(III)citrate pH 5	46	0	0
CFC-12	Ti(III)citrate pH 7	0	0	0
CFC-12	Ti(III)citrate pH 9	0	0	0

Table 34. CT pH variation experiment performance at 24 hours.

Chemical	Experimental conditions	Decline in CT concentration (%)	Carbon identified as transformation products (%)	Methane (μmol)	CF (μmol)	DCM (μmol)	CM (μmol)	PCE (μmol)	Ethene (μmol)
CT	Vitamin B ₁₂ and Ti(III)citrate at pH 5	100	22	4	0	3	6	11	0
CT	Vitamin B ₁₂ and Ti(III)citrate at pH 7	100	19	12	0	0	25	0	0
CT	Vitamin B ₁₂ and Ti(III)citrate at pH 9	100	25	13	0	0	0	0	10
CT	Vitamin B ₁₂ pH 5	3	0	0	0	0	0	0	0
CT	Vitamin B ₁₂ pH 7	0	0	0	0	0	0	0	0
CT	Vitamin B ₁₂ pH 9	4	0	0	0	0	0	0	0
CT	Ti(III)citrate pH 5	13	0	0	0	0	0	0	0
CT	Ti(III)citrate pH 7	10	2	0	4	0	0	1	0
CT	Ti(III)citrate pH 9	6	0	0	0	0	0	0	0

Table 35. Performance of reactions with super-nucleophilic B₁₂ and transformation products at 24 hours.

Chemical	Experimental conditions	Percent of starting chemical degraded
Chlorodifluoromethane	Vitamin B ₁₂ and Ti(III)citrate at pH 7	0
Chlorodifluoromethane	Vitamin B ₁₂ pH 7	0
Chlorodifluoromethane	Ti(III)citrate pH 7	0
Difluoromethane	Vitamin B ₁₂ and Ti(III)citrate at pH 7	0
Difluoromethane	Vitamin B ₁₂ pH 7	0
Difluoromethane	Ti(III)citrate pH 7	0
Fluoromethane	Vitamin B ₁₂ and Ti(III)citrate at pH 7	0
Fluoromethane	Vitamin B ₁₂ pH 7	0
Fluoromethane	Ti(III)citrate pH 7	0
Chlorofluoromethane	Vitamin B ₁₂ and Ti(III)citrate at pH 7	0
Chlorofluoromethane	Vitamin B ₁₂ pH 7	0
Chlorofluoromethane	Ti(III)citrate pH 7	0

CHAPTER V

CHARACTERIZATION OF SULFIDOGENIC ENRICHMENT CULTURE GROWN IN THE PRESENCE OF CARBON TETRACHLORIDE.

Abstract

Carbon tetrachloride (CT) is a recalcitrant groundwater contaminant that is susceptible to degradation by reactive iron species (*i.e.*, magnetite and ferrous sulfide). There is interest in the potential of biologically mediated abiotic degradation to tackle the CT problem. The basis for BMAD is the observation that some microorganisms form extracellular reactive iron species, which can then degrade CT. Unfortunately, the trichloromethyl radical that forms upon a 1-electron transfer to CT is inhibitory to many microbes, including dissimilatory iron-reducing bacteria, which are a group of organisms known for their ability to reduce ferric iron to ferrous iron. This study explored the ability of a precipitate-forming sulfidogenic enrichment culture to degrade CT, examined the enrichment cultures' microbial community composition with 16S rRNA gene amplicon sequencing, and characterized the precipitates formed in the presence of FeCl₂, NaSO₄, and Na₂S, FeCl₂ and Na₂S, FeCl₂, and NaSO₄, and FeCl₂ with X-ray powder diffraction. A decline in the amount of CT per bottle was greater in vessels with active enrichment culture than in corresponding heat-killed control incubations. An analysis of the 16S rRNA gene amplicon the sulfidogenic enrichment culture identified 11 bacterial classes, with the majority of sequences representing Campylobacteria, Clostridia, Spirochaetia, and Bacteroidia. Vivianite was the only mineral phase identified in the X-ray diffraction data of precipitates from inoculated enrichments and abiotic controls containing FeCl₂, FeCl₂, and Na₂S, FeCl₂, or NaSO₄, and the abiotic control containing FeCl₂, Na₂S, and NaSO₄. In the enrichments amended with FeCl₂, Na₂S, NaSO₄ and inoculum vivianite and mackinawite phases were both observed in the X-ray diffraction data.

Introduction

Carbon tetrachloride (CT) is a toxic chlorinated solvent, a predicted carcinogen, and an ozone-depleting agent.³⁷ It was widely used during the 20th century as a dry cleaning agent, industrial degreaser, grain fumigant, fire extinguisher, and in the synthesis of chlorofluorocarbons. Research into the health and environmental impacts of CT and other chlorinated solvents began to raise concerns in the 1960s.¹⁰ Awareness of the health and environmental impacts, combined with increased publicization of the prevalence of industrial dumpsites, led to public outcry for governmental regulatory action, which facilitated the creation of the Environmental Protection Agency in the United States. In the latter half of the 20th century, production and use of CT and other hazardous chemicals were phased out globally under the Montreal protocol.²⁰⁸ Currently, minimal amounts of CT are produced for use as an extraction solvent and as an intermediate for the production of other chemicals.¹¹ Unfortunately, CT is still found at many hazardous waste sites.¹²

Abiotic reductive dechlorination of CT by magnetite has been reported in the literature.^{76,136,149} The transformation of CT to nontoxic substances by magnetite may be of environmental significance. McCormick and Adriaens (2004) identified carbon monoxide, formate, and methane as transformation products of CT via the carbene-reduction pathway.¹⁶¹ Magnetite's chemical composition is Fe₃O₄. It is a mixed-valence

iron oxide that has a cubic closed-packed structure.²⁰⁹ The production of magnetite by dissimilatory iron-reducing bacteria (DIRB) is well established.^{210–213} However, DIRB cannot produce Fe₃O₄ in the presence of CT, due to the inhibitory effects of the trichloromethyl radical that forms from the reduction of CT by one electron.⁷⁶ Currently, only one study in the literature describes biogenic magnetite formation through an oxidative pathway.²¹⁴ The work by Chaunduri et al, describes magnetite formation by an organism that couples the oxidation of ferrous iron (Fe(II)) to the reduction of nitrate in an anoxic system. The biogenic production of magnetite or another reactive iron species could facilitate biologically mediated abiotic degradation of CT. This work characterized a sulfidogenic enrichment culture that forms iron containing precipitates in the presence of CT and Fe(II). This work explores the hypothesis that magnetite formation in this system occurs via a biogenic oxidative pathway in the presence of CT.

The enrichment cultures used in this work were developed by transferring microcosms established with sediment from a CT-contaminated railyard. Interest in the sediment from the CT-contaminated railyard developed because chloroform (CF) formation was observed after a wetland was installed. The aims of this study were i) to track chlorinated volatile organic compound (VOC) concentrations over time in the enrichment culture, ii) identify the phylogenetic diversity of the microbial consortium present in this culture, and iii) identify the precipitate formed by the anaerobic enrichment culture.

Methods

Chemicals.

CT (99%) and sodium sulfide nonahydrate were purchased from ACROS organics. Chloroform (CF) (99%), dichloromethane (DCM) (99.8%), chloromethane (CM) (99.5%), methane (99%), and sodium DL-lactate 60% w/w syrup were purchased from Sigma-Aldrich. Ferrous chloride (FeCl₂) (99.5%) and sodium sulfate anhydrous (NaSO₄) (99.0%) were purchased from Fisher Scientific. MilliQ water (18.2 MΩ.cm) was used to prepare all solutions and medium. All other chemicals used were reagent grade or better. The Hungate technique was used to make anoxic solutions.²¹⁵

Transformation of carbon tetrachloride in enrichment cultures.

Enrichment cultures and microcosms were established in an anaerobic glove box (Coy, Ann Arbor, MI) containing N₂/H₂ (97/3 vol/vol). The original microcosms contained ~10 g sediment (wet weight) from a CT-contaminated rail yard, 90 mL anoxic bicarbonate-buffered mineral salts medium, and 60 mL of headspace.²¹⁶ CT (10 μmol/bottle) was added to each microcosm using a Hamilton syringe equipped with a Chaney adapter. The microcosms were maintained over six transfers in anoxic bicarbonate-buffered mineral salts medium pH (7.2) amended with lactate (5 mM), FeCl₂ (5 mM), and NaSO₄ (5 mM), and CT (10 μmol/bottle).

This work reports observations of an enrichment culture which was developed by transferring microcosms established with sediment from a CT-contaminated railyard. The microcosms were established in 160 mL bottles containing inoculum (3%), 100 mL

of anoxic bicarbonate-buffered mineral salts medium, 60 mL of headspace containing a mixture of N₂/CO₂ (80/20, vol/ vol), lactate (5 mM), and CT (10 µmol/bottle). Details describing additional amendments of FeCl₂ (5 mM), NaSO₄ (5 mM), and NaS₂ (0.2 mM) are available in **Table 36**, Appendix E. The Hungate method was used to make anoxic stock solutions of FeCl₂ (1 M), NaSO₄ (1 M), and NaS₂ (100 mM). All amendments and the inoculum were transferred into the 160 mL bottles with 3 mL N₂ flushed syringes.

Analytical techniques.

CT, CF, DCM, chloromethane (CM), and methane concentrations were monitored by headspace injection (100 µl) into an Agilent 7890 gas chromatograph (GC) with a DB-624 capillary column (60 m × 0.32 mm × 1.8 µm) and a flame ionization detector (FID). Further details of the GC-FID method are found in **Table 37**, Appendix E. Quantification of CT, CF, DCM, CM, and methane was obtained by measuring standards with known amounts of each compound. Standards were prepared in 160 mL bottles with 100 mL of liquid and 60 mL of headspace. Dimensionless Henry's constants used to calculate the concentration of each chemical were as follows: CT (1.244), CF (0.150), DCM (0.0895), and CM (0.361).²¹⁷

Statistical analysis of chlorinated VOC data.

The effect of treatment on CT was examined using a one-way analysis of variance (ANOVA). Diagnostic analysis of the data was conducted to verify the model assumptions for normality and equal variance. Tukey's adjustment was used to carry out post hoc multiple comparisons. A threshold of 0.05 was used to determine statistical significance. SAS 9.4 TS1M7 for Windows 64x (SAS Institute Inc., Cary, NC) was used to perform these analyses.

Microbial community analysis via 16S rRNA gene amplicon sequence analysis.

A cell pellet was collected by vacuum filtration of 20 mL of liquid from a microcosm which contained FeCl₂, NaSO₄, and Na₂S. The cell pellet was collected on a sterile 0.22 µm membrane filter. DNA was extracted from the membrane filter using a PowerSoil kit (Qiagen, Hilden Germany). Extracted DNA was purified using the Genomic DNA Clean and Concentrator Kit (Zymo Research, Irvine CA). The V4 region of the 16S rRNA gene was amplified via polymerase chain reaction (PCR) using barcoded-primers F515/R806²¹⁸ Target amplicon length was 291 base pairs. The total volume of the PCR assays was 50 µL and contained 10 µL of sample DNA, 1 µL of barcoded primer (10 µM), and 39 µL of a master mix containing 22 µL de-ionized water (5 PRIME, Gaithersburg, MD), 1 µL CAP 515F primer (10 µM), 5 µL Invitrogen Pfx50™ buffer (Invitrogen, Carlsbad CA), 1 µL Invitrogen Pfx50™ Polymerase, 5 µL dNTP, and 5 µL of MgCl₂ (25 mM). The thermocycling program consisted of an initial denaturing at 94°C for 3 min, followed by 35 cycles of 94°C for 45 seconds, 55°C for 60 seconds, 72°C for 90 seconds, and a final extension of 10 minutes at 72°C. After PCR, the DNA concentration was determined on a NanoDrop 2000 and then pooled with DNA of similar concentration. Further purification of the pooled samples was performed with Solid Phase Reversible Immobilization (SPRI) magnetic beads (Beckman Coulter, Inc., Indianapolis IN). Purified pooled DNA was quantified and checked for quality and presence of primer

dimers on a Bioanalyzer 2100 (Agilent, Santa Clara CA) with a high-sensitivity DNA kit. The final amplicon library was diluted to 10 nM and prepared for paired-end sequencing and loaded onto the Illumina MiSeq (Illumina, Inc., San Diego CA) following the manufacturer's protocol. The default parameters of the MiSeq reporter were used to base call, demultiplex, and trim adapters.

Analysis of the raw 16S rRNA gene amplicon sequences was performed using the CLC Genomics Workbench (v11.0) microbial genomic module (v3.6.1) from Qiagen. Overlapping pairs were merged if they had a minimum alignment score of 8, and a gap penalty of 3. A mismatch penalty of 2 was applied. Merged reads were trimmed using a quality limit of 0.001 and an ambiguous nucleotide limit of 2. Adapter sequences were removed. Sequences shorter than 240 base pairs in length were discarded. Resulting sequences were clustered using a reference-based method, and taxonomic assignments were determined by comparison to the SILVA 16S v132 database. Sequences that did not have 97% or greater similarity to a sequence in the SILVA 16S v132 database were clustered based on the similarity of sequences within a given cluster to other sequences within the cluster at 97% similarity threshold. Clustering sequences based on the similarity of sequences within a sample is referred to as De Novo Clustering.²¹⁹ To generate a phylogenetic tree the sequences were then aligned using ClustalOmega,²²⁰ and a maximum likelihood tree was created using PhymL²²¹ with a bootstrap value of 100. Then, iTOL²²² was used to visualize and annotate the phylogenetic tree.

Precipitate characterization.

Microcosms for precipitate characterization were established in 2 L glass bottles with 1 L of anoxic bicarbonate-buffered mineral salts medium supplemented with vitamins, lactate (5 mM), and CT (103 $\mu\text{mol/bottle}$). The amendment scheme is shown in **Table 38**, Appendix E. Hamilton syringes equipped with reproducibility (Chaney) adapters were used to add CT to microcosms (Hamilton, Reno, NV). Microcosms were incubated for 120 days in the dark. After incubation, the precipitate phase was collected via gravity filtration using a 1 L Stericup-GP sterile vacuum filtration system (Millipore Sigma) in an anaerobic chamber (Coy, Ann Arbor, MI) containing N_2/H_2 (97/3 vol/vol). After 24 hours any liquid that had not passed through the filter was poured out of the filtration system as the precipitate phase had settled to the bottom. The filters were then transferred to Mason jars. Each mason jar was wrapped in parafilm prior to being transferred out of the anaerobic glove box and moved to the XRD facility at the Joint Institute for Advanced Materials (JIAM). At JIAM, each precipitate was transferred to a PANalytical sample holder and scanned in a PANalytical Empyrean powder XRD instrument with a co-tube and a PIXcel3D-Medipix3 1×1 detector. Scanning 1-D line mode was used. The XRD scan was continuous, with a starting position of 10.0129° , an ending position of 109.9869° , and a step size of 0.0263. Further details of the XRD collection scheme are found in **Table 39**, Appendix E. Highscore PDF4+ was used for qualitative phase identification, as displayed in **Figure 63–Figure 66**, Appendix E. The background signal of diffraction patterns was calculated and then subtracted from diffraction patterns using a bending factor of 50 and a granularity of 9 in Highscore PDF4+. Reference files for vivianite (04-015-9091) and mackinawite (04-007-8222) were obtained from the

International Center for Diffraction Data (ICDD). Prior to plotting data were normalized by dividing all peaks by the highest peak and multiplying by 100.

Results

Chlorinated VOC.

To determine if the presence of the inoculum led to a greater decline in CT concentration under four treatments **Table 36**, Appendix E, microcosms and heat killed controls were grown in the presence of CT. Head space measurements of cVOCs were taken to track the concentrations of CT and the predicted transformation products CF, DCM, CM, and methane. The results showed that the presence of the inoculum facilitated a greater decline in the concentration of CT under all four treatments, as seen in **Figure 60**, Appendix E and **Figure 61**, Appendix E, which depict chlorinated VOC concentrations over time for microcosms containing CT. The graphs show averaged data of the three replicates, the error bars show the standard error of the three replicates at each time point. Standard error was calculated by calculating the standard deviation of the three replicates and dividing by the square route of the sample size, which was 3 for all conditions.

Figure 61, Appendix E shows the average decline in μmol of CT per bottle for each treatment. The p-values indicate that there are statistically significant differences between the inoculated treatment and the corresponding heat-killed control for all treatments.

Microbial community analysis. In order to identify members of the Elkhart enrichment culture.

16S rRNA gene amplicons were sequenced. Processing the 16S rRNA gene amplicons from the FeCl_2 , Na_2SO_4 , and Na_2S microcosms resulted in 41,704 sequence reads after processing, which were grouped into 64 predicted operational taxonomic units (OTUs). Of these OTUs, 47 were based on the SILVA 16S v132 database, and 17 were De Novo OTUs. The average read length was 253 base pairs. **Figure 62**, Appendix E is a phylogenetic tree built using maximum likelihood method with a bootstrap value of 100. All of the 64 predicted OTUs are included on the phylogenetic tree. The GenBank accession numbers are included from OTUs based on the SILVA 16S v132 database.

This analysis identified 11 bacterial classes, as shown in **Figure 62**, Appendix E. In **Figure 62**, Appendix E, each representative of the 11 bacterial classes is highlighted with a different color: the corresponding colors and classes are identified in the legend on the upper-right-hand corner. In general, OTUs from the bacterial classes grouped together, with the exception of the Clostridia, which was intersected by the Bacilli and Alphaproteobacteria classes. The largest class of organisms identified in this analysis was the Clostridia, which has 29 representatives from 15 genera. The next largest class was the Campylobacteria, which has 12 representatives, all of which are from the *Sulfurospirillum* genus. The most identified organism was a *Sulfurospirillum*, which was identified 22,720 times. There were seven representatives from the Bacteroidia class, with representatives from three genera. There were five OTUs identified as Spirochaetia, all of which were unidentified at the genus level. Three Gammaproteobacteria representatives were identified, all from different genera. Two Alphaproteobacteria were

identified, one of which was from the *Pleomorphomonas* genera. A single OTU was identified from each of the Actinobacteria, *Dehalococcoidia*, Anaerolineae, and Oxyphotobacteria classes.

Precipitate characterization.

In order to determine the identities of the precipitates formed under four treatments **Table 38**, Appendix E, X-ray diffraction data were collected and analyzed. The results showed that vivianite ($\text{Fe}_3^{2+}(\text{PO}_4)_2 \cdot 8\text{H}_2\text{O}$) was the only mineral phase identified in the diffraction data collected on the precipitates from vessels that contained FeCl_2 , FeCl_2 , and Na_2S , and FeCl_2 and NaSO_4 **Figure 63–Figure 66**, Appendix E, and **Table 41**, Appendix E. The presence or absence of CT had no effect on the formation of the precipitate phase in any of the experimental vessels. Additionally, vivianite and mackinawite (FeS) were identified in the diffraction data collected on the the precipitate from the vessels that contained FeCl_2 , Na_2S , NaSO_4 , and cells **Figure 63**, Appendix E. Vivianite was only identified in the diffraction data collected on the precipitate from the vessels that contained FeCl_2 , Na_2S , NaSO_4 without cells. A reference pattern (04-007-8222) from the ICDD was used to identify FeS . Key peak alignments that suggest the presence of FeS in the diffraction pattern from the precipitate formed in the vessel that contained FeCl_2 , Na_2S , NaSO_4 , and cells are the split peak at 20° of 2-theta, the peak at 40° of 2-theta, and the forked peaks at 58° 2-theta.

Discussion

Biologically mediated abiotic degradation of CT via the formation or regeneration of reactive iron species in contaminated aquifers has the potential to facilitate degradation of this recalcitrant contaminant. A deeper understanding of the microbial processes that can generate reactive iron species in the presence of CT is needed. This study tracked the concentration of CT and its predicted transformation products in microcosms, used 16S rRNA amplicon sequencing to identify members of the microbial consortium, and identified the precipitate phases formed under different conditions. This study found that there was greater decline in CT concentration in microcosms that contained inoculum than in heat killed controls, identified FeS in microcosms that contained FeCl_2 , NaSO_4 , Na_2S and cells, and observed 16S rRNA gene sequences of sulfate reducing bacteria, which suggest that the formation of FeS could be a byproduct of sulfate reduction.

There were greater declines in the amount of CT per bottle in bottles that were inoculated with the enrichment culture than in the bottles that were inoculated with the enrichment culture and then heat-killed for all treatments. Despite the statistically significant difference between inoculated and heat-killed control vessels, stoichiometric amounts of transformation products were not observed. It is possible that transformation products that were not detectable by the GC-FID method were formed. For example, McCormick and Adriaens observed the formation of carbon monoxide and formate in their 2004 study, and some studies report the formation of carbon dioxide and phosgene.^{68,76} The

analytical methods used in this study were not established to capture the formation of phosgene or carbon dioxide or carbon monoxide.

The identification of vivianite as the only mineral phase is robust in vessels that contained FeCl_2 , FeCl_2 , and Na_2S , and FeCl_2 and NaSO_4 **Figure 63–Figure 66**, Appendix E, as all diffraction peaks in these samples match the vivianite diffraction pattern obtained through the ICDD (01-080-9696), and there are no unaccounted for peaks in these diffraction patterns suggesting a single phase. It seems that the formation of vivianite is an abiotic process, as vivianite was identified in samples both with and without cells. The ratio of phosphate to ferrous iron in vivianite is 2:3, as seen in vivianite's chemical formula $\text{Fe}_3(\text{PO}_4)_2(\text{H}_2\text{O})$. Further, the medium used in these experiments contained 1.47 mM of monobasic potassium phosphate (KH_2PO_4). Therefore, if all the KH_2PO_4 precipitated with ferrous iron, 2.2 mM of ferrous iron could be consumed. As 5.0 mM FeCl_2 was used in each experiment, this reaction could precipitate out half of the iron in each experimental vessel.

Identification of FeS in the vessels that contained FeCl_2 , Na_2S , NaSO_4 , and cells is not as robust as the identification of vivianite, as the other peaks associated with FeS are small and obscured by superposition with peaks from the vivianite diffraction pattern. Precipitate formation was the same in the inoculated vessels that contained FeCl_2 , Na_2S , and NaSO_4 whether or not CT was present. A possible explanation for the formation of FeS in vessels that contained FeCl_2 , Na_2S , NaSO_4 , and cells is that the activity of a sulfate-reducing organism (*i.e.*, *Desulfosporosinus meridiei* DSM 13257, which was identified in the 16S rRNA gene amplicon analysis) increased the amount of sulfide present in the system. An increase in sulfide concentration could lead to the formation of an identifiable amount of FeS , as sulfide precipitation with ferrous iron could form FeS .

Analysis of microbial community structure by 16S rRNA gene amplicon sequencing has limitations that are inherent in the methodology. Examples of these limitations are primer bias, sequencing error, and variation in starting DNA concentration.²²³ Primer bias can cause some sequences to be amplified more than others, leading to an overestimate of their prevalence in the population. Furthermore, all general primers will entirely miss some members of diverse microbial populations. During PCR amplification cycles, incorrect base pairing occurs, which creates OTUs that do not represent real organisms. This leads to an overestimation of OTUs in the sample. Finally, variation in starting concentration of DNA can select for increased amplification of more abundant DNA. Knowledge of these errors can be addressed to some extent in experimental design and data processing. For example, errors can be minimized by using high-quality primer sets, being precise with starting DNA concentration when preparing the amplicon library, and removing singly represented OTUs during data processing. Despite these limitations, benchmark studies like the one performed by D'Amore and colleagues (2016) suggest 16S rRNA gene amplicon sequencing still has some quantitative value.²²³ This report mentions the relative abundance of community members at the phylum level identified during 16S rRNA gene amplicon sequencing, with the understanding that there is error in the reported values.

The most frequently detected OTU represents an uncultured *Sulfurspirillum* sp. identified by a sequencing effort to characterize the microbial community of the Danshui river estuary of Northern Taiwan. The metabolic strategy of this uncultured *Sulfurspirillum* is unknown.²²⁴ Members of the *Sulfurspirillum* genus are known to use toxic compounds such as arsenate, selenite, and organohalides as electron acceptors coupled to the oxidation of pyruvate, hydrogen, and formate as electron donors.¹⁴³ Members of this genus can also use nitrate and oxidized sulfur compounds as electron donors and perform pyruvate fermentation, which produces H₂.²²⁵ *Sulfurspirillum* is commonly found in contaminated sites, and research suggests that menaquinones and cytochromes are involved in their anaerobic respiratory chains.¹⁴³ Menaquinones and cytochromes have been shown to facilitate CT transformation when produced by other organisms.^{101,109,124,226} It is possible that the menaquinones or corrinoid produced by *Sulfurspirillum* spp. could facilitate co-metabolic CT degradation. All of the representatives from the Campylobacteria class are *Sulfurospirillum* **Figure 62**, Appendix E.

The second most frequently identified OTU was a member *Spirochaetaceae* family with the GenBank accession number JN685463. The uncultured *Spirochaetaceae* was identified by a sequencing effort to characterize the microbial community in a water-flooded oil reservoir.²²⁷ Members of the Spirochaetia class can be free-living or host-associated, and they are generally motile, spiral-shaped organisms that can be obligate or facultative anaerobes.¹⁴³ A saccharolytic metabolism is often employed by members of the Spirochaetia class. The representative identified in the enrichment culture is of the *Spirochaetaceae* family, which contains the genera *Borrelia*, *Brevinema*, *Cristispira*, *Spirochaeta*, *Spironema*, and *Treponema*.

The third, fourth, and fifth most frequently identified OTUs were members of the Clostridia class. All members of the clostridia phylum are obligate anaerobes. The third most frequently identified OTU was *Desulfosporosinus meridiei* DSM 13257, which can be identified by the GenBank accession number CP003629. This bacterium was isolated from groundwater contaminated with motor fuel. *Desulfosporosinus meridiei* DSM 13257 is a gram-positive organism that utilizes sulfate reduction as a metabolic strategy.^{228,229} Other members of the *Desulfosporosinus* genus have been isolated from acid mine drainage and are slightly acidophilic.²²⁸ The fourth most frequently identified OTU is an ambiguous member of the *Caproiciproducens* genus, identified by the GenBank accession number CCFG01000002. Not much information is associated with this GenBank accession number. A cultured member of the *Caproiciproducens* genus, *Caproiciproducens galactitolivorans*, was isolated from a wastewater treatment plant and it produces caproic acid from galactitol.²³⁰ The fifth most frequently detected OTU was an ambiguous member of the *Ruminococcaceae* family, which was identified in samples from Chinese mines.²³¹ Members of the *Ruminococcaceae* family are obligate anaerobes, and the type strain of the *Ruminiclostridium* genera is *Ruminiclostridium cellobioparum*, which was isolated from bovine rumen and employs a fermentative metabolism.²¹⁵

Other less frequently detected members of the Clostridia class belonged to the genera: *Desulfitobacterium*, *Hydrogenoanaerobacterium*, *Intestimonas*, *Oscillibacter*, *Family XIII*, and *Lutispora*. Many organisms from these genera are known to utilize fermentative metabolisms.^{232–238} Of note are the members of the *Desulfitobacterium* genera, which are known to be metabolically diverse. Representatives of the *Desulfitobacterium* genera are known to utilize nitrate, sulfite, metals, humic acids, and man-made or naturally occurring halogenated organic compounds as electron acceptors.²³⁹

Fermentative metabolisms are also commonly observed in members of less frequently detected classes (*i.e.*, Bacilli, Actinobacteria, Anaerolineae). There are two representatives from the Bacilli class, both are from the *Bacillus* genus. Members of the *Bacillus* genus are known to be rod-shaped, gram-positive, spore-forming, aerobic, or facultatively anaerobic bacteria that use fermentative metabolic strategies.²⁴⁰ The representative of the Actinobacteria is unidentified at the species level. The type strain of the genus is *Propioniceella superfundia*, which was isolated from groundwater contaminated with chlorinated ethanes and vinyl chloride. *Propioniceella superfundia* is a gram-positive, rod-shaped, non-motile, non-spore-forming bacterium that is a facultative anaerobe and utilized a fermentative metabolic strategy.²⁴¹ The Anaerolineae representative sequence was originally identified as part of a methanogenic community developed using subsurface sediments and cultivated in a bioreactor prior to sequencing efforts.²⁴² The type strain of the *Pelolinea* genera is *Pelolinea submarina*, which is described as a filamentous anaerobic gram-negative bacterium that is non-motile and non-spore former that can grow on several carbohydrates in the presence of yeast extract.²⁴³

Two OTUs identified at low abundances that likely utilize distinct metabolic strategies were an uncultured *Dehalogenimonas* and a member of the Oxyphotobacteria class. Members of the *Dehalogenimonas* genus are capable of reductively dehalogenating halogenated alkanes, and some members can dehalogenate trichloroethene and vinyl chloride to the non-chlorinated end product ethene.^{155,244} The member of the Oxyphotobacteria class was identified as a *Cyanobium gracile* PCC 6307, which is described by Uniprot²⁴⁵ and Jgi²⁴⁶ as a non-motile unicellular gram-negative bacteria isolated from a freshwater system. Cyanobacteria are typically oxygenic phototrophic organisms.

Conclusions

This study sought to gain a deeper understanding of the processes that contribute to biologically mediated abiotic degradation of CT. The hypothesis that magnetite formation in this system occurs via a biogenic oxidative pathway in the presence of CT was not supported. Magnetite was not identified in these experiments, and the precipitate that did form did not contain oxidized (ferric) iron. FeS formation was observed in the vessels that contained FeCl₂, Na₂S, NaSO₄ and were inoculated with the enrichment culture. FeS was not identified under any other treatment condition. The formation of FeS was likely facilitated by a sulfate-reducing microbe. Additionally, the decline in CT concentration

was greater in the inoculated microcosms than in their corresponding heat-killed controls. The greater decline in CT concentration in inoculated bottles could be due to the activity of *Desulfosporosinus* and *Sulfurspirillum*, as their sulfate reducing metabolisms are likely responsible for the formation of FeS.^{247,248} Furthermore, the production of menaquinones and cytochromes by *Sulfurspirillum* may facilitate co-metabolic CT transformation.^{101,109,124,226}

Appendix E

Table 36. Amendment schemes for Elkhart microcosms.

ID	Inoculum	FeCl ₂	NaSO ₄	Na ₂ S	Lactate	CT
1 a, b, and c	3%	5 mM	5 mM	0.2 mM	5 mM	10 µmol/bottle
2 a, b, and c	3% HK	5 mM	5 mM	0.2 mM	5 mM	10 µmol/bottle
3 a, b, and c	3%		5 mM		5 mM	10 µmol/bottle
4 a, b, and c	3% HK		5 mM		5 mM	10 µmol/bottle
5 a, b, and c	3%	5 mM		0.2 mM	5 mM	10 µmol/bottle
6 a, b, and c	3% HK	5 mM		0.2 mM	5 mM	10 µmol/bottle
7 a, b, and c	3%	5 mM			5 mM	10 µmol/bottle
8 a, b, and c	3% HK	5 mM			5 mM	10 µmol/bottle

HK = heat kill

Table 37. Detailed instrument settings used for the analysis of headspace samples with gas chromatography.

Chlorinated compounds	Front Inlet	Inlet/Detector Gases	Detector (FID)	Program
PCE, 1,1-DCE, CT, CF, DCM, CM, methane	Heater = 200 °C Pressure = 23.193 psi Septum purge flow = 1.5 mL /min Split = Split ratio of 50:1 w/Split flow of 150 mL /min	He = inlet N ₂ = detector	Heater = 200 °C Air Flow = 400 mL /min H ₂ Fuel flow = 30 mL /min	Initial rate °C/min = 0 Initial value °C = 60 Initial hold time min = 2 Initial run time min = 2 Ramp 1 rate °C/min = 25 Ramp 1 value °C = 200 Ramp 1 hold time min = 1 Ramp 1 run time min = 8.6

Table 38. Amendment Schemes for X-ray diffraction microcosms.

Biotic or Abiotic	FeCl₂	NaSO₄	Na₂S	CT
Biotic	5 mM	5 mM	0.2 mM	10 µmol/bottle
Biotic	5 mM	5 mM	0.2 mM	10 µmol/bottle
Abiotic	5 mM	5 mM	0.2 mM	10 µmol/bottle
Biotic	5 mM		0.2 mM	10 µmol/bottle
Biotic	5 mM		0.2 mM	10 µmol/bottle
Abiotic	5 mM		0.2 mM	10 µmol/bottle
Biotic	5 mM	5 mM		10 µmol/bottle
Biotic	5 mM	5 mM		10 µmol/bottle
Abiotic	5 mM	5 mM		10 µmol/bottle
Biotic	5 mM			10 µmol/bottle
Biotic	5 mM			10 µmol/bottle
Abiotic	5 mM			10 µmol/bottle

Table 39. X-ray diffraction collection parameters

Radiation		
Co K α 1 [Å]		1.789
Co k α 2 [Å]		1.793
Tube voltage [kV]		45
Tube current [mA]		40
Collimation		
Divergence slit [°]	1/4	1/8
Anti-scatter slit [°]	1/2	1/4
Soller slit [Rad]	0.04	0.04
Environment		
Atmosphere		Inert
Temperature		Ambient
Pressure		Ambient
Collection		
Scan range [2θ °]	$10.0 \leq 2\theta \leq 90.0$	$10.0 \leq 2\theta \leq 90.0$
Step size [2θ °]	0.02	0.013
Counting time [s]	39.00	159.00
Spin time [s/rotation]	4	4

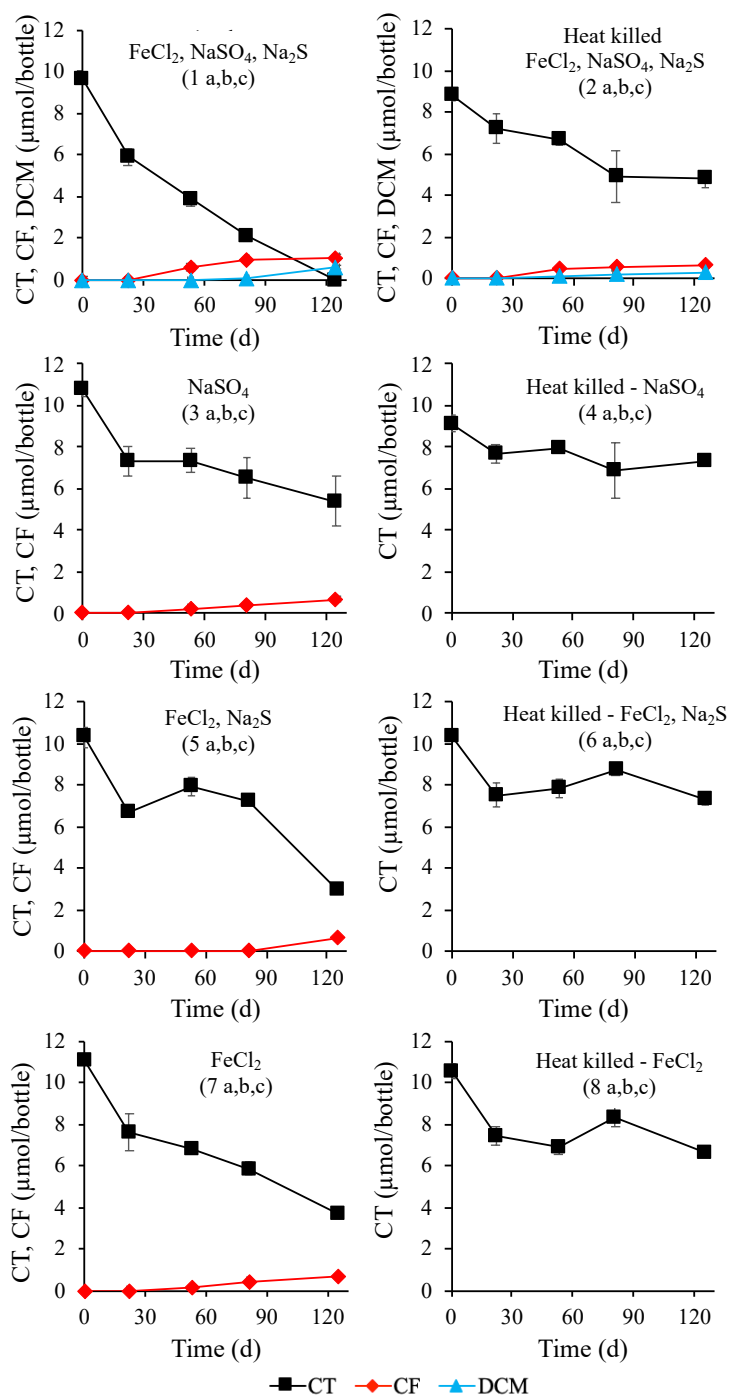


Figure 60. Chlorinated volatile organic compound concentrations over time in carbon-tetrachloride-containing microcosms.

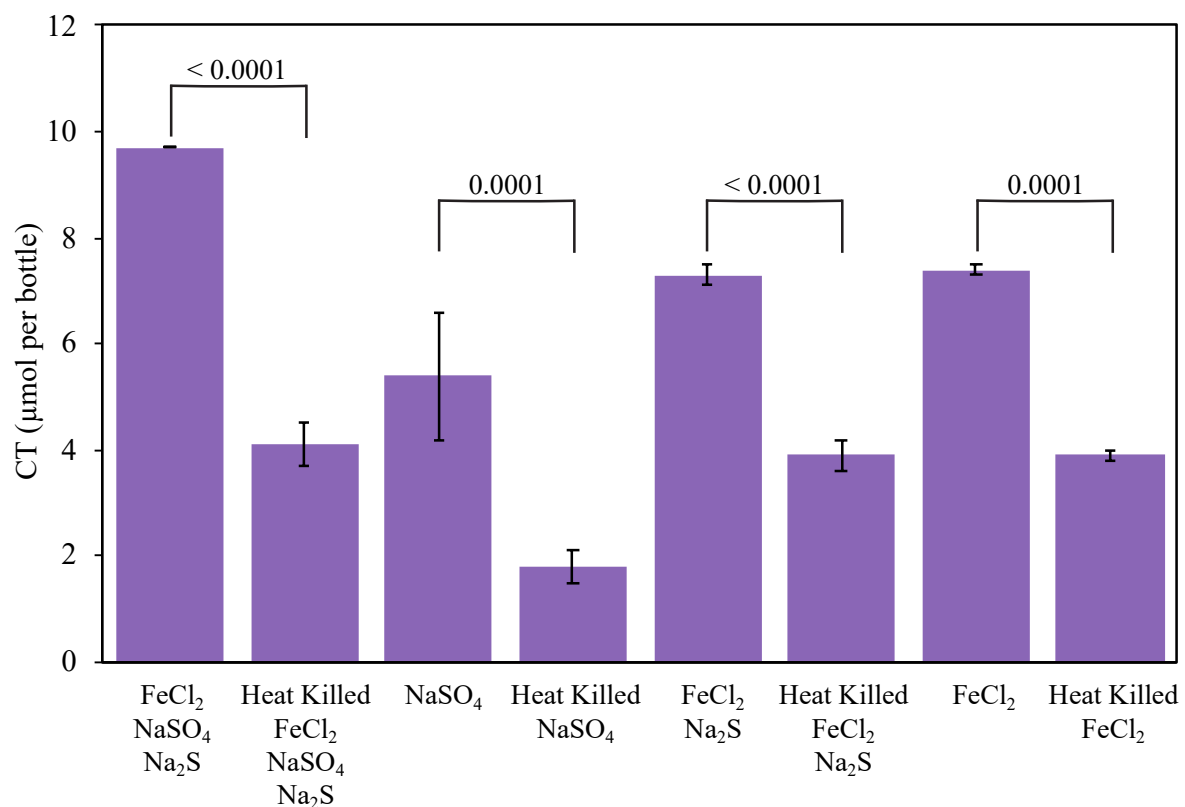


Figure 61. One-way analysis of variance of the effect of treatment on carbon tetrachloride decline. Each purple bar indicates the average decline in μmol of CT per treatment. The error bars show the standard error of each treatment. The p -value for each comparison is shown above the brackets which indicate the two treatments being compared.

Table 40. Pairwise comparisons for the effect of treatment on carbon tetrachloride.

treatment	treatment	Estimate	Standard Error	DF	t Value	Pr > t	Adj P
FeCl ₂	FeCl ₂ , Na ₂ S	0.1127	0.7026	16	0.16	0.8746	1.0000
FeCl ₂	FeCl ₂ , NaSO ₄ , Na ₂ S	-2.2252	0.7026	16	-3.17	0.0060	0.0860
FeCl ₂	Heat-Killed FeCl ₂	3.5208	0.7026	16	5.01	0.0001	0.0025
FeCl ₂	Heat-Killed FeCl ₂ , Na ₂ S	4.3940	0.7026	16	6.25	<.0001	0.0002
FeCl ₂	Heat-Killed FeCl ₂ , NaSO ₄ , Na ₂ S	3.3800	0.7026	16	4.81	0.0002	0.0037
FeCl ₂	Heat-Killed NaSO ₄	5.6333	0.7026	16	8.02	<.0001	<.0001
FeCl ₂	NaSO ₄	2.0843	0.7026	16	2.97	0.0091	0.1226
FeCl ₂ , Na ₂ S	FeCl ₂ , NaSO ₄ , Na ₂ S	-2.3378	0.7026	16	-3.33	0.0043	0.0642
FeCl ₂ , Na ₂ S	Heat-Killed FeCl ₂	3.4082	0.7026	16	4.85	0.0002	0.0034
FeCl ₂ , Na ₂ S	Heat-Killed FeCl ₂ , Na ₂ S	4.2813	0.7026	16	6.09	<.0001	0.0003
FeCl ₂ , Na ₂ S	Heat-Killed FeCl ₂ , NaSO ₄ , Na ₂ S	3.2673	0.7026	16	4.65	0.0003	0.0050
FeCl ₂ , Na ₂ S	Heat-Killed NaSO ₄	5.5207	0.7026	16	7.86	<.0001	<.0001
FeCl ₂ , Na ₂ S	NaSO ₄	1.9717	0.7026	16	2.81	0.0127	0.1611
FeCl ₂ , NaSO ₄ , Na ₂ S	Heat-Killed FeCl ₂	5.7460	0.7026	16	8.18	<.0001	<.0001
FeCl ₂ , NaSO ₄ , Na ₂ S	Heat-Killed FeCl ₂ , Na ₂ S	6.6192	0.7026	16	9.42	<.0001	<.0001
FeCl ₂ , NaSO ₄ , Na ₂ S	Heat-Killed FeCl ₂ , NaSO ₄ , Na ₂ S S	5.6052	0.7026	16	7.98	<.0001	<.0001
FeCl ₂ , NaSO ₄ , Na ₂ S	Heat-Killed NaSO ₄	7.8585	0.7026	16	11.18	<.0001	<.0001
FeCl ₂ , NaSO ₄ , Na ₂ S	NaSO ₄	4.3095	0.7026	16	6.13	<.0001	0.0003
Heat-Killed FeCl ₂	Heat-Killed FeCl ₂ , Na ₂ S	0.8732	0.7026	16	1.24	0.2319	0.9067
Heat-Killed FeCl ₂	Heat-Killed FeCl ₂ , NaSO ₄ , Na ₂ S	-0.1408	0.7026	16	-0.20	0.8437	1.0000
Heat-Killed FeCl ₂	Heat-Killed NaSO ₄	2.1125	0.7026	16	3.01	0.0084	0.1144
Heat-Killed FeCl ₂	NaSO ₄	-1.4365	0.7026	16	-2.04	0.0577	0.4852
Heat-Killed FeCl ₂ , Na ₂ S	Heat-Killed FeCl ₂ , NaSO ₄ , Na ₂ S	-1.0140	0.7026	16	-1.44	0.1683	0.8248

Table 40. Continued.

treatment	treatment	Estimate	Standard Error	DF	t Value	Pr > t	Adj P
Heat-Killed FeCl ₂ , Na ₂ S	Heat-Killed NaSO ₄	1.2393	0.7026	16	1.76	0.0968	0.6501
Heat-Killed FeCl ₂ , Na ₂ S	NaSO ₄	-2.3097	0.7026	16	-3.29	0.0046	0.0691
Heat-Killed FeCl ₂ , NaSO ₄ , Na ₂ S	Heat-Killed NaSO ₄	2.2533	0.7026	16	3.21	0.0055	0.0800
Heat-Killed FeCl ₂ , NaSO ₄ , Na ₂ S	NaSO ₄	-1.2957	0.7026	16	-1.84	0.0838	0.6026
Heat-Killed NaSO ₄	NaSO ₄	-3.5490	0.7026	16	-5.05	0.0001	0.0023

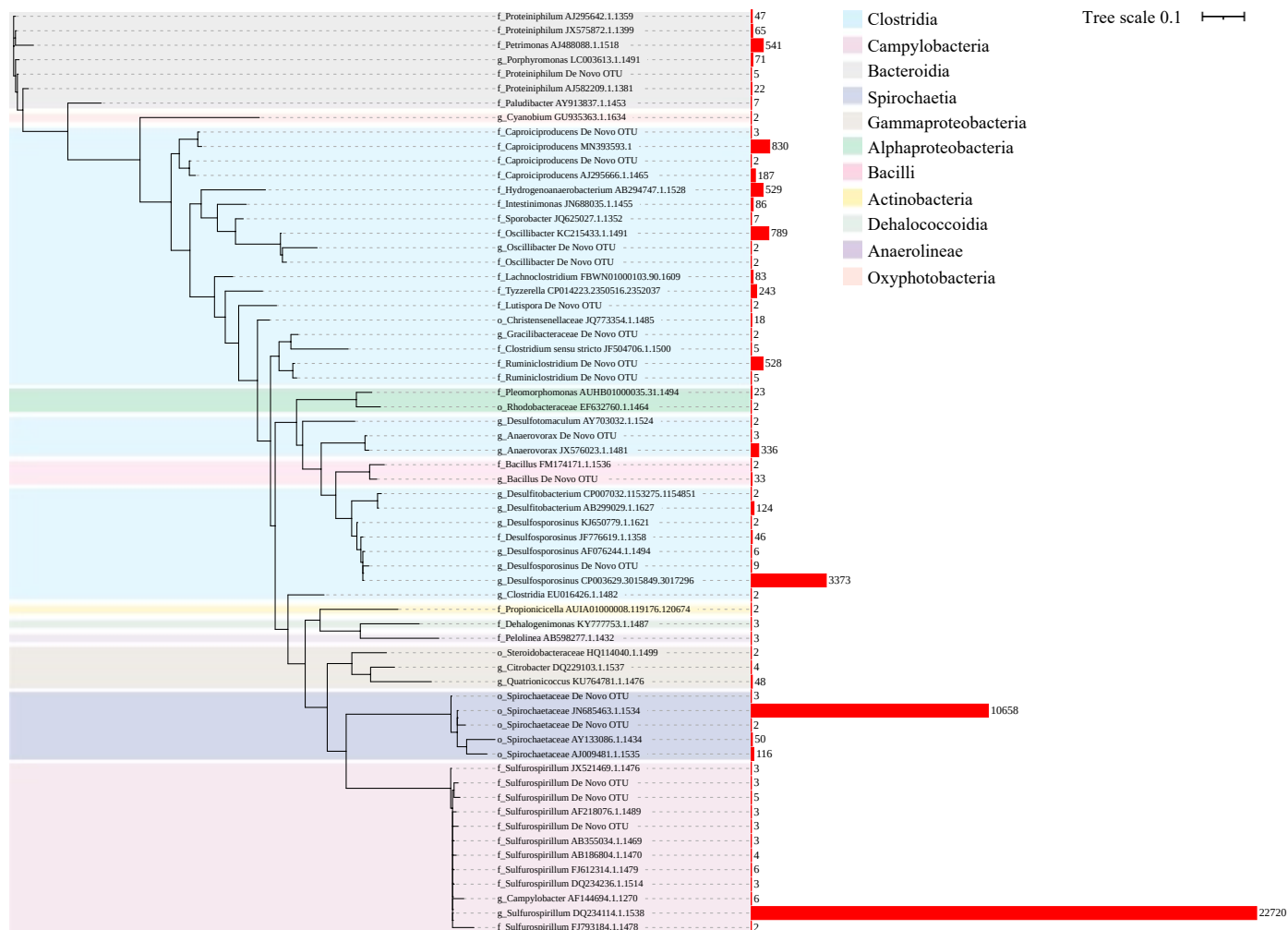


Figure 62. Maximum likelihood tree built from predicted Elkhart operational taxonomic unit.

In Figure 62, each class is highlighted by a different color, as shown in the legend. The lowercase letter before each the operational taxonomic unit name identified the level of phylogenetic identification that was determined for each operational taxonomic unit. Additionally, the red bars on the right-hand side of the tree report how many times each operational taxonomic unit was identified.

Table 41. Description of precipitate formation in microcosms.

Biotic or Abiotic	FeCl₂	NaSO₄	Na₂S	CT	Mineral Phase
Biotic	5 mM	5 mM	0.2 mM	10 µmol/bottle	Vivianite, Mackinawite
Biotic	5 mM	5 mM	0.2 mM	0 µmol/bottle	Vivianite, Mackinawite
Abiotic	5 mM	5 mM	0.2 mM	10 µmol/bottle	Vivianite
Biotic	5 mM		0.2 mM	10 µmol/bottle	Vivianite
Biotic	5 mM		0.2 mM	0 µmol/bottle	Vivianite
Abiotic	5 mM		0.2 mM	10 µmol/bottle	Vivianite
Biotic	5 mM	5 mM		10 µmol/bottle	Vivianite
Biotic	5 mM	5 mM		0 µmol/bottle	Vivianite
Abiotic	5 mM	5 mM		10 µmol/bottle	Vivianite
Biotic	5 mM			10 µmol/bottle	Vivianite
Biotic	5 mM			0 µmol/bottle	Vivianite
Abiotic	5 mM			10 µmol/bottle	Vivianite

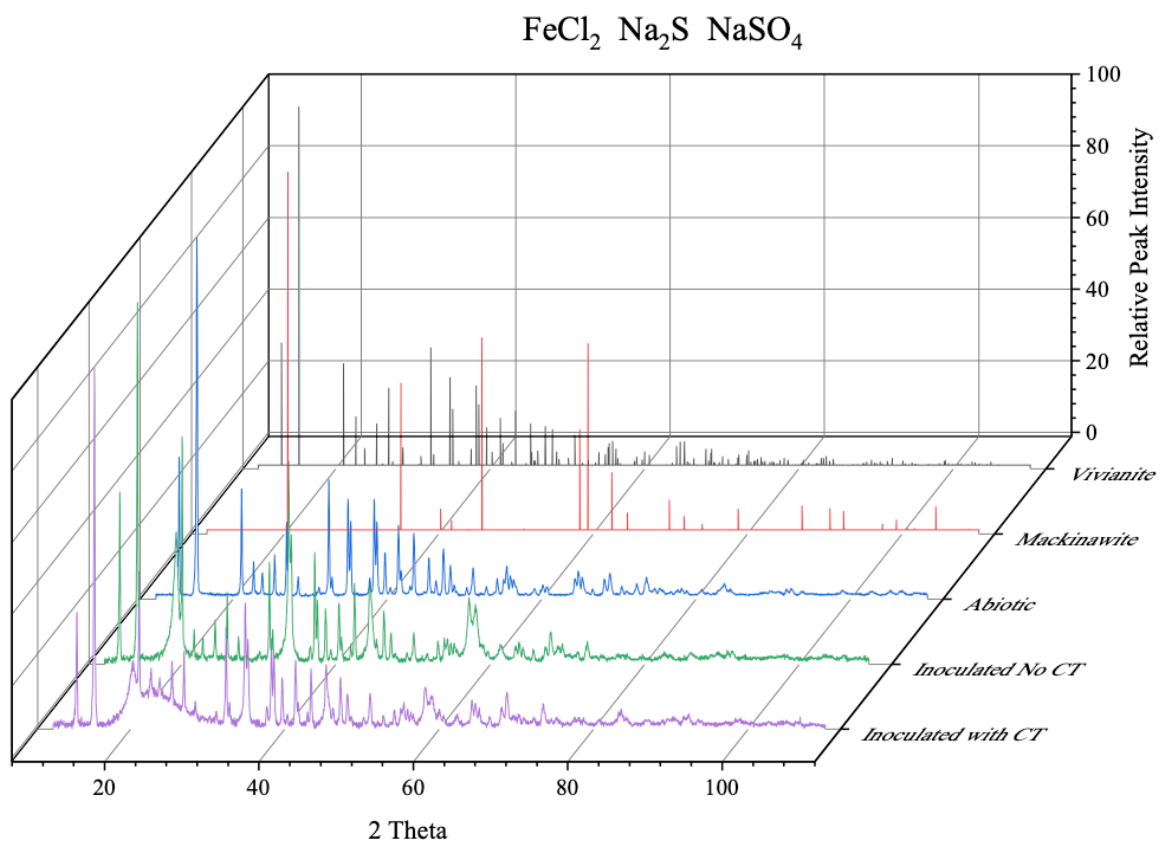


Figure 63. Diffraction patterns of the FeCl_2 , Na_2S , and NaSO_4 amended microcosms.

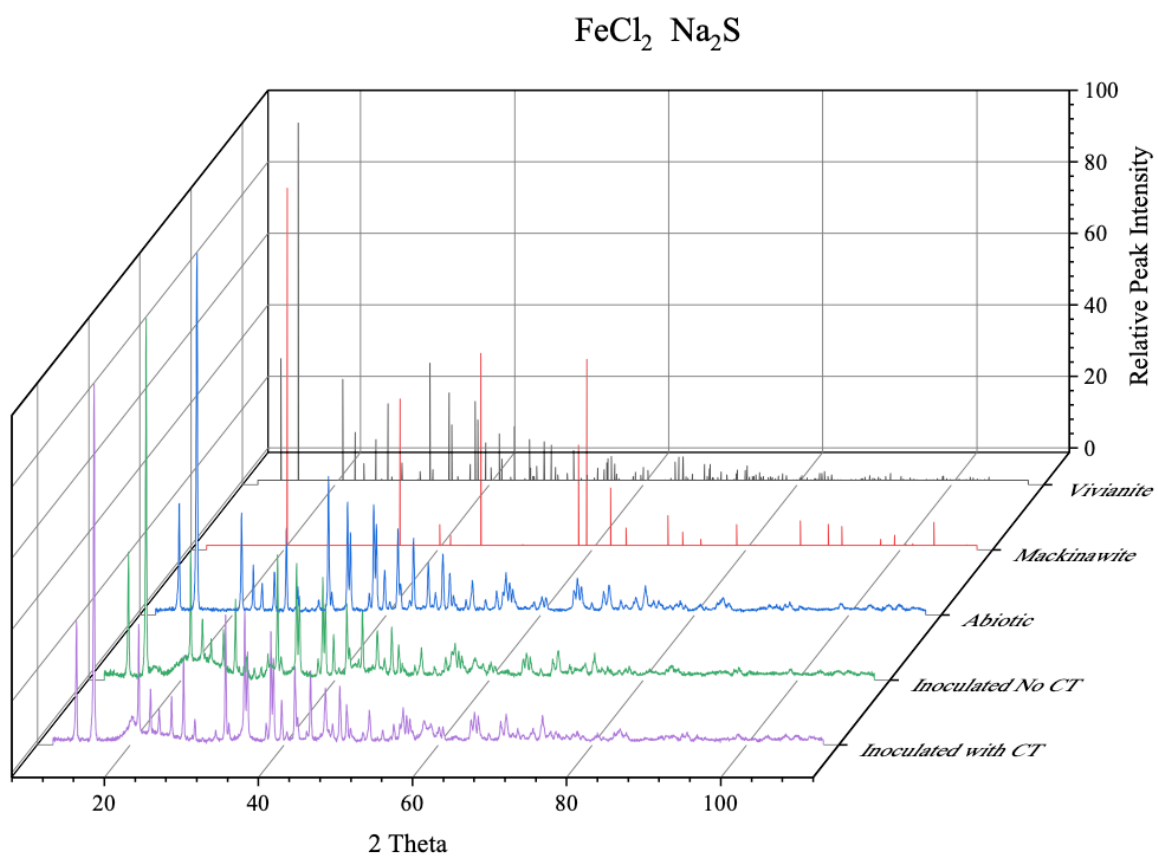


Figure 64. Diffraction patterns of the FeCl_2 and Na_2S amended microcosms.

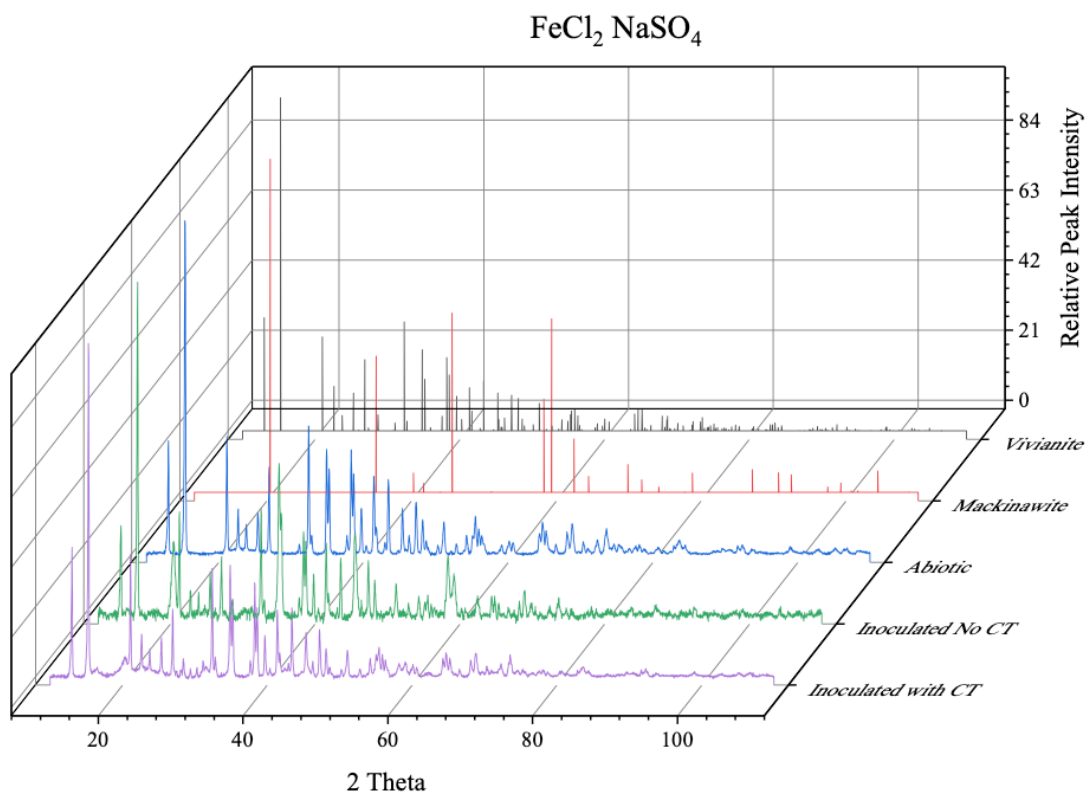


Figure 65. Diffraction patterns of the FeCl_2 and Na_2SO_4 amended microcosms.

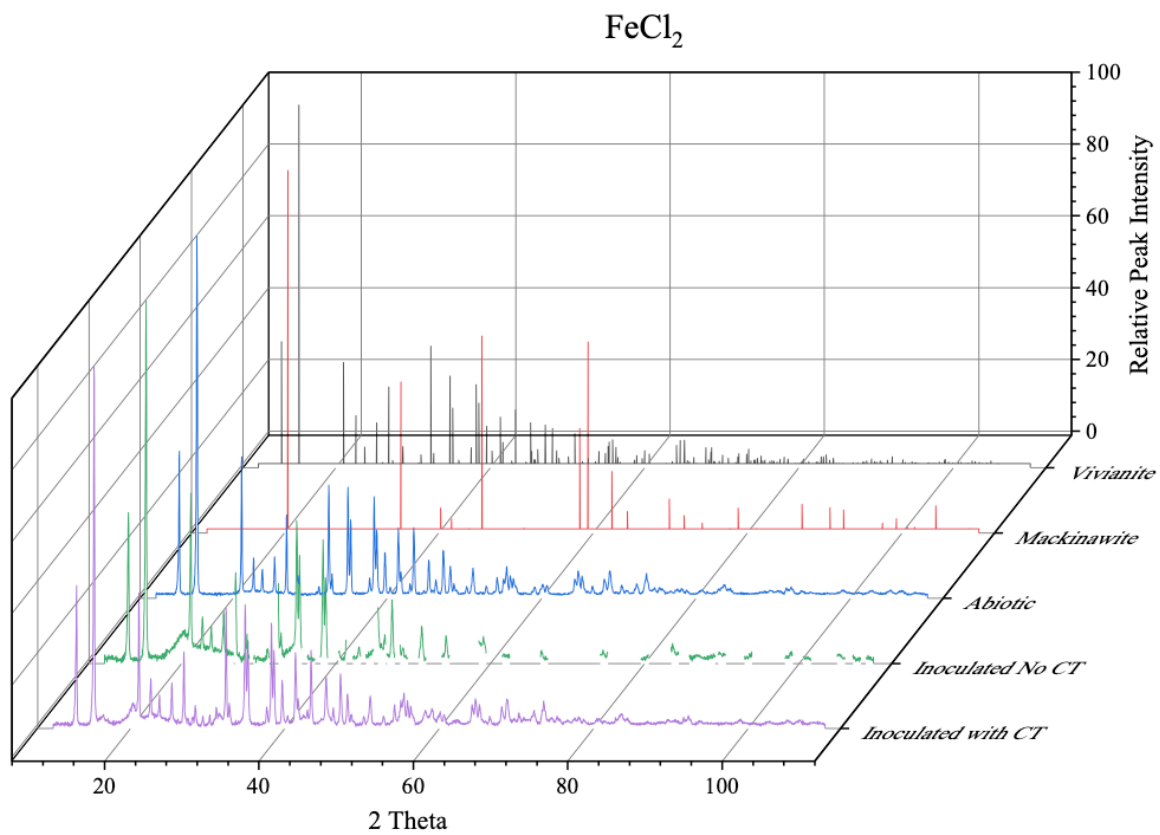


Figure 66. Diffraction patterns of the FeCl_2 amended microcosms.

CONCLUSIONS

CFC-11, CFC-12, and CT are greenhouse gasses, ozone depleting substances and groundwater pollutants found globally.⁴⁻⁶ Though CFC-11 and CFC-12 are not very toxic they do inhibit the biodegradation of other contaminants, and their transformation products can be more toxic, carcinogenic, and flammable than they are, for these reasons it is important to understand how these chemicals can be degraded in the subsurface.⁵⁸ It is also important to understand the mechanisms of degradation for CT, as it is a toxic substance, suspected carcinogen, and inhibitory to dehalogenating bacteria.^{8,20,21,26}

Data from two treatability studies contaminated with CFC-11, CFC-12, and CT and other chemicals was presented in this dissertation. At the first contaminated site a soil gas survey (SGS) and membrane interface hydrologic profiling tool (MiHPT) were used to characterize contaminant distribution at the site prior to taking sediment and groundwater samples (Chapter 2). Data from the SGS survey and MiHPT transect revealed that contaminant concentration was highest along the diagonal southern boundary of the contaminated site. This finding was consistent with historical information regarding land use at the site, and was used to inform sediment and groundwater sampling locations. Analysis of the volatile organic carbons in the groundwater indicate that the borehole A3 from 19.70–24.70 mbgs contains the most contaminants, as PCE, 1,1-DCE, CFC-11, CFC-12, and CT were present in this sample. A successful remediation strategy for CFC-11 or CFC-12 was not identified by the treatability study. However, the data from the treatability study did suggest that bioaugmentation combined with biostimulation and the addition of zero valent iron or ferrous sulfide would transform CT into non-halogenated transformation products. The treatability study data also suggested that bioaugmentation would be an effective remediation strategy for PCE and 1,1-DCE. However, degradation of CFC-11, CFC-12, and CT is recommended prior to implementing bioaugmentation as a remediation strategy, as these chemicals are likely to inhibit the activity of the bioaugmentation culture.

It appeared that the aquifer material from the second contaminated site was oxic, because repeated additions of sulfide were needed to reduce the microcosms set up with aquifer material from this site. The microcosm data from the second contaminated site suggest that a component in the ground water is inhibitory to the bioaugmentation cultures tested; degradation of PCE and CF was achieved in bioaugmentation microcosms that contained reduced anoxic mineral salts medium but not in microcosms that contained groundwater. It is possible that the inhibitory component in the groundwater is CFC-11, CFC-12 or CT, as these chemicals are known to be inhibitory to bacteria and were present at the site. Similarly to the finding at the first contaminated site, it is recommended that degradation of CFC-11, CFC-12, and CT occur prior to an effort to degrade PCE or CF via bioaugmentation. CT was transformed to CF in microcosms set up with aquifer material and additional zero valent iron.

In chapter four the effect of pH on the dehalogenation of CFC-11, CFC-12 and CT by super-nucleophilic B₁₂ was explored. Experiments in chapter four indicate that pH does have an effect on the dehalogenation of CFC-11, CFC-12 and CT by super-nucleophilic

B₁₂. The formation and distribution of transformation products changes as pH increased. Additionally, it was observed that super-nucleophilic vitamin B₁₂ is unable to dehalogenate chlorodifluoromethane, difluoromethane, fluoromethane, chlorofluoromethane.

In chapter five a precipitate forming sulfidogenic enrichment culture was characterized. The data from this chapter indicate that the presence of active enrichment culture facilitated a greater decline in CT concentration than was observed in heat killed controls. Additionally, the two precipitate phases were identified in microcosms that contained FeCl₂, NaSO₄, Na₂S and enrichment culture. The two mineral phases were, mackinawite (FeS) and vivianite (Fe₃²⁺(PO₄)₂ · 8H₂O). Vivianite formation occurred via precipitation of added ferrous iron and the phosphate present in the medium, as this mineral phase was observed in all treatments including heat killed controls. Mackinawite formation was only observed in the treatments that contained FeCl₂, NaSO₄, Na₂S and enrichment culture, and likely occurred via precipitation of ferrous iron and hydrogen sulfide formed as a byproduct of sulfate reduction. Sulfate reducing bacteria were identified in 16S rRNA gene sequences from the enrichment culture.

This work indicates that there is a need to implement effective remediation strategies for the CFC-11, CFC-12, and CT, as they inhibit the degradation of other contaminants (*i.e.*, chlorinated ethenes). However, an understanding of the transformation products that will be produced is important, as some transformation products are more hazardous than the starting chemicals.⁵⁸ Stimulation of microbial communities like the sulfate reducing community characterized in chapter five may help facilitate transformation of CFC-11, CFC-12, and CT, but the literature indicates that more hazardous transformation products are likely to form.^{73,74,132} Development of a remediation strategy that utilizes B₁₂ may be effective, as carbon monoxide is reportedly the predominant transformation product. Some barriers that would have to be overcome in order to develop a remediation strategy that utilizes B₁₂ would be preparing an environment that has the capacity to reduce B₁₂ to the super-nucleophilic state, and the necessity of immobilizing B₁₂ in the subsurface, so that it would not diffuse, get taken up by microbes, or travel away from the prepared reducing environment.

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VITA

Briana M. McDowell was born at Beale Air Force Base in Yuba County, California, to Lisa R. Bryan and Glenn A. McDowell. Growing up, she was passionate about musical theater and regularly performed in theatrical productions and choral ensembles. During her high school years, she became beguiled by the wildness and beauty of the Yuba and American rivers. She frequented these rivers as often as possible, spending entire days climbing up their granite pool drop rapids, exploring their nooks and crannies, and learning about the relentless power of these and all rivers. She became a raft guide on the American River after high school and spent two summers rafting and exploring as many rivers as possible. Feeling the need to explore more of the world, she moved to Boston in 2011, where she attended Suffolk University.

At Suffolk University, Briana acquired a B.S. in Biochemistry. During her undergraduate career, she was selected to participate in the McNair Scholars Program, where she was encouraged to find undergraduate research opportunities and attend graduate school. With the McNair Scholars advisors' encouragement, she gained a position as a Visiting Scientist at MIT Sea Grant. She worked with Dr. Julia Simpson on carbon sequestration in coastal eelgrass meadows. After graduating from Suffolk University in 2015, Briana entered graduate school in the Department of Microbiology at the University of Tennessee in Knoxville, where she joined the lab of Dr. Frank Löffler and began studying the degradation of halogenated methanes.