Carbon tetrachloride (CT) and chloroform (CF) were transformed in batch reactor experiments conducted with anaerobic dechlorinating cultures and supernatant (ADC+S) harvested from continuous flow reactors. The Evanite (EV-5L) and Victoria/Stanford (VS-5L) cultures capable of respiring trichloroethene (TCE), 1,2-cis-dichloroethene (cDCE), and vinyl chloride (VC) to ethene (ETH) were grown in continuous flow reactors receiving an influent feed of saturated TCE (10 mM; 60 mEq) and formate (45 mM; 90 mEq) but no CT or CF. In all experiments, cells and supernatant were harvested from the chemostats and inoculated into batch reactors. Transformation of various concentrations of CT (0.86, 2.6, or 8.6 µM), CF (2.1 or 21.1 µM), dichloromethane (DCM; 23.1 µM), and TCE (50 µM) was examined. CT transformation was complete and exhibited pseudo-first order kinetics with CF as the primary measured transformation product in all treatments. Lesser amounts of DCM and carbon disulfide (CS₂) were measured leading to an overall mass balance of 20-40% of the original mass as CT accounted for. An analytical first order solution was developed to model CT degradation and product formation under multiple conditions. Cells poisoned with 50 mM sodium azide (NaN₃) catalyzed rapid and complete CT transformation suggesting a greater importance of redox active cofactors than live cells in the abiotic and cometabolic transformation. DCM and CS₂ however were not produced in the poisoned treatments. TCE and CT simultaneous transformation
occurred with an approximately two-fold increase in the CT degradation rate while maintaining complete TCE respiration to ETH. During the initial round of TCE respiration, the rate limiting step was VC to ETH, which was impacted by the presence of CT and CF. A subsequent addition of 50 µM TCE showed a substantial decline in the rates of reductive dechlorination owing to the inhibitory effects of long term exposure to CF. The results clearly demonstrate that transformation can be promoted by anaerobic dechlorinating cultures and supernatant not previously acclimated to CT and CF. However, abiotic reactions account for much of the observed transformation.

The role of CF inhibition on H₂ utilization by the culture was also explored. Sodium formate was provided as a rapid release substrate, providing H₂ as an electron donor. H₂ partial pressures were tracked throughout the course of the kinetic experiments. The rapid transformation of CT to CF made it not possible to determine if CT inhibited H₂ use by the anaerobic dechlorinating cultures. However, the rapid buildup and subsequent slow transformation of CF was found to reversibly inhibit H₂ consumption for homoacetogenesis. It was found that an aqueous CF concentration above 0.4 µM or 0.6 µM inhibited H₂ consumption by the EV-5L and VS-5L cultures, respectively. This result differed for the VS-5L culture when metabolizing TCE in the presence of CT and CF. The VS-5L culture consumed H₂ at CF concentrations as high as 1.3 µM. The culture may have been partially inhibited at CF concentrations greater than 0.6 µM, which is shown by slower consumption of H₂ than controls that did not contain CF. The results demonstrate that CF reversibly inhibits the consumption of H₂ by the anaerobic dechlorinating cultures, and that more research is required to determine if it is through a chemical inhibition or toxicity.
Transformation of Carbon Tetrachloride and Chloroform by Trichloroethene
Respiring Anaerobic Mixed Cultures and Supernatant

by
Kyle E. Vickstrom

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APPROVED:

Major Professor, representing Environmental Engineering

Head of the School of Chemical, Biological, and Environmental Engineering

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Kyle E. Vickstrom, Author
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CHAPTER 1: INTRODUCTION

Carbon tetrachloride (CT) and chloroform (CF) are toxic and recalcitrant groundwater pollutants with a long history of industrial use and improper disposal leading to widespread contamination (Doherty, 2000; Knox and Canter, 1996). Subsurface remediation of these compounds is of interest since they have been shown to deplete stratospheric ozone and are probable or known carcinogens (US Environmental Protection Agency, 2016). CT and CF are highest priority groundwater pollutants and are often found in mixtures with tetrachloroethene (PCE) and trichloroethene (TCE) due to their successive use as degreasers and dry cleaning agents (Bagley et al., 2000; Knox and Canter, 1996). Mixtures of chlorinated aliphatic hydrocarbons (CAHs) complicate bioremediation strategies using the organohalogen respiring bacteria (OHRB) *Dehalococcoides mccartyi* due to inhibition exerted by low concentrations of CT and CF on reductive dehalogenation (Bagley et al., 2000; He et al., 2005; Maymó-Gatell et al., 2001). This can lead to the buildup of the toxic metabolite vinyl chloride (VC), a known carcinogen (Agency for Toxic Substances and Disease Registry, 2006). In order for the bioremediation of mixtures of CT and TCE to be effective, further understanding of the dynamics of CT and CF transformation by OHRB needs to be developed.

The transformation of CT and CF in anaerobic environments involves parallel pathways catalyzed by biotic and abiotic mechanisms leading to the formation of dichloromethane (DCM), chloromethane (CM), methane (CH₄), carbon disulfide (CS₂), carbon monoxide (CO), carbon dioxide (CO₂), and formate (Cappelletti et al., 2012; Criddle and McCarty, 1991; de Best et al., 1998; Hashsham and Freedman, 1999). Reduced iron sulfides (Butler and Hayes, 2000; Kriegman-King and Reinhard, 1994, 1992), biogenic iron minerals (McCormick et al., 2002; McCormick and Adriaens, 2004), and metallo-coenzymes (Chiu and Reinhard, 1995; Gantzer and Wackett, 1991; Krone et al., 1989a, 1989b) can catalyze the abiotic transformation of
CT. Additionally, cometabolic CT and CF transformation has been found to occur in methanogenic (Bouwer and McCarty, 1983; Novak et al., 1998a), acetogenic (Egli et al., 1988; Hashsham and Freedman, 1999), sulfate reducing (de Best et al., 1998; Egli et al., 1987), iron reducing (Maithreepala and Doong, 2008; McCormick et al., 2002), and fermenting (Criddle et al., 1990b; Hashsham et al., 1995) environments. Product formation and degradation rates are controlled predominantly by the reductant and coenzymes present in the system. The addition of cofactors such as cobalamins greatly enhances degradation rates and shifts product formation away from chlorinated metabolites (Hashsham et al., 1995; Workman et al., 1997). CT and CF have been found to inhibit numerous anaerobic processes including methanogenesis (Bauchop, 1967a; Yu and Smith, 2000), autotrophy (Egli et al., 1988), acetogenesis (Liu et al., 2010; Scholten et al., 2000), and reductive dehalogenation (Bagley et al., 2000; He et al., 2005; Maymó-Gatell et al., 2001).

The ability to couple detoxification of TCE and CT would be advantageous for the in situ bioremediation of co-contaminated sites, despite that CT transformation is largely driven by abiotic processes. A recent study examined the transformation of CT and PCE in a continuous flow column bioaugmented with the Evanite (EV) culture, an anaerobic dechlorinating mixed culture enriched in D. mccartyi strains (Behrens et al., 2008; Marshall et al., 2014) that can transform PCE to ethene (ETH) (Yu et al., 2005). CT (0.015 mM) and PCE (0.1 mM) were transformed simultaneously, but the process was highly dependent on the electron donor (Azizian and Semprini, in press). When formate (1.5 mM) was provided, PCE was transformed to VC (20%) and ETH (80%) along with complete CT transformation to CF (20%) and an unknown fraction (80%). When the electron donor was switched to the fermenting substrate lactate (1.1 mM), PCE dehalogenation decreased with the formation of cDCE (48%), VC (36%), and ETH (7%). Long-term exposure to CF impacted propionate fermentation, thus reducing the amount of available H2 (Azizian and Semprini, in press).

While CT and CF transformation has been studied in numerous systems, a rigorous investigation with chlorinated ethene respiring cultures has not been undertaken owing to their inhibitory effects on reductive dehalogenation. The goals of the present study were to (1) determine the extent of CT and CF transformation by cells
and reduced media obtained from chemostats containing TCE respiring anaerobic mixed cultures not previously acclimated to these compounds; (2) develop a kinetic model for CT degradation and product formation; and (3) explore the dynamics of CT and TCE simultaneous transformation in highly controlled batch reactor systems.
CHAPTER 2: LITERATURE REVIEW

2.1 Carbon Tetrachloride

Carbon tetrachloride (CT) is an industrial chemical with a long history of use and environmental contamination. CT is a slightly soluble, semivolatile compound with a water saturation concentration of 810 mg/L and a dimensionless Henry’s coefficient ($H_{cc}$) of 0.949 at 20 °C (National Center for Biotechnology Information, 2016a; Staudinger and Roberts, 2001). It is also a lipophilic compound with a moderate octanol-water partitioning coefficient ($\log k_{ow} = 2.83$), which may allow it to cause damage to cellular membranes (National Center for Biotechnology Information, 2016a; Penny et al., 2010). CT is regulated under the National Primary Drinking Water Regulations (NPDWRs) by the United States Environmental Protection Agency (EPA) with a Maximum Contaminant Level (MCL) of 5 µg/L, and is also considered a probable human carcinogen (US Environmental Protection Agency, 2016). Furthermore, CT is fairly recalcitrant with an abiotic hydrolysis half-life of 7000 years in water at 20 °C (Mabey and Mill, 1978).

Carbon tetrachloride was manufactured and used extensively in the 20th century for a variety of applications until 1970 when it was officially banned from all United States consumer goods due to its toxicity and probable animal carcinogenicity.
(Doherty, 2000; Kroschwitz and Howe-Grant, 1991). These links have been well researched and established in numerous animal models, including microorganisms (Eastmond, 2008; WHO, 2004a). Before being banned in the United States, CT was used in a variety of ways as an industrial solvent, a dry cleaning agent, a grain fumigant, and as a component in floor waxes, furniture polishes, paints, and varnishes. However, after its removal from consumer goods, CT was still used heavily in portable fire extinguishers and as an intermediate in the production of chlorofluorocarbons (CFCs). As awareness grew about the larger environmental impacts of carbon tetrachloride and CFCs through the depletion of stratospheric ozone, CT was phased out under the Montreal Protocol with a complete ban on its production and use going into effect on January 1, 2000 (Doherty, 2000; Petrisor and Wells, 2008).

Due to a long history of industrial use and improper disposal, substantial CT contamination of soil and groundwater has occurred in the United States (Knox and Canter, 1996). Knox and Canter (1996) have listed CT as a “highest priority” groundwater contaminant due to its ubiquity at contaminated sites and its human and environmental toxicity (Knox and Canter, 1996). Contamination of groundwater with CT occurred most frequently with its use as an industrial degreaser and as a dry cleaning agent (Doherty, 2000). The high volatility and low solubility of CT generated an early misconception that the safest method for disposal was to remove the spent degreasing fluids to an uninhabited area where it was dumped onto dry earth and then ignited (Petrisor and Wells, 2008). In addition, early dry cleaning facilities were unable to recover the majority of their spent carbon tetrachloride, which then accumulated in soils and sediments. CT is a dense non-aqueous-phase liquid (DNAPL) with a density of 1.59 g/mL, and it will sink into groundwater aquifers where it dissolves slowly and creates plumes of contaminated water (National Center for Biotechnology Information, 2016a; Penny et al., 2010).

As a greater understanding of the environmental and health impacts of carbon tetrachloride was developed, CT was replaced predominantly by tetrachloroethylene (PCE) as the chlorinated solvent of choice (Petrisor and Wells, 2008). This led to further aquifer contamination before proper disposal practices were implemented,
creating complex contaminated sites that require more sophisticated remediation strategies (Bagley et al., 2000; Koenig et al., 2012).

2.2 Chloroform

Chloroform (CF), or trichloromethane, is a naturally occurring and synthetically produced chloromethane that is a degradation product of the reductive dechlorination of carbon tetrachloride. Chloroform is a slightly soluble (8,090 mg/L at 20 °C) DNAPL (1.48 g/mL) that is also semivolatile (H_{cc} = 0.126) (National Center for Biotechnology Information, 2016b; Staudinger and Roberts, 2001). Like CT, chloroform is also lipophilic with a high octanol-water partitioning coefficient (log k_{ow} = 1.97) (National Center for Biotechnology Information, 2016b). It is regulated by the United States EPA under the NPDWRs as a trihalomethane with an MCL of 80 µg/L, and is also considered a probable human carcinogen (US Environmental Protection Agency, 2016). Chloroform differs from carbon tetrachloride in that it is also of natural origin in addition to being synthetically produced. The total global environmental flux of chloroform is approximately 600,000 tonnes per year with greater than 90% of the emissions being natural in origin from marine and terrestrial environments (Gribble, 2004; Laturnus et al., 2002).

Figure 2.2: The chemical structure of chloroform (Source: Wikimedia Commons, Public Domain).
Chloroform has previously been used as an inhaled anesthetic, an extraction solvent, an intermediate in the production of refrigerants, in fire extinguishers, and as a fumigant (Agency for Toxic Substances and Disease Registry, 1997; Wawersik, 1997; WHO, 2004b). Currently, the predominant industrial uses of chloroform are its reaction with hydrogen fluoride in order to produce monochlorodifluoromethane (CFC-22), which is a precursor in the production of Teflon® (polytetrafluoroethylene), and as the reagent source of dichlorocarbene (\(\text{:CCl}_2\)) (Rossberg et al., 2000; Srebnik and Laloë, 2001). The majority of the CF that has entered the environment has been due to improper handling, storage, and disposal practices in addition to its formation as a Disinfection Byproduct (DBP) during the chlorination of drinking water and wastewater (Ivahnenko and Zogorski, 2006; Laturnus et al., 2002; McCulloch, 2003). Further groundwater contamination has occurred due to the degradation of carbon tetrachloride and subsequent formation of chloroform in anoxic environments (Kriegman-King and Reinhard, 1992; Semprini et al., 1992; Vogel et al., 1987). Along with CT, chloroform has been classified as a highest priority groundwater contaminant (Knox and Canter, 1996). Sites contaminated with CT usually contain CF as a transformation product, along with other chlorinated ethenes and ethanes, thus complicating possible remediation strategies (Semprini et al., 1992; Vogel et al., 1987).

2.3 Transformation of Carbon Tetrachloride

Despite the recalcitrance and long hydrolytic half-life for CT, it has the potential to form a broad range of transformation products in anaerobic environments through numerous biotic and abiotic processes (Fig 2.3). However, since the carbon in CT is fully oxidized, it is not readily degraded in aerobic environments, and therefore typically only undergoes transformation in anoxic environments (Criddle and McCarty, 1991; Penny et al., 2010; Semprini et al., 1992; Vogel et al., 1987). CT can be directly hydrolyzed to carbon dioxide (\(\text{CO}_2\)), and in reduced sulfide environments abiotically transformed to carbon disulfide (\(\text{CS}_2\)), which is easily mineralized to \(\text{CO}_2\). While the direct hydrolysis of CT is possible, the first step in reducing environments is typically a one-electron reduction catalyzed by redox-active electron shuttles giving a trichloromethyl radical (\(\text{CCl}_3\)) and a chloride ion (Criddle and McCarty, 1991; Penny
et al., 2010; Vogel et al., 1987). From here, the trichloromethyl radical can be reduced to form chloroform, undergo a second one-electron transfer forming dichlorocarbene (:\text{CCl}_2\text{)}, or dimerize and form hexachloroethane (HCE), which is only a minor pathway (Criddle and McCarty, 1991). Hexachloroethane can undergo dihaloelimination and be reduced to tetrachloroethene (PCE), which is then reductively dechlorinated by \textit{Dehalococcoides mccartyi} sp. to ethene (Criddle et al., 1986; Maymó-Gatell et al., 1997; Vogel et al., 1987). However, one of the more prevalent biochemical pathways involve chloroform being reductively dechlorinated to dichloromethane (DCM), which can then be further reduced to chloromethane (CM) and methane (MET) in highly reduced environments (Cappelletti et al., 2012; Criddle and McCarty, 1991; de Best et al., 1998). This reductive pathway is the hydrogenolysis of CT, similar to the reductive dechlorination of PCE and TCE. Both CF and the trichloromethyl radical can undergo one-electron transfers in which they are reduced to dichlorocarbene, which rapidly hydrolyzes to form carbon monoxide (CO) and formic acid (HCOOH), thus forming CO\textsubscript{2} (Cappelletti et al., 2012; Criddle and McCarty, 1991). Carbon dioxide is the most desirable end product for CT transformation due to the toxicity of the chlorinated intermediates and CS\textsubscript{2}. 
2.4 Abiotic Transformation Mechanisms

2.4.1 Electrolytic Transformation

There are numerous compounds that can facilitate the abiotic transformation of CT in reducing environments. In addition, it is possible to induce CT transformation abiotically by creating an electrochemical gradient using an electrolytic cell (Criddle and McCarty, 1991). Criddle and McCarty (1991) created a highly controlled
electrolysis cell consisting of an anode and cathode separated by a proton permeable membrane, which balanced the charge in the cell. Water was oxidized at a platinum diode, releasing molecular oxygen, electrons, and protons. The electrons then traveled along an external circuit to a silver cathode where they were used in reductive reactions. A potentiostat controlled the reduction potential at the cathode, which was deoxygenated by sparging with argon or zero-grade helium.

In order to test CT transformation at varying reduction potentials, Criddle and McCarty (1991) introduced 16 µmoles CT into the liquid phase of the cathode compartment and allowed it to equilibrate with the gas phase. A potential of -0.93 V or -1.15 V (versus the Ag/AgCl/Na₂SO₄ reference electrode) was applied to the electrolysis cell for a 6-hour period, and CT transformation was measured. At a reduction potential of -0.93 V, CT was transformed partially to CF (~15%) via hydrogenolysis and trace amounts of carbon monoxide. A reduction potential of -1.15 V was then applied over a 16-hour period, and 32 µmol CT were added to the system in two separate spikes, one at time zero and the second after 6 hours of electrolysis. CT was transformed again partially to CF and trace amounts of CO. However, dichloromethane was also detected due to the more reduced nature of the electrolysis cell. During this phase of the experiment, measurements of formate and released chloride ion (Cl⁻) were made in order to quantify the non-chlorinated products of CT transformation. They found that 23 µmol of CT were removed yielding 1.4 µmol CF, 100 µmol Cl⁻, and 17 µmol formate. Therefore in the more reduced conditions, reduction of CF accounted only for ~6% of the transformed CT while the reduction to formate accounted for ~75% of the CT added to the system (Criddle and McCarty, 1991).

In order to be reduced to formate and CO, CT must undergo a two-electron transfer to dichlorocarbene, which is thermodynamically favorable when an electron donor is oxidized that has a reduction potential of +0.2 V or less (Criddle and McCarty, 1991). Furthermore, the formation of the trichloromethyl radical is not favorable unless it is coupled with an electron donor that is oxidized at a potential less than or equal to -0.15 V. Thus, in reducing environments the formation of dichlorocarbene may be competitive with formation of the trichloromethyl radical leading to the presence of
parallel pathways for the reduction of CT (Criddle and McCarty, 1991). While more highly reduced environments can shift the CT transformation pathway away from CF production and towards formate and CO, it also allows for the formation of DCM, albeit in smaller amounts.

2.4.2 Reduced Iron and Iron Sulfide Compounds

Numerous studies have also been conducted to determine the ability of various reduced iron and iron sulfide compounds to transform CT abiotically. Kriegman-King and Reinhard (1992) explored the environmental parameters that govern the rate of CT transformation in heterogeneous environments containing sulfide (calculated as HS⁻), biotite, and vermiculite; the latter two are common iron-containing sheet silicate minerals found in subsurface environments. Previous research found that the transformation rate of CT would be a function of the mineral surface area (Kriegman-King and Reinhard, 1991). In their system, the mechanism of CT transformation was hypothesized to be affected by the sheet silicates and sulfide in three ways: 1) CT will undergo electron transfer with the ferrous iron in the sheet silicates and the oxidized iron will then be reduced by sulfide; 2) CT will react with the sulfide that becomes adsorbed to the sheet silicates; 3) sulfide will react with the dissolved iron released into solution by mineral dissolution, thus forming iron sulfides which can react with CT.

In order to test these hypotheses, Kriegman-King and Reinhard (1992) created treatments containing HS⁻ and biotite or vermiculite. CT transformation followed pseudo-first order kinetics and treatments containing mineral solids catalyzed faster CT transformation, with biotite being more reactive than vermiculite. From their rate analyses, the CT half-life with 1 mM HS⁻ was calculated to be 2600, 160, and 50 days for the homogeneous, vermiculite, and biotite systems, respectively. The transformation products measured were carbon disulfide (CS₂) (81-86%), chloroform (5-15%), formate (3-6%), and carbon monoxide (1-2%). CT transformation rates were also dependent on the type of mineral surface present, the solid surface area, and temperature. Biotite facilitated CT transformation at a higher rate than vermiculite did,
and rates increased with higher mineral surface areas and temperatures. However, CT transformation rates were independent of pH and [HS⁻] above 0.5 mM.

This was followed by experiments exploring the capacity for pyrite to transform CT in aqueous environments (Kriegman-King and Reinhard, 1994). Pyrite (FeS₂) is an iron sulfide mineral that is commonly found in sulfate-reducing environments. Upon transformation with CT, an iron oxide coating forms on the surface of the mineral deactivating it. However, sulfide may be able to regenerate the pyrite surface through a reductive dissolution of the iron oxide coating (Dos Santos Afonso and Stumm, 1992; Peiffer et al., 1992). Kriegman-King and Reinhard (1994) found that pyrite facilitates the transformation of CT in both aerobic and anaerobic environments, and that greater than 90% of the initial CT mass was transformed within 12-36 days in the presence of 1.2-1.4 m²/L pyrite at 25 °C. The anaerobic environment facilitated more rapid CT transformation than did the aerobic environment, but approximately 50% of the CT mass was transformed to chloroform anaerobically while >70% was transformed to CO₂ under aerobic conditions. Furthermore, both environments saw small amounts of CS₂ and formate as transformation products. The CT degradation data fit a zero order model (R² ~ 0.9) better than a first order model (R² ~ 0.7) across all treatments, showing a diminished dependence between transformation and CT concentration. A zero order dependence on CT concentration is expected when a heterogeneous reaction is controlled by surface chemistry rather than by diffusion (Goldhaber, 1983), such as when the pyrite surface is catalyzing the transformation of CT.

Another common soil mineral capable of CT transformation is iron (II) sulfide (FeS). Butler and Hayes (2000) investigated the transformation kinetics of nine different halogenated aliphatic compounds including CT by FeS solids in anaerobic aqueous solutions. A uniform set of experimental conditions were created in order to control against inconsistent results found by other researchers in which free sulfide and bound Fe(II) could not catalyze dechlorination (Doong and Wu, 1992). Butler and Hayes (2000) conducted their kinetic experiments for up to 4 months in 5 mL flame-sealed glass ampules with 10 g/L FeS (0.005 m²/g), pH of 8.3, and an ionic strength of 0.1 M. CT transformation was complete and followed pseudo-first order kinetics with an observed rate constant of (6.39 ± 0.79) x 10⁻² h⁻¹ with 46% of the mass recovered as
CF. They did not measure any additional products from CT degradation, but hypothesized that a portion of the unknown mass could be measured as CS$_2$. Devlin and Müller (1999) also found that freshly precipitated FeS catalyzed the transformation of CT to CF and CS$_2$ in an approximately 2:1 ratio at a near neutral pH (Butler and Hayes, 2000; Devlin and Müller, 1999). Additional research has shown that zero valent iron (ZVI) and nano-scale ZVI (nZVI) can effectively transform CT to less chlorinated and non-chlorinated products, and has been employed extensively for the remediation of numerous halogenated aliphatic compounds (Gillham and O’Hannesin, 1994; Huo et al., 2015; Jiao et al., 2009; Schreier and Reinhard, 1994).

2.4.3 Metallo-coenzyme Catalyzed Transformation

Numerous mammalian and microbial enzymes contain transition-metal coenzymes as prosthetic groups such as cobalamins (Vitamin B$_{12}$ derivatives containing cobalt), heme (an iron porphyrin complex), and cytochrome F$_{430}$ (nickel-centered porphinoid found only in methanogens) (Chiu and Reinhard, 1995). These compounds, along with biomimetic cobalt macrocycles (Ukrainczyk et al., 1995), have been implicated in the abiotic transformation of CT in anaerobic environments. Gantzer and Wackett (1991) conducted early research on the transformation of carbon tetrachloride and polychlorinated ethylenes and benzenes catalyzed by vitamin B$_{12}$, coenzyme F$_{430}$, and hematin in a titanium (III) citrate solution (Gantzer and Wackett, 1991). All three transition metal co-factors are found in anaerobic bacteria cultures, and previous research with methanogens has suggested that the reduction of CT to methane is mediated nonspecifically by cobalamin, F$_{430}$, or by both co-enzymes (Krone et al., 1989a, 1989b). In order to directly test the role of the metallo-coenzymes in the transformation of CT, Gantzer and Wackett (1991) established anaerobic reactor vials containing 2.2 µmol of CT, 27 µmol titanium (III) citrate as the reductant, and 46 nmol of the tested co-factor at pH 8.2 (Gantzer and Wackett, 1991; Krone et al., 1989a, 1989b). They found that all three co-factors mediated the reductive dechlorination of CT to chloroform following pseudo-first order kinetics, but they did not track product distribution beyond the formation of CF. Kinetic analyses determined that coenzyme F$_{430}$ mediated the fastest transformation of CT followed by vitamin B$_{12}$ and hematin...
with pseudo-first order rate constants of $100 \pm 4 \text{ h}^{-1}$, $74 \pm 4 \text{ h}^{-1}$, and $2.4 \pm 0 \text{ h}^{-1}$, respectively.

Furthering the work of Gantzer and Wackett (1991), Chiu and Reinhard (1995) focused specifically on the transformation of CT catalyzed by vitamin B$_{12}$ and hematin in an aqueous titanium (III) citrate solution over a range of pH values (Chiu and Reinhard, 1995). They found that vitamin B$_{12}$ was a more stable and effective catalyst than hematin for CT transformation. Hematin had a fairly low turnover number with 27 CT molecules transformed per molecule of hematin deactivated at pH 8.0 and 42 CT molecules transformed per molecule of hematin deactivated at pH 9.9. This same deactivation behavior was not observed with vitamin B$_{12}$, which maintained its transformation capacity over time. By conducting a spectroscopic analysis of the vitamin B$_{12}$ spectrum, Chiu and Reinhard (1995) found that the titanium (III) citrate solution was able to instantaneously reduce B$_{12a}$ [Co(III)] to B$_{12r}$ [Co(II)] and B$_{12s}$ [Co(I)]. The more reduced forms of cyanocobalamin are responsible for providing the reducing power required for CT transformation. As B$_{12s}$ is oxidized to B$_{12r}$ during the redox process, Ti(III) will instantly reduce it back to B$_{12s}$ thus owing to the high transformation capacity of vitamin B$_{12}$ (Chiu and Reinhard, 1995). Increasing concentrations of vitamin B$_{12}$ catalyzed faster transformation of CT exhibiting zero order kinetics with respect to $[\text{CCl}_4]_{\text{aq}}$ and first order kinetics with respect to vitamin B$_{12}$. However, chloroform was the primary transformation product ranging from 58% (pH 7.3) to 95% (pH 10.3), and the presence of vitamin B$_{12}$ or hematin did not influence the yield. Instead, CF production was a function of pH, titanium (III) concentration, and organic content, and increased as these factors increased. The reducing agents studied here play a significant role in the transformation of CT, but the results obtained from one reductant system cannot be extrapolated to a system that uses a different reducing agent due to additional factors that control CT transformation and product formation.

This was studied in depth by Lewis et al. (1996) who looked at the cobalt corrin-catalyzed transformation of CT using titanium (III) citrate, dithiothreitol (DTT), and the S$_2$-/cysteine reducing pair as reductants (Lewis et al., 1996). Cobalt corrins are heterocyclic compounds that contain the transition metal cobalt in various oxidation
states. The Co-corrins used were vitamin B₁₂ (cyanocobalamin), cobinamide dicyanide (Cd), and aquocobalamin (AqC). In these reducing systems, the different reductants produce Co-corrins with Co at different oxidation states. Ti(III) produces the most reduced corrin [Co(I)] from Co(III) while DTT and S²⁻/cysteine produce a less reduced form, Co(II). In the Ti(III)/Co(I) system, CT was transformed completely and predominantly by hydrogenolysis with the primary products being chloromethane (CM) and methane (CH₄), which differed from previous results with this system (Chiu and Reinhard, 1995). CM constituted 44-71% of the original CT mass with 7-17% methane and smaller amounts of CO, CO₂, and nonvolatile products. The highly reduced nature of the Co(I) corrins likely catalyzed the reduction of CT beyond CF and DCM leading to the accumulation of CM and CH₄. The thiol reductants, DTT and S²⁻/cysteine, produced more halogenated products, possibly due to the less reduced nature of the Co(II) in the corrins. DTT and Co(II) also transformed CT completely with the primary transformation products being DCM (20-31%), carbon monoxide (11-39%), and nonvolatile products (17-47%). The S²⁻/cysteine reductants were the least effective at transforming CT, with AqC unable to fully degrade the initial CT present (16% remaining). The primary transformation products were CF (0-27%), DCM (0-15%), CS₂ (3-4%) and nonvolatile products (23-62%). It seems that Co(II) is not capable of catalyzing reductive dehalogenation beyond DCM, or does so very slowly. However, the most important determining factors in the transformation pathways and products of CT degradation are the reductants and their interactions in the reducing environment, shown clearly by the differences between the Ti(III) and thiol environments.

Another example of a transition metal catalyzing CT transformation is the use of homogeneous and mineral-supported biomimetic cobalt macrocycles in aqueous solution (Ukrainczyk et al., 1995). A biomimetic Co macrocycle is a cyclic macromolecule containing cobalt that catalyzes specific biochemical processes such as reductive dehalogenation. This research is of interest because it explores the use of mineral substrates for the catalyst to adsorb to, thus more closely representing groundwater environments for possible in situ remediation. In order to get the Co macrocycle to adsorb to the mineral surface, the compound must permanently carry a charge. Two Co macrocycles were used in this study, cobalt tetrakis (N-methyl-4-
pyridiniumyl) porphyrin (CoTMPyP) cation and cobalt tetrasulfophthalocyanine (CoPcTs) anion in solution and supported on high surface area minerals. In homogeneous experiments, a Co macrocycle (0.1 mM) was added to an anoxic solution containing DTT (0.1 M) and CT (1.0 mM). The heterogeneous experiments included a silica supported CoTMPyP, a CoPcTs-layered double hydroxide, CoTMPyP-hectorite and CoTMPyP-fluorohectorite as mineral substrate surfaces for adsorption. Short-term experiments were conducted over a two-hour time period, in which CF and DCM accounted for less than 30% of the CT transformed, with the remaining mass as nonvolatile products. CF was the only detectable product in the heterogeneous experiments even at reaction times greater than two hours. CoTMPyP was more active relative to its heterogeneous catalysts, while the supported (heterogeneous) CoPcTs degraded more CT than the homogeneous catalyst, which was deactivated due to aggregation. Ukrainczyk et al. (1995) hypothesize that the lack of DCM formation in the heterogeneous systems is most likely due to a change in the reduction potential of the Co macrocycles to a more positive value relative to the aqueous macrocycles, which occurred because of adsorption onto the mineral surfaces.

Long-term experiments were also conducted over a period of three days in order to test the catalytic activity of the homogeneous versus the mineral bound Co macrocycles. These experiments were conducted with lower amounts of catalyst (0.002 mM) and higher CT concentrations (2.3 and 4.6 mM) in order to examine catalyst stability under deactivating conditions. CoPcTs catalyzed the fastest CT transformation at 2.3 mM CT with a pseudo-first order rate constant 0.75 d⁻¹. The catalysts did begin to lose activity after one day of the experiment, however it appears this was not due to adsorption onto the mineral surface. The silica-supported CoTMPyP ($k_{obs} = 0.57$ d⁻¹) was more active than the homogeneous catalyst ($k_{obs} = 0.49$ d⁻¹), suggesting that adsorption onto the physical substrate helped stabilize the catalyst and provide activity over longer time scales. It is important to note that CT transformation occurred at very high concentrations (353 and 706 mg/L), which normally would inhibit microbial mediated CT degradation. For comparison, the concentrations of carbon tetrachloride at the Hanford site in Richland, WA, a highly contaminated groundwater site, averages between 1-5 mg/L CT (Truex et al., 2001).
2.5 Microbial Carbon Tetrachloride Transformation

A multitude of microorganisms have been implicated in the transformation of carbon tetrachloride in anoxic and anaerobic environments. However, despite the potential energy to be gained by using CT as a terminal electron acceptor, only cometabolic transformation has been found to occur and a microbe able to grow on CT as sole carbon and energy source is yet to be discovered (Penny et al., 2010). The cometabolic nature of CT degradation and the multitude of biochemical pathways through which CT is transformed complicate the characterization and understanding of the transformation mechanisms carried out by different pure and mixed cultures. Furthermore, CT is a biocidal compound that has been shown to strongly inhibit a variety of environmentally significant metabolic processes such as methanogenesis (3 \( \mu \text{M} \)), reductive dehalogenation (1-10 \( \mu \text{M} \)), and autotrophy (80 \( \mu \text{M} \)) (Bagley et al., 2000; Bauchop, 1967a; Egli et al., 1988; He et al., 2005). Since CT is also a lipophilic compound with a moderate octanol-water partition coefficient (\( \log k_{ow} = 2.83 \)), it may also cause damage to cellular membranes, further impacting the growth of microorganisms (National Center for Biotechnology Information, 2016a; Penny et al., 2010). CT degradation can catalyze the formation of intermediate compounds, such as highly active radicals, that can have inhibitory or toxic effects on cellular processes. Despite these complications, a large consortium of microbial cultures contributes to the transformation of CT in methanogenic, acetogenic, sulfate (\( \text{SO}_4^{2-} \)) reducing, iron (Fe) reducing, nitrate (\( \text{NO}_3^- \)) reducing, dechlorinating, and fermenting environments. However, CT transformation solely by cometabolism is relatively slow compared to degradation catalyzed by abiotic mechanisms. Numerous studies have shown that microbial cultures help facilitate rapid and complete CT transformation by producing and excreting small extracellular compounds (Doong et al., 2014; Hashsham et al., 1995; Hashsham and Freedman, 1999; McCormick et al., 2002; Novak et al., 1998a, 1998b; Workman et al., 1997). As shown by the abiotic transformation experiments, environmental conditions are a strong determinant of the CT metabolite product distribution, which continues to hold in microbial systems.
2.5.1 Methanogenic Environments

The first experiments with microorganisms capable of carrying out the transformation of CT were conducted using a methanogenic mixed culture enriched from anaerobic digester sludge (Bouwer and McCarty, 1983). Experiments were conducted in batch reactors and continuous-flow columns packed with 3 mm diameter glass beads in order to test the biological transformation of low concentrations of numerous 1- and 2-carbon halogenated aliphatic compounds. Bouwer and McCarty (1983) also utilized \[^{14}C\] carbon tetrachloride in order to completely track product formation by the methanogenic culture. Batch cultures were established with 45 µg/L CT (0.29 µM), which was completely degraded by the methanogenic culture after 16 days, while sterile controls showed no appreciable decline in CT concentration. Experiments conducted with \[^{14}C\] CT found that 99 ± 2% of the radioactivity associated with the \(^{14}CCl_4\) was recovered as \(^{14}CO_2\).

Experiments were also conducted in a continuous-flow column with a two-day retention time containing a mixture of chlorinated aliphatic compounds including CF and CT (Bouwer and McCarty, 1983). Acetate served as the electron donor to the methanogenic culture, and CT was added at a concentration of 17 µg/L (0.11 µM) in the column influent. The column did not require an acclimation period to CT, and it was fully transformed with >99% removal under steady state operating conditions. Transformation of \(^{14}CCl_4\) resulted in 99 ± 2% of the radioactivity recovered as \(^{14}CO_2\) in the column effluent. The absence of the formation of chlorinated metabolites in this study is desirable. However, the concentrations examined are an order of magnitude below the concentration found to be inhibitory to the growth of methanogens (3 µM CT) (Baucho, 1967a). Higher concentrations of CT that are inhibitory to methanogens could possibly shift the transformation pathways to produce chlorinated products (CF and DCM) in addition to CO\(_2\).

CT transformation by pure cultures of the methanogens *Methanosarcina barkeri*, *Methanosarcina thermophila*, and *Methanosaeta concillii* has also been studied extensively (Novak et al., 1998a, 1998b). The three species were chosen due to their differing abilities to grow on hydrogen (H\(_2\)) and CO\(_2\) (hydrogenotrophic methanogenesis): *M. barkeri* grows readily on H\(_2\)-CO\(_2\) with no acclimation period; *M.*
thermophila grows poorly on H$_2$-CO$_2$ after an approximately 9-day lag period; *M. concillii* is unable to grow on H$_2$-CO$_2$. Due to the gaseous nature of H$_2$, it is difficult to add to and control in subsurface environments. However, elemental iron (Fe$^0$) can serve as an electron donor for the hydrogenolytic transformation of CT to CF (Johnson et al., 1996), and for the growth of methanogens since it produces H$_2$ through the corrosion of Fe$^0$ to Fe$^{2+}$. When incubated in the presence of Fe$^0$, all three species exhibited faster CT degradation compared to treatments containing cells only, cells and H$_2$, methanol (MeOH) fed cells, and Fe$^0$ only systems. CF was the primary transformation product, and in systems that degraded CF, about 50% was transformed to DCM. Despite the inability of *M. thermophila* to grow on H$_2$-CO$_2$ during the time frame of these experiments, it exhibited the most rapid transformation of CT and CF compared to the other two species.

Due to the enhanced transformation of CT and CF found by treatments containing cells and Fe$^0$, the researchers hypothesized that an excreted biomolecule might be responsible for some or all of the enhanced CT and CF transformation. The supernatant from treatments where organisms were grown in the presence of iron were exchanged with those where organisms were grown only in media. There was a significant difference in the rates of CT transformation between treatments that contained cells and supernatant grown in the presence of Fe$^0$ versus those that did not. These results prompted the researchers to explore the transformation of CT and CF solely by the supernatant from *M. thermophila* grown in the presence and absence of Fe$^0$ (Novak et al., 1998b). Treatments in which the supernatant was filtered with a 0.22 µm filter did not alter the patterns of CT or CF transformation. Additionally, CT transformation was rapid and complete in supernatants from cultures grown both in the presence and absence of Fe$^0$. However, the supernatant from cultures grown without Fe$^0$ were not able to subsequently catalyze the transformation of CF. There was also no significant difference in CT transformation in treatments that were autoclaved versus those that weren’t; however, no CF transformation occurred in autoclaved treatments. It was hypothesized that the excreted biomolecule catalyzing CT and CF transformation could possibly be a protein structure with a metal center. The metal center catalyzing CT transformation would not be affected by heat treatment while the protein would be.
The excreted biomolecule also had a high transformation capacity with no observed reduction in rates after multiple spikes of CT dechlorination totaling approximately 2.1 µmol/bottle over 12 days.

Characterization of the cellular exudates from *M. thermophila* showed that they were from the < 10 kDa (Dalton) molecular weight fraction, which is too small to be attributed to proteins. Fractionation through a C_{18} column showed elevated levels of iron, zinc, and cobalt, which led researchers to hypothesize that the exudates are porphorinogen-type molecules containing these three transition metals (Koons et al., 2001). The iron and cobalt containing exudates are likely heme and cobalamins (vitamin B_{12} homologs) since the enhanced dechlorination activity of the exudates at different pH and temperature values matched results found previously with these compounds (Assaf-Anid et al., 1994; Chiu and Reinhard, 1995; Holliger et al., 1992; Krone et al., 1989b). Additionally, the zinc-containing exudates could likely be novel Zn porphorinogens capable of carrying out dechlorination reactions. In order to test this theory, two Zn porphorinogens and a model quinone were tested for their dechlorination activity. Both of the Zn porphorinogens catalyzed rapid and complete CT transformation while the quinone was only capable of very slow transformation. This confirms the possibility that the unknown Zn-containing cellular exudates from *M. thermophila* could be novel Zn porphorinogens. The ability of methanogenic bacteria to degrade CT and CF through small extracellular molecules demonstrates the role that microbes play in facilitating CT degradation by abiotic mechanisms.

### 2.5.2 Acetogenic Environments

Complete and rapid transformation of CT by pure cultures of *Acetobacterium woodii* and *Clostridium thermoaceticum* has also been found to occur. These two species are acetogenic bacteria that produce acetate from CO_{2} with fructose (*A. woodii*) or glucose (*C. thermoaceticum*) salts serving as electron donors (Egli et al., 1988). Egli et al. (1988) found that *A. woodii* and *C. thermoaceticum* degraded approximately 80 µM CT completely within three days with CF forming as a transient intermediate (maximum 20 µM) and 8 µM DCM. Experiments were also conducted with ^14^CCl_{4} in
order to track product formation and determine if the radioactivity (\(^{14}\text{C}\)) introduced into acetate from \(^{14}\text{CCl}_4\) proceeded through \(^{14}\text{CO}_2\) or through the more reduced C1 compounds CF and DCM. Growing cultures of \(A.\ woodii\) that were spiked with 40 µM \(^{14}\text{CCl}_4\) converted 92% of the initial mass to nonhalogenated products. Ninety-nine percent of the original radioactivity was recovered with 13% \(\text{CO}_2\), 38% acetate, 10% pyruvate, 8% DCM, 6% cellular material, 10% hydrophobic material, and 14% an unknown fraction. The high percentage of nonhalogenated product formation shows that the majority of the CT is undergoing a substitutive transformation to \(\text{CO}_2\), which is then used for acetate production or is incorporated into cells. Pulse experiments were conducted to measure the fate of radioactivity from \(^{14}\text{CCl}_4\). After 1 minute there was eight times more \(^{14}\text{C}\) label found in \(\text{CO}_2\) than in acetate and little radioactivity found in CF. It was hypothesized that \(\text{CO}_2\) is the first intermediate in CT degradation by \(A.\ woodii\) and that it is subsequently transformed to acetate via the Acetyl-CoA pathway.

Based on work with two cultures lacking the Acetyl-CoA pathway (\(\text{Desulfobacter hydrogenophilus}\) and an autotrophic nitrate-reducing bacterium) that were unable to transform CT, Egli et al. (1988) hypothesized that a correlation exists between CT degradation and microbes containing this biochemical pathway. While this pattern is not definitive, microbes that contain the Acetyl-CoA pathway contain high levels of corrinoids (e.g. vitamin B\(_{12}\)) (Dangel et al., 1987; Krautler, 1988), which have been shown to catalyze CT transformation independent of microbial culture (Chiu and Reinhard, 1995; Gantzer and Wackett, 1991; Lewis et al., 1996; Ukrainczyk et al., 1995). In order to test the mechanisms by which CT is transformed, live cells, cell-free extracts, and autoclaved cells of \(A.\ woodii\) were tested (Egli et al., 1990). It was found that CT degradation by cell-free extracts was similar to that by live cells, but the transformation occurred at a slower rate. Furthermore, when \(A.\ woodii\) cells were autoclaved, the reductive dechlorination of CT to CF was partly abolished while substitutive transformation to \(\text{CO}_2\) was unaffected. It was also found that chloroform was oxidized by both live and autoclaved \(A.\ woodii\) cells to \(\text{CO}_2\) at a rate about 20 times less than the transformation of CT to \(\text{CO}_2\), showing that CF is not an intermediate in this step. Since it is likely that CT reductive dehalogenation to CF is catalyzed by metallo-coenzymes in the presence of \(A.\ woodii\), it is strange that this pathway would
be partly abolished when cells were autoclaved. It is possible that under these conditions, reductive dehalogenation depends on protein-mediated electron transport from \( \text{H}_2 \) rather than on electrons supplied by cysteine and sulfide from the incubation media.

Two teams of researchers further explored the role of vitamin \( \text{B}_{12} \) homologs in CT transformation by \( A. \text{woodii} \) (Hashsham and Freedman, 1999; Stromeyer et al., 1992). While working with aquocobalamin [\( \text{Co(II)} \)], Stromeyer et al. (1992) found that whole cells of \( A. \text{woodii} \) alone and aquocobalamin alone demonstrated similar CT transformation rates and products. However, as the concentration of aquocobalamin was increased, the percentage of chlorinated products decreased. After 20 hours, DCM was measured at the following amounts (% of initial \( ^{14}\text{CCl}_4 \)) in the different treatments: live cells, 17%; autoclaved cells, <0.5%; 15 nmol aquocobalamin, 10%; 74 nmol aquocobalamin, 1%. All of the treatments contained less than 3% CF with the major chlorinated product being DCM. The treatment containing 74 nmol aquocobalamin also showed the highest percentage of \( ^{14}\text{CO}_2 \) (29%) compared to the cells (8-9%) and the 15 nmol aquocobalamin (8%). These results show that cellular vitamin \( \text{B}_{12} \) could be responsible for all of the CT reactions that are catalyzed by cells. However, reductive dechlorination of CF to DCM and CM could be driven by cellular processes. As the reducing power of a system is increased by higher concentrations of vitamin \( \text{B}_{12} \) and its homologs, two electron transfers and the substitutive transformation of CT to \( \text{CO}_2 \) becomes more thermodynamically favorable, thus shifting the product distribution in that direction (Criddle and McCarty, 1991).

Hashsham and Freedman (1999) demonstrated that complete transformation of very high aqueous concentrations of CT (470 \( \mu \text{M} \); 72 mg/L) can be achieved by \( A. \text{woodii} \) over a short time span (2.5 days) when the culture was amended with 10 \( \mu \text{M} \) hydroxycobalamin (\( \text{OH-Cbl; vitamin B}_{12a}; \text{Co(III)} \)) and 25.2 mM fructose. The addition of \( \text{OH-Cbl} \) and fructose to both live and dead \( A. \text{woodii} \) cells and medium catalyzed a 30-fold increase in CT transformation over those that did not receive \( \text{OH-Cbl} \). In treatments that received \( \text{OH-Cbl} \) but were missing either live \( A. \text{woodii} \) cells or fructose, a 5-fold increase in CT transformation was still observed. In addition to increasing the rate of CT transformation, \( \text{OH-Cbl} \) and fructose also shifted the
transformation products to more non-chlorinated compounds. Treatments containing live *A. woodii* along with OH-Cbl and fructose showed only the transient formation of CF (3.4% after 2.5 days) without the subsequent accumulation of DCM. In this treatment, 31% of the original $^{14}\text{CCl}_4$ was recovered as $^{14}\text{CO}_2$ produced through the carbon monoxide (CO) pathway. However, the abiotic formation of carbon disulfide (CS$_2$) was observed due to the reduced sulfide media. This observation differed from previous research conducted with *A. woodii* and could explain some of the unaccounted mass (Egli et al., 1990, 1988; Stromeyer et al., 1992). These results suggest that live cells play an important role in shaping the product distribution from CT. Treatments with live *A. woodii*, fructose and OH-Cbl produced only 13% CS$_2$ while the other treatments containing OH-Cbl but lacking either live cells or fructose produced approximately 30% CS$_2$. In contrast, live *A. woodii* without both OH-Cbl and fructose catalyzed incomplete CT transformation after 13 days but produced only 1.9% CS$_2$. It is clear that OH-Cbl is an important component in catalyzing the transformation of CT, but in order to maximize the percentage of desirable end products an additional electron donor such as fructose is required.

2.5.3 Sulfate Reducing Environments

Sulfate (SO$_4^{2-}$) reducing bacteria (SRB) are commonly found in groundwater systems due to the high occurrence of SO$_4^{2-}$ in the environment. The reduced sulfide (HS$^-$ and S$^2-$) produced by SRB can catalyze the transformation of CT and CF (Butler and Hayes, 2000; Kriegman-King and Reinhard, 1994, 1992). Pure culture studies conducted with *Desulfobacterium autotrophicum* showed that complete CT transformation (80 µM) could occur within 18 days with approximately 70% of the mass measured as CF and DCM (Egli et al., 1988, 1987), with the remainder as unidentified water-soluble products (Egli et al., 1990). The accumulation of such high percentages of CF and DCM is undesirable. Heterotrophic growth of *D. autotrophicum* was unaffected by the presence of CT and CF, but completely inhibited autotrophic growth of the culture. Furthermore, autoclaving *D. autotrophicum* cells abolished dechlorination activity suggesting the importance of heat labile compounds, such as cytochromes, that can catalyze CT transformation. Cytochromes, which consist of
heme bonded to a protein group, of the b and C type have been reported in *D. autotrophicum* (Widdel, 1987).

In order to study the transformation of CT in a system that more closely replicated groundwater environments, de Best et al. (1998) constructed a continuous flow sulfate reducing anaerobic packed-bed reactor and operated it under different conditions of electron donor, CT, and SO$_4^{2-}$ (de Best et al., 1998). The packed bed reactor was inoculated with digested sludge (20% v/v) from a wastewater treatment plant and initially fed 510 µM SO$_4^{2-}$, 2.5 µM CT, and 1 mM acetate as electron donor. CT was completely transformed with 51.8% as CF (1.3 µM), 15.9% as DCM (0.44 µM), and the remainder unknown (32.3%; 0.77 µM). After 23 weeks of operation methane was detected in the reactor at 672 µM and SO$_4^{2-}$ reduction was reduced to 350 µM (68.6%). CT transformation (2.5 µM) was still complete, but a dramatic shift in the product distribution coincided with the onset of methanogenesis. CT was no longer reductively dechlorinated to CF and DCM and instead was completely mineralized to CO$_2$, which had been found previously in a methanogenic culture but only at very low (0.11 µM) CT concentrations (Bouwer and McCarty, 1983). When sulfate concentrations were increased to 5 mM, methanogenesis ceased and CT transformation continued to be complete, but the product distribution shifted back to chlorinated compounds (CF, 46.0%; DCM, 13.1%). The results suggest that high concentrations of SO$_4^{2-}$ don’t impact the transformation of 2.5 µM CT, but the chemical dynamics play a large role in the formation of products.

Further experiments were conducted in the reactor with 1 mM SO$_4^{2-}$ and acetate, and various CT concentrations (2.5, 11.8, 10.9, 29.6, and 56.6 µM). At all concentrations except 56.6 µM, CT transformation was complete and formed between 34-46% CF and 3-21% DCM. At concentrations above 11.8 µM CT, DCM formation was partially inhibited due likely to the higher concentration of CF formed. At 56.6 µM CT, sulfate reduction was inhibited and 42% (23.5 µM) of the original CT was measured in the reactor effluent with no formation of CF and DCM. However, 114 µM chloride (Cl$^-$) was measured, suggesting that the transformed CT was completely dechlorinated either to CS$_2$ or CO$_2$. In order to test the role of the sulfate reducing bacteria, selective inhibitors were introduced to culture harvested from the packed bed
reactor and inoculated into batch systems. Molybdate and vancomycin, which selectively inhibit sulfate reduction and gram-positive bacteria, respectively, were both found to completely inhibit CT transformation, showing that the $\text{SO}_4^{2-}$ reducing bacteria play an important role in CT degradation. Further inhibition experiments were conducted in order to test the inhibition of CT on sulfate reduction. Sulfate reduction was not impacted up to 18.2 $\mu\text{M}$ CT, was partially inhibited from 20-38.2 $\mu\text{M}$ CT, and was completely inhibited above 38.2 $\mu\text{M}$ CT. This is approximately half of the CT concentration found to inhibit the autotrophic growth of $\text{D. autotrophicum}$ (Egli et al., 1988). At CT concentrations where partial or full inhibition of $\text{SO}_4^{2-}$ reduction occurred, CT transformation was substantially impacted with no degradation occurring at 76.3 $\mu\text{M}$ CT. These results show the high sensitivity of SRB to increasing concentrations of CT, but also confirm the ability of these subsurface microbes to readily transform CT to less chlorinated products.

2.5.4 Iron Reducing Environments

Iron (III) oxides are ubiquitous in subsurface environments where their reduction to Fe(II) by dissimilatory iron-reducing bacteria (DIRB) such as $\text{Geobacter metallireducens}$, $\text{G. sulfurreducens}$, $\text{G. lovleyi}$, $\text{Shewanella putrefaciens}$, $\text{S. alga}$, and $\text{Klebsiella pneumoniae}$ can be coupled to the oxidation of organic matter (Bae and Lee, 2012; Caccavo et al., 1994; Li et al., 2009; Lovley et al., 1987; Sung et al., 2006). The DIRB form biogenic iron compounds such as magnetite ($\text{Fe}_3\text{O}_4$), maghemite ($\gamma\cdot\text{Fe}_2\text{O}_3$), goethite ($\alpha\cdot\text{FeO(OH)}$), and dissolved Fe(II) which can catalyze the subsequent transformation of CT and is enhanced by the addition of quinones such as anthraquinone-2,6-disulfonate (AQDS) (Amonette et al., 2000; Doong et al., 2014; McCormick et al., 2002).

McCormick et al. (2002) examined the relative kinetics of the biotic ($\text{G. metallireducens}$) and abiotic (magnetite) transformation of CT under iron reducing conditions (McCormick et al., 2002). Biogenic magnetite produced by $\text{G. metallireducens}$ was collected magnetically, and was washed and sonicated in order to remove cells and cell debris. Kinetic experiments revealed that the primary
transformation product of CT was CF ranging from 15-30% and 35-45% in the biotic and abiotic experiments, respectively; DCM and CM formation was not observed in either system. In the biotic systems containing *G. metallireducens*, CT transformation was pseudo-first order. The observed CT transformation rates ($k_{\text{CT,obs}}$) decreased as the initial aqueous CT concentration increased. Additionally, as *G. metallireducens* biomass (mg-protein/L) increased, $k_{\text{CT,obs}}$ increased as well. The abiotic experiments with the biogenic magnetite found that a linear relationship existed between the surface area loading ($m^2/L$) of the solids and observed pseudo-first order rates with a concomitant increase in rates as magnetite loading increased. This suggests a strong dependence on available particle surfaces for CT transformation. In order to assess the relative contribution of biotic and abiotic mechanisms, experiments were conducted with cells and magnetite present together. The yield of total protein and magnetite surface area formed during *G. metallireducens* growth were used as a comparison for the biotic/abiotic contributions, leading to the estimate that the mineral-mediated CT transformation was 60-260 fold faster than the biotic reaction.

Characterization of the biogenic magnetite particles by transmission electron microscopy (TEM), selected area electron diffraction (SAED), and X-ray diffraction (XRD) found that they consisted predominantly of ultra-fine particles ranging in size from 5-30 nm in diameter with rings and spots consistent with randomly oriented magnetite crystals. Further experimentation was conducted with the biogenic magnetite focusing on the product formation and reaction mechanisms in sulfide-free, purely abiotic systems (McCormick and Adriaens, 2004). Once again, CF (46%) was the main transformation product of CT, but CO (38%), CH$_4$ (9%) and trace amounts of PCE (0.1%) were also measured. In order to determine if free radical or carbene intermediates were formed during CT transformation, a radical trapping experiment was conducted using 2,3-dimethyl-2-butene (DMB) as a radical/carbene trap (Choi and Hoffmann, 1996). Mass spectra from this experiment confirm that both the trichloromethyl free radical and dichlorocarbene intermediates are formed in the magnetite catalyzed transformation of CT. This finding helps explain the parallel pathways observed in CT transformation. The measured CF and CO are products of the hydrogenolysis of the trichloromethyl free radical and hydrolysis of dichlorocarbene,
respectively. Through the radical analysis it was also determined that the formation of methane (9% of initial CT) was not due to complete reductive dechlorination of CT, but rather was a product of the reduction of dichlorocarbene. The absence of DCM and CM coupled with the linearity found between CF and CH₄ in a differential plot analysis points to CH₄ formation occurring as a parallel pathway alongside CF and CO formation.

As shown previously, vitamin B₁₂ is an important extracellular molecule capable of catalyzing rapid transformation of CT, even at high concentrations (Chiu and Reinhard, 1995; Gantzer and Wackett, 1991; Hashsham et al., 1995; Hashsham and Freedman, 1999; Lewis et al., 1996; Stromeyer et al., 1992). It has been hypothesized that the most rapid CT transformation, which produced lower concentrations of the chlorinated intermediates CF, DCM, and CM, occurs when the cobalt present in cobalamins is reduced to either Co(II) or Co(I) (Chiu and Reinhard, 1995; Lewis et al., 1996). When enough reducing power is provided to the system, CT will undergo a two electron transfer to dichlorocarbene which then hydrolyzes to CO and formate (Criddle and McCarty, 1991; McCormick and Adriaens, 2004). The DIRB *Shewanella alga* strain BrY is a metal reducing microbe that also has the ability to reduce B₁₂a to B₁₂r (Workman et al., 1997). When B₁₂a was incubated in PIPES buffered media alone, with lactate, with H₂, or with strain BrY, only modest amounts of B₁₂r were formed (3-14%). However, in the presence of BrY and lactate or H₂, 85% and 84% of the B₁₂a was reduced to B₁₂r, respectively. In treatments containing B₁₂, BrY, and lactate, CT transformation was measured over a period of 20 days with 91.9% of the mass recovered as CO and 1.43% CF. The B₁₂ and dithiothreitol (DTT) treatment transformed CT substantially (55% CO and 20% DCM), while the others did not (B₁₂ and lactate; BrY and lactate; B₁₂ and BrY). The formation of >90% CO in this system demonstrates the ability to control the oxidation state of B₁₂ using a DIRB and shift the pathway away from hydrogenolysis in iron reducing environments.

*Geobacter sulfurreducens* is another common DIRB found in subsurface environments that is also capable of reducing sulfur and using either hydrogen (H₂) or acetate as the sole electron donor (Caccavo et al., 1994). It is closely related to *G. metallireducens*, and is therefore likely capable of mediating the transformation of CT
through the production of biogenic iron species. The degradation of CT in the presence of *G. sulfurreducens*, biogenic iron species, copper ions, ferrihydrite, and naturally occurring quinones was examined in depth (Doong et al., 2014; Maithreepala and Doong, 2008). In all treatments, CT was transformed predominantly to CF and DCM with a carbon mass balance of 42-56% of the original CT (Doong et al., 2014). The percentage of CF and DCM formed agrees with other studies in iron reducing environments, and the unknown carbon is likely in nonvolatile products, CO, CH₄ or cell material (Bae and Lee, 2012; McCormick and Adriaens, 2004; Penny et al., 2010). The results show that CT transformation was primarily driven by *G. sulfurreducens* through the formation of biogenic surface-bound iron species produced during the reductive dissolution of Fe(III) ferrihydrite. The dissolution of ferrihydrite was enhanced with 10 µM AQDS, which served as a surrogate for natural organic matter (NOM) and as an electron shuttling compound. CT transformation in the presence of ferrihydrite and AQDS was three times faster than with *G. sulfurreducens* alone. Interestingly, when 0.3-0.5 mM Cu(II) was introduced to the system with ferrihydrite, it slightly inhibited the growth of *G. sulfurreducens* and decreased Fe(II) formation. However, the rate of CT degradation increased by 2.1-4.2 fold over the system without Cu(II). It was hypothesized that the reduction of Cu(II) to Cu(I) was facilitated by AQDS and was linked to the oxidation of Fe(II), thus forming secondary iron minerals (e.g. goethite), and different compositions of iron (III) oxides that accelerated the reduction of CT.

In order to enhance the transformation of CT in iron reducing environments with *G. sulfurreducens*, four different naturally occurring quinones were investigated for their ability to increase degradation rates through an increased dissolution of ferrihydrite and subsequent formation of Fe(II) (Doong et al., 2014). Quinones are a component of NOM and act as electron shuttling compounds. AQDS was used as a model compound for comparison with the naturally occurring quinones lawsone (LQ), ubiquinone (UQ), juglone (JQ), and 1,4-napthoquinone (NQ). It was found that AQDS, NQ and LQ enhanced Fe(II) production while UQ and JQ had little effect. This in turn led to an accelerated rate of CT transformation in systems containing AQDS, NQ, or LQ over those without quinones. Relatively low concentrations (10 µM) of the
quinones were sufficient to stimulate enhanced ferrihydrite dissolution which in turn catalyzed CT transformation. The Fe(II) species in this system appear to be only moderately successful at transforming CT. However, the addition of these naturally occurring quinones to iron reducing environments seem to sustain long term ferrihydrite dissolution, which could be coupled with other processes in order to achieve complete CT dechlorination.

2.5.5 Fermenting and Other Environments

Additional microbial species and reducing environments have also been implicated in the transformation of CT. Gälli & McCarty (1989) isolated *Clostridium* sp. TCAIIB from two anaerobic suspended-growth bioreactors that were known to biotransform TCE to vinyl chloride (VC) and trichloroethane (TCA) to dichloroethane (DCA) and chloroethane (Gälli and McCarty, 1989). After enriching strain TCAIIB, it was found to readily degrade small amounts of CT (100 µg/L; 0.65 µM) to CF (55%) and DCM (8%). It was also capable of catalyzing the transformation of CF (100 µg/L; 0.84 µM) to DCM (20%) which was not degraded further. However, the mineral medium in which it was grown contained Fe(II) porphyrins and ions in solution which both catalyze CT transformation, therefore making it difficult to separate the biotic and abiotic contributions in this system.

Another anaerobic enrichment culture capable of CT transformation was grown on dichloromethane as the sole carbon and energy source (Hashsham et al., 1995). The enrichment culture oxidized a portion of the DCM to CO₂ and H₂, with the remainder being fermented to acetate. The acetate, CO₂, and H₂ were then converted to CH₄ by acetoclastic and hydrogenotrophic methanogens. This DCM respiring and fermenting culture was able to completely transform 20 µM CT (3.1 mg/L) within 20 days forming CF (17%), CS₂ (21%) and CO₂ (21%). When 2 µM cyanocobalamin (CN-Cbl) was added to the reactors, the 20 µM CT was completely degraded within 2 days, an approximate 10-fold rate increase, and the product distribution shifted to 59% CO₂, 11% CS₂, and less than 1% CF. These results agree with multiple other studies conducted with vitamin B₁₂ homologs, and thus confirm the high transformation
capacity in a number of reducing environments. Rapid transformation of multiple additions of CT as high as 340 µM were sustained with CN-Cbl concentrations as low as 10 µM and 200 µM additions of H₂ over a period of 200 days with less than a 1% accumulation of CF and DCM.

The nitrate reducing *Pseudomonas stutzeri* KC isolated from groundwater aquifer solids was capable of completely transforming ¹⁴CCl₄ to ¹⁴CO₂ and an unidentified water soluble fraction (Criddle et al., 1990a). Additionally, pure cultures of *Escherichia coli* K-12 were grown in different environments in order to determine the role that electron acceptor conditions played in CT transformation with the same organism. *E. coli* K-12 was grown under aerobic, low oxygen (~1%), nitrate respiring, fumarate respiring, and fumarate fermenting conditions. No CT degradation occurred under aerobic or NO₃⁻ respiring conditions, while significant transformation was observed in the other three electron acceptor environments. The low oxygen acceptor environment transformed CT with the following distribution: CT, 51%; CF, 3.9%; CO₂, 17.4%; nonvolatile fraction, 4.6%; cell fraction, 21.8%. The fumarate respiring and fumarate fermenting conditions fully transformed CT but did so with an accumulation of CF (16% and 37.5%) and less CO₂ (11.1% and 2.1%).

Due to the prevalence of CT as a high priority groundwater contaminant and the numerous environments, microorganisms, and extracellular factors that can catalyze its transformation, there are a plethora of studies examining its degradation. These studies look at strictly abiotic, purely biotic, and a spectrum of biotic/abiotic interactions that run the gamut of reducing environments implicated in CT transformation. Due to the large number of CT degradation studies to date examining a wide range of concentrations and conditions, it is difficult to compare kinetic parameters across systems. While CT transformation rates vary substantially under different reducing conditions, the addition of extracellular factors such as corrins, porphyrins, and quinones enhance the degradation rates above those mediated solely by cometabolic transformation and reduced sulfide mineral media. Furthermore, the reducing conditions of an environment greatly impact the product distribution, and cobalamins have been shown repeatedly to shift the pathway away from hydrogenolysis (CF and DCM) to dichlorocarbene hydrolysis (CO and formate). In order to develop
effective remediation strategies, the complexities of CT transformation needs to be understood and manipulated in order to limit the formation of undesirable products.

2.6 Transformation of Chloroform

Chloroform is the first hydrogenolytic product of CT reductive dechlorination in anaerobic systems, and frequently comprises a large portion of the transformed CT mass. However, CF behaves differently in the environment due to the reduced nature of the carbon atom (+2) in chloroform compared to the completely oxidized carbon (+4) in CT. Unlike CT, chloroform can be biologically transformed by cometabolism in aerobic environments as well as anaerobic. CF is cometabolized in aerobic environments primarily by monooxygenase (MO) enzymes, which introduce one oxygen atom to CF forming CO, which is subsequently mineralized to CO$_2$ (Cappelletti et al., 2012). The primary products of CF cometabolism in aerobic environments are CO$_2$ and free chloride (Cl$^{-}$). A wide range of MO enzymes can cooxidize CF and many other chlorinated aliphatic hydrocarbons (CAHs) due to the nonspecificity of the enzymes (Cappelletti et al., 2012). However, the same MO can exhibit variable activities for different chlorinated compounds (Chang and Alvarez-Cohen, 1996; Colby et al., 1977; Kim et al., 2000). CF cometabolism has been found to be mediated by the following monooxygenase enzymes: methane (Aziz et al., 1999; Chang and Alvarez-Cohen, 1996; Oldenhuis et al., 1989; Speitel et al., 1993; Speitel and Leonard, 1992; van Hylckama et al., 1996), propane (Aziz et al., 1999; Frascari et al., 2008, 2003; Malachowsky et al., 1994), butane (Ciavarelli et al., 2011; Frascari et al., 2012, 2007, 2006; Hamamura et al., 1997), hexane (Frascari et al., 2006), toluene (Chauhan et al., 1998; McClay et al., 1996), and ammonia (Ely et al., 1997; Wahman et al., 2007, 2006, 2005).

However, in anaerobic environments CF is more recalcitrant and transformation can typically stall at lower chlorinated methanes unless sufficient reducing power is provided to the system (Mikesell and Boyd, 1990). There are three main pathways for anaerobic CF transformation (Fig. 2.3): hydrolysis and subsequent oxidation, cometabolic reductive dechlorination, and dehalorespiration (Cappelletti et al., 2012).
CF can undergo a direct hydrolysis to CO, which will then be oxidized by H₂O to form CO₂. The half-life for CF hydrolysis under purely hydrolytic conditions is 3100 years (Mabey and Mill, 1978). Chloroform can also be dechlorinated to form the unstable dichlorocarbene and chlorocarbene radicals, which will rapidly hydrolyze to CO with a subsequent oxidation to CO₂ (Cappelletti et al., 2012). Methanogens, sulfate reducing bacteria, fermenting bacteria such as Clostridium sp. and Enterobacter sp., and one species of homoacetogen (Acetobacterium woodii) have been found to cometabolically degrade CF under anaerobic conditions (Bouwer and McCarty, 1983; Egli et al., 1988; Guerrero-Barajas and Field, 2005; Shan et al., 2010a; Yu and Smith, 2000). The cometabolic transformation of CF often resulted in the accumulation of DCM with small amounts of chloromethane (CM) and methane (CH₄) detected as well (Egli et al., 1988; Krone et al., 1989a, 1989b; Mikesell and Boyd, 1990). The reductive dechlorination of DCM to CM and CH₄ occurred at very slow rates due to the less favorable thermodynamics of the lower chlorinated compounds.

To date, two mixed cultures have been found that are capable of transforming CF via dehalorespiration using it as a terminal electron acceptor (Grostern et al., 2010; Lee et al., 2012). The first was a highly enriched anaerobic mixed culture originating from contaminated sediment containing greater than 60% Dehalobacter sp. (Grostern et al., 2010). The culture (Dhb-TCA) was found to grow via the reductive dechlorination of trichloroethane (TCA) to chloroethane (CA) via 1,1-dichloroethane (1,1-DCA). When experiments were conducted attempting to quantify the inhibitory effect of CF on the TCA respiring culture, it was found that Dhb-TCA immediately began to reductively dechlorinate the CF to DCM. The culture metabolized CF to DCM as rapidly as or faster than TCA, with a maximum rate of 360 µM/d when maintained on 1 mM CF. A second anaerobic mixed culture enriched from subsurface soil was found to completely dechlorinate CF via two metabolic processes (Lee et al., 2012). CF was respired to DCM via dehalorespiration with a subsequent fermentation to H₂, CO₂, and acetate. Complete CF dechlorination of at least 360 µM was achieved at rates of 40 µM/d. Pyrosequencing of the separated CF respiring culture and the DCM fermenting culture revealed that both were also enriched in Dehalobacter species. Subsequent growth experiments revealed that the growth of Dehalobacter lineages was
linked directly to the dehalorespiration of CF and the dehalofermentation of DCM. The complete dechlorination of CF by metabolic processes proves promising for the remediation of contaminated sites. For both anaerobic mixed cultures, the transformation of CF to DCM followed a 1:1 stoichiometry with further dechlorination of DCM by the second culture (Lee et al., 2012).

Similar to carbon tetrachloride, the rates and products of CF transformation are dependent on the type of reducing environment, growth substrates, microorganisms, CF concentration, and coenzymes present (Freedman et al., 1995; Guerrero-Barajas and Field, 2005; Gupta et al., 1996a, 1996b). Furthermore, the addition of small extracellular compounds such as cobalamins (vitamin B$_{12}$ homologs) (Egli et al., 1988; Krone et al., 1989a, 1989b), coenzyme F$_{430}$ (Gantzer and Wackett, 1991; Krone et al., 1989a), iron-containing porphyrins such as heme (Klečka and Gonsior, 1984), and zinc-containing porphorinogen-type cell exudates (Koons et al., 2001; Novak et al., 1998b) can catalyze the cometabolic and abiotic transformation of CF. Analogous to systems with CT, the addition of vitamin B$_{12}$ homologs significantly increased the rate of CF transformation and shifted the pathway from DCM formation to CO formation in methanogens, sulfate reducers, and *Acetobacterium woodii* (Becker and Freedman, 1994; Freedman et al., 1995; Guerrero-Barajas and Field, 2005; Hashsham and Freedman, 1999).

However, CF is a highly inhibitory compound exerting complete yet reversible inhibition of numerous microbial processes in anaerobic environments (Hickey et al., 1987; Rhee and Speece, 1992). Yu and Smith (2000) found that low levels of CF (0.09 mg/L; 0.76 µM) completely inhibited methanogenesis in an enrichment culture from a wastewater anaerobic digester (Yu and Smith, 2000). Additionally, while corrinoids catalyze CF reductive dechlorination, they also appear to be the target moieties by which CF inhibits methanogenesis (Yu and Smith, 1997). It’s been suggested that this occurs by a competitive inhibition of the corrinoids due to the structural similarity of CF with the methyl groups that bind the transition metal co-factors during methanogenesis (Bauchop, 1967a). CF has also been found to inhibit homoacetogens and acetate-consuming, sulfate-reducing bacteria that use the Acetyl-CoA pathway (Liu et al., 2010; Scholten et al., 2000). The Acetyl-CoA pathway has been implicated
in the transformation of CT and CF, and involves a cobalamin-containing enzyme as a carrier for methyl groups (Egli et al., 1988). Therefore the addition of vitamin B$_{12}$ was supposed to mitigate the toxic effects of CF on the cobalamin-containing enzymes in this pathway (Hashsham and Freedman, 1999; Stromeyer et al., 1992). An *Enterobacter* sp. lacking the Acetyl-CoA pathway was able to catalyze the transformation of high concentrations of CF (500 mg/L) when vitamin B$_{12}$ was added forming CO, CO$_2$, and organic acids. In order to deal with the toxicity associated with high concentrations of CF, the *Enterobacter* sp. was found to alter its membrane fluidity (Shan et al., 2010a).

Low concentrations of chloroform have also been shown to inhibit microbes that respire chlorinated ethenes. PCE degradation was completely inhibited by 4 µM CF in an anaerobic ethanol enrichment culture (Bagley et al., 2000). PCE dehalogenases isolated from *Dehalospirillum multivorans* were found to be 50% inhibited at 25 µM CF (Neumann et al., 1996). PCE and 1,2-*cis*-DCE dechlorination by *Dehalococcoides ethenogenes* were completely inhibited by 8.4 µM and 1.6 µM CF, respectively (Maymó-Gatell et al., 2001). CF may inhibit dechlorinating organisms directly through enzyme competition (Neumann et al., 1996) or through a general inhibition of important metabolic processes (Cappelletti et al., 2012).

### 2.7 Remediation of Carbon Tetrachloride and Chloroform

The remediation of carbon tetrachloride and chloroform is complicated by the diverse range of metabolites that can be formed during their transformation. Typically, remediation strategies comprise physical and chemical approaches such as soil excavation, pump and treat groundwater stripping, and venting. However, these approaches are typically energy and cost intensive, are usually only somewhat effective, and can have undesirable environmental consequences such as mobilizing CT and CF in the atmosphere where they can deplete stratospheric ozone (Schwarzenbach et al., 2006; US Environmental Protection Agency, 2008). An alternative strategy has been natural attenuation in which a contaminated groundwater site is not manipulated and instead relies on natural processes and long-term monitoring.
in order to track contaminant depletion. Natural attenuation of contaminated sites leverages processes such as dispersion, sorption, and biotic and abiotic degradation occurring due to the intrinsic physical, chemical, and biological characteristics of an environment (Wiedemeier et al., 1999). In a strongly reducing groundwater environment, a CT DNAPL was found to be 90% degraded to CF, DCM, and CS$_2$ over an 11-year period without any stimulation of the groundwater environment (Davis et al., 2003). While the reduction of CT observed is substantial, a secondary remediation strategy needs to be employed for the removal of CF and DCM.

Bioremediation affords the possibility of transforming contaminants in situ with two different strategies, biostimulation and bioaugmentation. Biostimulation is the injection of amendments into contaminated environments in order to stimulate contaminant degradation by the indigenous microbial community whereas bioaugmentation is the injection of native or non-native microbes to a contaminated area to encourage degradation (US Environmental Protection Agency, 2010). Bioaugmentation strategies typically also involve the biostimulation of the contaminated area in order to create favorable conditions for the growth of the injected culture. In anaerobic systems, carbon and energy-rich compounds such as vegetable oil or molasses are injected to promote the growth of anaerobic microbes. Semprini et al. (1992) demonstrated how biostimulation could effectively degrade CT and other halogenated compounds in a model sandy aquifer system (Semprini et al., 1992). Acetate (as a growth substrate and electron donor) along with nitrate and sulfate (electron acceptors) were injected into the aquifer, which stimulated the native microbial community to degrade 95% of the initial CT within 2 m of travel in the test zone. When the NO$_3^-$ amendment was ceased, CT degradation increased, with 30-60% of the CT transformed to CF. Devlin and Müller (1999) also conducted a successful field demonstration of biostimulation in order to degrade CT (Devlin and Müller, 1999). A sandy aquifer was injected with acetate in order to stimulate the growth of sulfate reducing bacteria. CT was added to the test aquifer at approximately 1 mg/L and was completely transformed to CF and CS$_2$ in a 2:1 ratio.

Remediation strategies and pilot tests that couple bioaugmentation with biostimulation have been more successful at completely dechlorinating CT. Most pilot
scale and field demonstrations of bioaugmentation have used the denitrifying bacteria *Pseudomonas stutzeri* KC, which is capable of completely transforming CT to CO$_2$ and nonvolatile organic acids under anoxic conditions (Criddle et al., 1990a; Dybas et al., 2002, 1998; Mayotte et al., 1996; Pfiffner et al., 2000). A CT (2.3–46.5 µg/L) and nitrate (1.87–63.7 mg/L) contaminated aquifer in Schoolcraft, MI was successfully remediated by the deployment of a full-scale biocurtain containing *P. stutzeri* KC and the addition of acetate, nitrate, and phosphate as growth supporters. Approximately 18,600 m$^3$ of contaminated groundwater was treated with a 98% average reduction of CT with only transient formation of CF (< 20 µg/L) and CS$_2$ (< 2 mg/L). The remediation of CT in this system was sustained efficiently over a four year period with intermittent addition of substrate in closely spaced wells (Dybas et al., 2002).

Additionally, bioremediation potential was examined for a contaminated site in California with concentrations of CT (8.8 mg/L), CF (500 mg/L), and trichlorofluoromethane (CFC-11; 26 mg/L) generally deemed too high for biological treatment (Shan et al., 2010b). Pilot scale tests in continuous flow columns explored the possibility of employing different biostimulation and bioaugmentation treatments at the contaminated site. Biostimulation alone (injection of corn syrup, emulsified vegetable oil, and lactate), biostimulation (corn syrup) with addition of cyanocobalamin (vitamin B$_{12}$), and bioaugmentation with catalytic levels of cyanocobalamin were studied for CT transformation potential. The bioaugmentation cultures were lactate-grown sulfate reducers, ethanol-grown sulfate-reducers, and a corn syrup-grown fermenting culture. Complete transformation of the three compounds was achieved by the biostimulation with B$_{12}$ and bioaugmentation treatments, with the lactate-grown sulfate reducers and the fermenting culture being the most effective. Additionally, the vitamin B$_{12}$ catalyzed the transformation to produce CO, CO$_2$ and organic acids even at the high concentrations of halogenated compounds.

Due to differences in the transformation of carbon tetrachloride versus the lesser chlorinated methanes (CF and DCM), multiple remediation strategies can be applied in concert to achieve complete dechlorination in contaminated groundwater. Zero valent iron (ZVI; Fe$^0$), has been found to transform CT and CF to DCM, CH$_4$, formate, CO, and CO$_2$ when installed as a permeable reactive barrier in the flow path of contaminated
groundwater (Gillham et al., 2010; Gillham and O’Hannesin, 1994; Johnson et al., 1996; Matheson and Tratnyek, 1994; TÁmara and Butler, 2004). However, CT has been found to strongly inhibit Dehalobacter sp. capable of transforming CF and DCM via dehalorespiration and dehalofermentation (Justicia-Leon et al., 2014). Therefore, it would be feasible to install a ZVI permeable reactive barrier near the source of CT contamination followed by a subsequent biostimulation and bioaugmentation of Dehalobacter sp. capable of transforming CF and DCM. Justicia-Leon et al. (2014) conducted a pilot test treatability study in order to determine the value of biostimulation and bioaugmentation using CF contaminated sediment (Justicia-Leon et al., 2014). When microcosms were inoculated with a CF respiring culture (Dhb-CF) and a DCM degrading consortium (RM), complete dechlorination of CF and DCM occurred with HCO$_3^-$ serving as the only amendment required. Additionally, Lee et al. (2015) found that Fe$^0$ in the presence of a Dehalobacter sp. was able to degrade CF and form DCM at 8-fold faster rates and 14 times higher amounts, respectively, compared to systems with Fe$^0$ alone (Lee et al., 2015). The DCM was subsequently fermented to CO$_2$, H$_2$, and acetate, thus completely dechlorinating the original CF. Sustainable and effective long term remediation applications will require the use of multiple strategies that leverage both biological and chemical transformation mechanisms.

2.8 Anaerobic Dechlorinating Cultures

The Evanite (EV), Victoria/Stanford (VS), and Point Mugu (PM) anaerobic mixed cultures are enrichment cultures capable of respiring TCE and PCE to ethene. The EV and PM cultures were enriched from contaminated sediment and groundwater at the Evanite Corporation site in Corvallis, OR and Point Mugu Naval Weapons Facility, CA, respectively (Yu et al., 2005). The VS culture was enriched from aquifer material at a PCE-contaminated site in Victoria, TX (Cupples et al., 2003). The three cultures were enriched under batch conditions for 10 years and subsequently inoculated into continuous flow reactors (chemostat) with a 2 L and 5 L size (Berggren et al., 2013; Cupples et al., 2003; Marshall et al., 2014; Yu et al., 2005). The anaerobic dechlorinating cultures were inoculated into chemostat reactors in September 2007 (EV-2L), July 2007 (EV-5L), July 2008 (VS-2L), July 2009 (VS-5L), February 2008
PM-2L), and July 2009 (PM-5L) and have a hydraulic retention time of approximately 50 days. They are grown in a basal anoxic mineral media (Yang and McCarty, 1998) modified to double the buffering capacity of the system (1 g/L K$_2$HPO$_4$ and 3 g/L Na$_2$CO$_3$) (Yu et al., 2005). The EV and VS cultures receive an influent solution containing saturated TCE (10 mM) and 45 mM formate as electron acceptor and donor, respectively (Marshall et al., 2014). The PM cultures receive an influent of saturated PCE (1.12 mM) as electron acceptor and lactate (4.3 mM) as a fermenting electron donor, while the PM-2L chemostat also receives 1.0 mM SO$_4^{2-}$ as a competing electron acceptor for dehalogenation (Berggren et al., 2013).

Molecular analyses of the continuous flow reactors have shown that the cultures are highly enriched in Dehalococcoides mccartyi, which comprises 90% of the microbial community (Berggren et al., 2013; Marshall et al., 2014). Other microorganisms present in the reactors are Geobacter sp., and microbes of the orders Desulfuromonadales, Selenomonadales, and Spirochaetales (Semprini, unpublished data). Recent work by Semprini (unpublished data) has shown that the EV and VS chemostats contain roughly equal amounts of the 16s rRNA transcripts encoding for D. mccartyi strain tceA and D. mccartyi strain vcrA whose relative abundance can change based on the concentration of electron donor present in the system. The enzymes tceA and vcrA are reductive dehalogenases capable of catalyzing the transformation of TCE/DCE (Magnuson et al., 2000) and DCE/VC, respectively (Müller et al., 2004). When the culture is enriched in strain vcrA, VC is completely transformed to ethene while an enrichment in strain tceA allows for the accumulation of VC to occur (Marshall et al., 2014).

A previous study showed that the bioaugmentation of the EV culture into a continuous flow column packed with Ottawa quartz sand could achieve simultaneous transformation of PCE and CT (Azizian and Semprini, in press). However, the amount and type of electron donor provided dramatically affected the transformation of both compounds. When sufficient formate (1.5 mM) or lactate (1.1 mM) was provided as an electron donor, PCE (0.1 mM) was transformed to VC (11-17%) and ethene (81-85%). At lower lactate concentrations (0.67 mM), chlorinated ethene transformation was impacted shown by the accumulation of cDCE (100%) at pseudo-steady state. When
CT was introduced to the column at a concentration of 0.015 mM, its transformation was complete with 20% found as CF and trace amounts of DCM and CM. Ethene production and \( \text{SO}_4^{2-} \) reduction were not impacted, but formate (1.5 mM) utilization and acetate production were inhibited by the presence of CT and CF. Additionally, when the electron donor was switched to lactate, a fermenting substrate, propionate buildup occurred along with a drop in \( \text{H}_2 \) concentrations. Propionate was not an effective electron donor, which affected PCE dehalogenation leading to cDCE (48%) and VC (36%) accumulation. It is likely that the long term exposure to CF impacted the microbes responsible for fermenting propionate to \( \text{H}_2 \).

The column was reaugmented with the EV culture after 1950 days and supplied with 0.1 mM PCE and 1.1 mM lactate in the absence of CT in order to encourage the growth of the fermenting population once again. Under these conditions, PCE transformation proceeded completely to ethene (98%) with only a small amount of VC (2%). The addition of CT (0.015 mM) did not impact chlorinated ethene transformation, lactate fermentation, or acetate formation. CT was completely transformed, and resulted in a transient buildup of approximately 20% CF which then disappeared. Increasing concentrations of CT (0.03, 0.06, and 0.1 mM) demonstrated similar behavior in the column. The use of \(^{13}\text{CCl}_4\) showed that under lactate fermenting conditions CT was transformed to \(^{13}\text{CO}_2\) (80%) and non-volatile products (9%) with trace amounts of CF (Azizian and Semprini, 2016). Decreasing lactate concentrations (0.67 mM) affected PCE and CT transformation, acetate formation and \( \text{H}_2 \) production. CF was observed to be the main transformation product under these conditions.

There are numerous sites in the United States co-contaminated with chlorinated ethenes and methanes, thus creating a need for a better understanding of the biochemical dynamics of complex mixtures (Knox and Canter, 1996; Petrisor and Wells, 2008). In the present study, CT transformation by the PCE and TCE respiring anaerobic mixed cultures was studied extensively in batch reactor systems in order to elucidate the pathways and kinetics of the transformation process. Modeling analyses were also conducted in order to predict the degradation of CT and subsequent product formation under multiple treatments. Transformations followed first order kinetics.
leading to the development of five analytical first order solutions that were solved simultaneously using a nonlinear least squares method.

As shown by this literature review, CT transformation has been studied in depth for a number of different reducing systems involving both biotic and abiotic mechanisms. However, CT transformation by chlorinated ethene respiring cultures has not been thoroughly examined owing to the inhibitory effects of CT and CF on dehalogenation. Inhibition of H2 consumption by the mixed cultures due to the presence of CF was also quantified in order to attempt to analyze the complex electron donor dynamics characteristic of anaerobic dechlorinating cultures. These facts, along with the complicated nature of the dynamics between chlorinated ethenes, methanes, and dehalogenating microbes make this research of interest.
CHAPTER 3: MATERIALS AND METHODS

3.1 Chemicals

All chemicals used in the transformation studies or for external standards were analytical grade: CT, 99.9% (Sigma-Aldrich); CF, 99.9% (OmniSolv); DCM, 99.9% (Fisher Chemical); CS₂, 99.9% (Alfa Aesar); CM, 99.5% (Sigma-Aldrich); CH₄, 99.9% (Air Liquide); CO, 99.0% (Sigma-Aldrich); TCE, 99.9% (Macrom Fine Chemicals); cDCE, 99.0% (TCI America); VC, 99.5% (Sigma-Aldrich); ETH, 99.5% (Airgas); formate, 99.0% (Alfa Aesar); lactate, 60% w/w syrup (J.T. Baker).

3.2 Anaerobic Dechlorinating Cultures

Experiments were conducted with the Evanite (EV) and Victoria/Stanford (VS) mixed anaerobic dechlorinating cultures that are grown in continuous flow reactors and contain at least two strains (tceA and vcrA) of Dehalococcoides mccartyi (Marshall et al., 2014). The two cultures are capable of respiring PCE to ETH (Yu et al., 2005) and are grown in a 2 liter and a 5 liter replicate chemostat (Marshall et al., 2014) in a basal anaerobic mineral media (Yang and McCarty, 1998) modified to double the buffering capacity of the system (1 g/L K₂HPO₄ and 3 g/L Na₂CO₃). The EV and VS 5 liter chemostats (designated EV-5L and VS-5L) have been growing under chemostat conditions since July 2007 and July 2009, respectively with a hydraulic retention time of approximately 50 days (Marshall et al., 2014). They receive an influent feed of saturated TCE (10 mM; 60 mEq) as electron acceptor and formate (45 mM; 90 mEq) as electron donor. Previous molecular characterization has found that the EV-5L and VS-5L chemostats are highly enriched (approximately 90%) in D. mccartyi strains containing the reductive dehalogenase (rdhA) enzymes tceA, vcrA, and bvcA (Marshall et al., 2014), which are responsible for the respiration of TCE/DCE (Magnuson et al., 2000) and DCE/VC (Krajmalnik-Brown et al., 2004; Müller et al., 2004) to ETH. Additionally, the EV-5L and VS-5L cultures contain operational transcription units (OTUs) for Geobacter and Desulfitobacterium strains (Marshall et al., 2014; Mayer-Blackwell et al., 2014). Neither culture has been previously acclimated to CT, CF, or their transformation products.
3.3 Batch Transformation Studies

Saturated solutions of CT, CF, DCM, CS$_2$, and cDCE were prepared in anaerobic mineral media (Yu et al., 2005) and used in the batch transformation studies or for analytical standards. Saturated TCE was anaerobically transferred from the chemostat influent media for the same purposes (Marshall et al., 2014). Cells and supernatant from the EV-5L or VS-5L chemostat were transferred to anaerobically-prepared 125 mL Wheaton Type I media bottles sealed with butyl rubber septa and plastic screw caps. The batch reactors comprised a two phase system with a headspace gas phase (108 mL) and a liquid phase (50 mL). Residual chlorinated ethenes were sparged from the batch reactors with a furnace treated mixed gas (75% N$_2$; 25% CO$_2$) prior to the onset of an experiment. Varying initial concentrations of CT (0.86, 2.6, or 8.6 µM) and CF (2.1 or 21.1 µM) along with DCM (23.2 µM) and TCE (50 µM) were added individually as slug inputs to the batch reactors. Controls were conducted in the sterile anaerobic mineral media in the absence of cells. In all experiments, formate (2 mM) was provided in excess as an electron donor. Reactors were incubated in the dark at 20 °C and 200 rpm with 30 minutes equilibration before the initial measurement assuming Henry’s Law equilibrium. Dimensionless Henry’s Law coefficients were used to track chemical transformation in both compartments over time (Sander, 2015; Staudinger and Roberts, 2001).

The relative importance of live cells in the transformation of CT and CF was tested in batch reactors poisoned with an anaerobically-prepared sodium azide (NaN$_3$) solution, a known biocide (Lichstein and Soule, 1944). Since CT and CF can both undergo abiotic transformation, the appropriate dose of NaN$_3$ required to poison the microbial culture was determined using the dehalorespiration of TCE as a marker of cell integrity. Reductive dechlorination of TCE is a predominantly biotic process when catalyzed by *D. mccartyi* in anaerobic environments (Maymó-Gatell et al., 1997). It was found that 50 mM NaN$_3$ was a sufficient dose to kill the cells and stop TCE reductive dechlorination. In the NaN$_3$ treatment, batch reactors established with 2.6 µM CT were poisoned with 50 mM NaN$_3$.

Simultaneous transformation of TCE and CT was also examined. Kinetic tests were conducted in batch reactors established with 2.6 µM CT and 50 µM TCE. Control
reactors received TCE but no CT in order to measure the impact of CT and CF on TCE reductive dehalogenation. The TCE batch kinetic tests were analyzed using a numerical model described previously (Berggren et al., 2013). A subsequent slug addition of TCE was added after 14 days in order to test the effects of long-term exposure to CF on the culture. Unless otherwise specified, all experiments were carried out in triplicate.

3.4 Analytical Methods

All compounds were quantified by headspace analysis in the two phase batch reactor systems. Hamilton gas tight syringes (1700 series) were used to extract 100 µL headspace samples which were manually injected onto gas chromatographs (GC). CT, CF, DCM, and CS₂ were measured on an HP-6890 GC equipped with an electron capture detector (ECD), a capillary column (Agilent 30 m x 0.32 mm GS-Q), and with helium (He) as the carrier gas (8.0 mL/min). Detection limits were as follows: CT, 10 nM; CF, 30 nM; DCM, 140 nM; CS₂, 20 nM. Higher concentrations of CT were measured by injecting 10 µL headspace samples under the same conditions. CM and CH₄ were quantified on an HP-6890 GC equipped with a flame ionization detector (FID), a capillary column (Agilent 30 m x 0.53 mm GS-Q+PT), He carrier gas (1.0 mL/min), and with detection limits of 95 nM (CM) and 20 nM (CH₄). CO was measured on an HP-5890 GC with a thermal conductivity detector (TCD), a packed column (Supelco 15’ x 1/8” SS support 60/80 Carboxen 1000), He carrier gas (30 mL/min), and with a detection limit of 20 nM. TCE, cDCE, VC, and ETH were quantified on an HP-6890 GC FID with a capillary column (Agilent 30 m x 0.53 mm GS-Q), He carrier gas (15 mL/min), and with detection limits as follows: TCE, 270 nM; cDCE, 270 nM; VC, 80 nM; ETH, 10 nM. Electron donor concentrations were tracked by measuring hydrogen (H₂) produced from formate. H₂ was quantified on an HP-5890 GC TCD with a packed column (Supelco 15’ x 1/8” SS support 60/80 Carboxen 1000), argon (Ar) carrier gas (20 mL/min), and with a detection limit of 20 nM.
3.5 First Order Rate Analyses and Transformation Model

Rate coefficients were estimated for CT and CF degradation from experimental data using a first order transformation model and a natural log-linear regression. Pseudo-first order kinetics were applied based on experimental results and previous studies (Butler and Hayes, 2000; Kriegman-King and Reinhard, 1992; McCormick et al., 2002; Novak et al., 1998a; Vogel et al., 1987). A subsequent analytical model was developed to simultaneously estimate the rate of transformation for CT and its metabolites by varying the rate coefficients (k₁–k₆) and fitting data from the batch kinetic experiments (Figure 3.1). CT degradation and product formation was modeled using a series of first order analytical solutions derived from the following ordinary differential equations:

\[
\begin{align*}
\frac{dM_{CT}}{dt} &= -(k_1 + k_3 + k_4)M_{CT} \\
\frac{dM_{CF}}{dt} &= k_1M_{CT} - (k_2 + k_5)M_{CF} \\
\frac{dM_{DCM}}{dt} &= k_2M_{CF} \\
\frac{dM_{CS2}}{dt} &= k_3M_{CT} - k_6M_{CS2} \\
\frac{dM_{CO2}}{dt} &= k_4M_{CT} + k_5M_{CF} + k_6M_{CS2}
\end{align*}
\]

Transformation rate coefficients were estimated by a non-linear least squares regression performed using the Solver Tool Pack (Microsoft Excel, 2016). Model validity was confirmed by comparing the observed CT transformation rates (k_{CT,obs}) with those generated by the analytical solution (k_{CT,model} = k_1+k_3+k_4). Due to the complex nature of the mixed culture, the differences between treatments, and the inability to separate the relative biotic and abiotic contributions to CT degradation, a more complicated transformation rate model would not necessarily facilitate a better kinetic analysis. For these reasons, a first order model was employed to examine the differences between treatments.
The use of $[^{13}\text{C}]$-labeled $^{13}\text{CCl}_4$ in transformation experiments was attempted in order to measure $^{13}\text{CO}_2$ and quantify the complete product distribution of CT degradation. However, this was unsuccessful due to the high background concentration of $^{13}\text{CO}_2$ (390 µM) present in the anaerobic mineral media compared to the maximum CT concentration (30 µM) tested. Despite this, numerous sources have confirmed the formation of CO$_2$ from CT degradation as shown in Figure 2.3 (Bouwer and McCarty, 1983; Cappelletti et al., 2012; Criddle and McCarty, 1991; Hashsham and Freedman, 1999). Therefore, the kinetic model incorporates a mass balance approach and assumes that the observed unknown fraction is $M_{\text{CO}_2} = M_{\text{CT},0} - (M_{\text{CT}} + M_{\text{CF}} + M_{\text{DCM}} + M_{\text{CS}_2})$ at any given time.

Figure 3.1: Simplified biochemical pathway representing measured compounds and an unknown fraction (postulated as CO$_2$ based on previous studies). Arrow weights represent relative proportion of product formed.
CHAPTER 4: CARBON TETRACHLORIDE AND CHLOROFORM TRANSFORMATION

4.1 CT Degradation and Product Formation

CT transformation by the EV-5L and VS-5L cultures and supernatant was complete (below 10 nM analytical detection limit) and followed pseudo-first order kinetics in all experimental treatments (Figures 4.1, A.2.1, A.2.2, and A.2.3). Additionally, the sterile anaerobic mineral media (CT Control) catalyzed partial CT transformation in the absence of cells, albeit at substantially slower rates (Figure A.1.1). This was likely due to the presence of precipitated iron sulfides (FeS) in the reduced sulfide media (Butler and Hayes, 2000; Devlin and Müller, 1999). Due to the comparatively rapid transformation of CT in the presence of the anaerobic dechlorinating cultures and supernatant (ADC+S), further analyses of the CT Control treatments were not conducted. As the initial aqueous CT concentration ([CT₀]ₐq) increased, first order transformation rates (kₜₜₜ,obs) showed a non-linear decrease, which has been reported previously (McCormick et al., 2002). When working with the DIRB G. metallireducens, McCormick et al. (2002) found that protein-normalized pseudo-first order rate constants were dependent on [CT₀]ₐq and that the kinetics fit a two-site Michaelis-Menten kinetic model. However, they do state that no mechanistic interpretation should be made from the analysis. While this analysis was not conducted with the ADC+S, kₜₜₜ,obs decreased as [CT₀]ₐq increased in a qualitatively similar manner. When multiple additions of 0.86 µM CT were added to the ADC+S, kₜₜₜ,obs and kₜₜₚ,obs decreased with each subsequent addition (Figures A.3.1, A.3.2, and A.3.3). This confirms that CT transformation by the ADC+S is abiotic and cometabolic, and the system has a transformation capacity that depletes over time.
Figure 4.1: Pseudo-first order CT transformation rates by the EV-5L and VS-5L ADC+S. As \([\text{CT}_0]_{\text{aq}}\) increased the observed degradation rates \((k_{CT,\text{obs}})\) decreased in a non-linear manner. Error bars represent one standard deviation.

CF was the predominant transformation product measured in all treatments, with lesser amounts of DCM and CS\(_2\) also detected (Figures 4.2, 4.3 and 4.4). CF, DCM, and CS\(_2\) are undesirable end products due to their status as probable carcinogens, and the potential for CS\(_2\) neurotoxicity in humans (US Environmental Protection Agency, 2016, 2000). CF and CS\(_2\) were subsequently transformed while no appreciable degradation of DCM occurred. CM, CH\(_4\), and CO were not detected throughout any of the experiments. Mass balance analyses revealed that only 20-40% of the original CT mass was detected as volatile products. It is likely the unknown mass is being transformed to CO\(_2\). This result is in agreement with previous research conducted with CT and the Evanite culture in a continuous flow column (Azizian and Semprini, in press). However, the absence of CM and the presence of DCM and CS\(_2\) in the batch reactor systems differs from the results of Azizian and Semprini (in press). The absence of CS\(_2\) in the column is likely due to the low iron content (28.7 mg/kg solids) of the Ottawa quartz sand (Azizian and Semprini, in press) compared to the anaerobic media (Yang and McCarty, 1998), thus preventing the formation of FeS solids which can
catalyze CS$_2$ formation from CT (Butler and Hayes, 2000; Devlin and Müller, 1999). Changes in the microbial cultures could also be responsible for the observed differences. The Evanite culture inoculated into the continuous flow column was grown on PCE in sequential batch mode with butanol as a fermenting substrate (Azizian and Semprini, in press). Furthermore, a shift in the microbial community occurred in the EV and VS cultures when they were inoculated into chemostat systems under different electron donor and acceptor conditions from their previous batch incubation (Behrens et al., 2008; Marshall et al., 2014).

The VS-5L ADC+S catalyzed faster CT transformation than the EV-5L ADC+S in all treatments (Figure 4.1; Table 4.1). The observed differences in CT transformation rates cannot be explained by experimental differences nor due to differing electron donor or acceptor conditions. The microbial community structure of the two cultures could be responsible for the differences in CT transformation rates. Previous analyses have shown that the EV-5L and VS-5L cultures contain roughly equal relative abundances of $D$. mccartyi strains containing the reductive dehalogenase enzymes tceA and vcrA (Marshall et al., 2014). Despite this, there are additional microbes such as Geobacter species present in the chemostats that could be contributing to this phenomenon (Mayer-Blackwell et al., 2014). Early work with CT and CF in acetate-grown biofilms (Bouwer and McCarty, 1985) and in an anoxic biofilm column (Bouwer and Wright, 1988) found that the fastest rates of CT and CF dechlorination occurred in the most reduced cultures. Additionally, CT transformation in an electrolysis cell was also more rapid at lower reduction potentials (Criddle and McCarty, 1991). One possibility is that CT transformation rates, and the subsequent degradation of CF, are likely higher in the VS-5L ADC+S compared to the EV-5L ADC+S due to a difference in reduction potentials, with VS-5L being more reduced.
Table 4.1: Comparison of CT first order transformation rate constants for the EV-5L and VS-5L ADC+S. Rates were calculated from experimental CT data ($k_{CT,obs}$) and from an analytical first order model ($k_{CT,model}$). All treatments initially contained 2.6 µM CT. Error estimate calculated as one standard deviation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$k_{CT,obs}$ (d⁻¹)</th>
<th>$k_{CT,model}$ (d⁻¹)ᵃ</th>
<th>$k_{CT,obs}$ (d⁻¹)</th>
<th>$k_{CT,model}$ (d⁻¹)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT Only</td>
<td>0.59 ± 0.06</td>
<td>0.57 ± 0.02</td>
<td>0.81 ± 0.09</td>
<td>0.79 ± 0.07</td>
</tr>
<tr>
<td>50 mM NaN₃</td>
<td>0.40 ± 0.01</td>
<td>0.39 ± 0.0004</td>
<td>1.05 ± 0.15</td>
<td>1.06 ± 0.10</td>
</tr>
<tr>
<td>50 µM TCE</td>
<td>1.05 ± 0.13</td>
<td>1.58 ± 0.12</td>
<td>1.73 ± 0.36</td>
<td>2.52 ± 0.65</td>
</tr>
</tbody>
</table>

ᵃ$k_{CT,model} = k_1 + k_3 + k_4$ from the first order analytical solution

4.2 Transformation of Chloroform and Dichloromethane

The VS-5L ADC+S also catalyzed faster transformation of biogenic CF than the EV-5L ADC+S in all treatments (Table 4.2). Transformation of CF was only complete when low concentrations (0.3 µM) were formed from the degradation of 0.86 µM CT (Figures A.3.1 and A.3.2). All other treatments did not catalyze complete CF transformation, even at long time scales (Figures 4.2, 4.3, and 4.4). It is possible that the VS-5L ADC+S is less susceptible to the inhibitory effects of CF leading to faster transformation. Additionally, it is possible that the VS-5L ADC+S is a more reduced system compared with the EV-5L ADC+S.

Table 4.2: Comparison of CF first order transformation rate constants for the EV-5L and VS-5L ADC+S. Rates were calculated from experimental CF data ($k_{CF,obs}$) and from an analytical first order model ($k_{CF,model}$). All treatments initially contained 2.6 µM CT. Error estimate calculated as one standard deviation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$k_{CF,obs}$ (d⁻¹)</th>
<th>$k_{CF,model}$ (d⁻¹)ᵃ</th>
<th>$k_{CF,obs}$ (d⁻¹)</th>
<th>$k_{CF,model}$ (d⁻¹)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT Only</td>
<td>0.029 ± 0.009</td>
<td>0.019 ± 0.003</td>
<td>0.045 ± 0.002</td>
<td>0.045 ± 0.004</td>
</tr>
<tr>
<td>50 mM NaN₃</td>
<td>0.017 ± 0.003</td>
<td>0.005 ± 0.001</td>
<td>0.021 ± 0.003</td>
<td>0.015 ± 0.001</td>
</tr>
<tr>
<td>50 µM TCE</td>
<td>0.028 ± 0.002</td>
<td>0.032 ± 0.005</td>
<td>0.043 ± 0.008</td>
<td>0.045 ± 0.010</td>
</tr>
</tbody>
</table>

ᵃ$k_{CF,model} = k_2 + k_5$ from the first order analytical solution
The ability of the ADC+S to transform CF and DCM not of biogenic origin was also tested. CF (2.1 µM) was significantly transformed by the EV-5L (97% degradation) and VS-5L (98% degradation) ADC+S (Figures A.4.1 and A.4.2). DCM was the measurable transformation product, amounting for 42-60% of the original mass as CF. When DCM is formed from 2.6 µM CT, it accounts for only 6-11% of the original mass as CT. It is likely that the remainder of the CF mass is being mineralized to CO₂ (Cappelletti et al., 2012). Transformation of CF followed first order kinetics, with the two ADC+S catalyzing CF degradation at similar rates (EV-5L, 0.073±0.023 d⁻¹; VS-5L, 0.072±0.007 d⁻¹) (Tables A.4.1 and A.4.2; Figure A.4.3).

Subsequent experiments were conducted with VS-5L at higher concentrations of CF (21.1 µM) and DCM (23.2 µM) to test the potential for transformation, and to determine if Dehalobacter species capable of CF respiration and DCM fermentation (Grostern et al., 2010; Justicia-Leon et al., 2012) were present in the D. mccartyi enriched mixed culture system. Partial CF transformation was observed after 100 days with 27% of the original CF mass remaining while DCM was not substantially transformed during the same time period (Figure A.4.4). In this experiment, the CF first order transformation rate was 0.014 ± 0.0004 d⁻¹ (Figure A.4.5). When compared to cultures capable of direct metabolism (Grostern et al., 2010; Justicia-Leon et al., 2012), the inability of the EV-5L and VS-5L ADC+S to rapidly degrade CF or DCM in the absence of CT suggests that transformation is cometabolic or not possible (DCM). The longer term exposures without achieving an accelerated rate of transformation indicate a Dehalobacter species was not stimulated.

### 4.3 Kinetics of Biotic and Abiotic Transformation

In the presence of 50 mM NaN₃, CT Transformation rates were not substantially affected in either the EV-5L or VS-5L ADC+S (Table 4.1). The VS-5L culture catalyzed slightly faster CT transformation when poisoned with 50 mM NaN₃ compared to a control group containing live cells. The opposite occurred in the EV-5L ADC+S, which catalyzed slightly slower CT transformation when poisoned with 50 mM NaN₃ compared to its control group. Due to numerous mechanisms that catalyze...
abiotic CT degradation, the presence of live cells appears to not be critical to achieve high transformation rates in the anaerobic dechlorinating cultures and supernatant. McCormick et al. (2002) found that CT transformation catalyzed by biogenic magnetite was 60-260 fold faster than that by the DIRB *G. metallireducens* alone. Additionally, an anaerobic enrichment culture grown on DCM experienced a 10-fold increase in CT transformation rates when amended with cyanocobalamin, pointing to the importance of enzymatic cofactors in catalyzing CT degradation (Hashsham et al., 1995). Numerous redox-active cofactors and microbial metal chelators exist that have been shown to catalyze CT transformation and enhance degradation rates (Penny et al., 2010), which are likely responsible for the transformation of CT in the NaN$_3$ poisoned anaerobic dechlorinating cultures.

While CT transformation rates were relatively unaffected, the product distribution was significantly impacted in the poisoned cells treatment (Figures 4.2 and 4.3). CS$_2$ formation was not observed in batch reactors poisoned with 50 mM NaN$_3$. Trace amounts of transient CS$_2$ were measured at the onset of the experiments, but disappeared within the first few hours. The initial CS$_2$ in these treatments was likely due to its presence as an impurity in the CT stock solution, rather than through formation from CT during the experiment. To confirm this, analytical CT standards were prepared in autoclaved Nanopure DI water (Barnstead NANOPure II; 16.5 megohm). The CT standards contained trace amounts of CS$_2$ comparable to the levels detected during the onset of the 2.6 µM CT and 50 mM NaN$_3$ treatment.

The absence of CS$_2$ in the poisoned cells treatment is unusual since numerous studies have confirmed CS$_2$ as an abiotic CT transformation product in reduced sulfide media (Hashsham et al., 1995; Hashsham and Freedman, 1999; Kriegman-King and Reinhard, 1992). It is possible that the formation of CS$_2$ occurred, but was not measurable due to the high concentration of NaN$_3$ present and the potential condensation reaction between CS$_2$ and the azide anion (Lieber et al., 1963). The use of an anaerobically-prepared formaldehyde solution (1% v/v) as a biocide was also attempted. Formaldehyde successfully shut down TCE reductive dehalogenation, but irreversibly bonded with the redox indicator (resazurin) in the media, thus creating a false positive for the presence of oxygen. Therefore, it was not possible to completely
confirm the anaerobic nature of the reactors with formaldehyde. Additionally, cells and 
supernatant were anaerobically centrifuged and filtered (0.22 µm filter) under glovebox 
conditions (95% N2/5% H2) to create cell-free extracts. However, the presence of FeS 
 solids successively clogged the filters making it unfeasible to filter 50 mL of 
supernatant and completely confirm the absence of cells.

Trace DCM was detected after approximately 80 days (EV-5L) and 90 days 
(VS-5L) of incubation with NaN3 (Figures 4.2 and 4.3). In the live cells treatment, 
DCM was detected as a transformation product as CT was nearing complete 
degradation. The live cells also subsequently catalyzed more rapid CF transformation 
than the poisoned treatment (Tables 4.2). The decrease in the CF transformation rate is 
likely driven by the absence of DCM, which is shown by an approximate order of 
magnitude reduction in the CF → DCM rate coefficient (k2) for both ADC+S (Tables 
4.3 and 4.4). It is possible that the live cells facilitate CF degradation by creating redox-
active cofactors that drive transformation (Cappelletti et al., 2012). This mechanism 
was likely shut down due to effects of the biocide, and any CF degradation and 
subsequent DCM formation occurred from the residual transformation capacity of the 
system after complete CT degradation.
Figure 4.2: Transformation of 2.6 μM CT by a) EV-5L and live cells, and b) EV-5L and 50 mM NaN₃. Application of biocide greatly diminished formation of CS₂ and DCM. Error bars represent one standard deviation.
Figure 4.3: Transformation of 2.6 µM CT by a) VS-5L and live cells, and b) VS-5L and 50 mM NaN₃. Application of biocide greatly diminished formation of CS₂ and DCM. Error bars represent one standard deviation.
Table 4.3: First order transformation rates for the EV-5L ADC+S estimated from the analytical solutions predicting CT degradation and product formation (Figure 3.1). All treatments contained 2.6 µM CT. Error estimate calculated as one standard deviation.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Treatment</th>
<th>$k_1 (d^{-1})^a$</th>
<th>$k_2 (d^{-1})^b$</th>
<th>$k_3 (d^{-1})^a$</th>
<th>$k_4 (d^{-1})^a$</th>
<th>$k_5 (d^{-1})^b$</th>
<th>$k_6 (d^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCl₄ → CHCl₃</td>
<td>CT Only</td>
<td>0.18 ± 0.008</td>
<td>0.009 ± 0.0006</td>
<td>0.053 ± 0.005</td>
<td>0.34 ± 0.009</td>
<td>0.01 ± 0.002</td>
<td>0.049 ± 0.02</td>
</tr>
<tr>
<td>CHCl₃ → CH₂Cl₂</td>
<td>50 mM NaN₃</td>
<td>0.12 ± 0.001</td>
<td>0.0007 ± 0.0005</td>
<td>0 ± 0</td>
<td>0.27 ± 0.001</td>
<td>0.004 ± 0.002</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>CCl₄ → CS₂</td>
<td>50 µM TCE</td>
<td>0.40 ± 0.048</td>
<td>0.015 ± 0.004</td>
<td>0.18 ± 0.016</td>
<td>0.99 ± 0.074</td>
<td>0.017 ± 0.008</td>
<td>0.041 ± 0.007</td>
</tr>
<tr>
<td>CHCl₃ → CO₂</td>
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<tr>
<td>CCl₄ → CO₂</td>
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<tr>
<td>CS₂ → CO₂</td>
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</tbody>
</table>

$^a k_{CT, model} = k_1 + k_3 + k_4$

$^b k_{CF, model} = k_2 + k_5$

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Table 4.4: First order transformation rates for the VS-5L ADC+S estimated from the analytical solutions predicting CT degradation and product formation (Figure 3.1). All treatments contained 2.6 µM CT. Error estimate calculated as one standard deviation.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Treatment</th>
<th>$k_1 (d^{-1})^a$</th>
<th>$k_2 (d^{-1})^b$</th>
<th>$k_3 (d^{-1})^a$</th>
<th>$k_4 (d^{-1})^a$</th>
<th>$k_5 (d^{-1})^b$</th>
<th>$k_6 (d^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCl₄ → CHCl₃</td>
<td>CT Only</td>
<td>0.31 ± 0.008</td>
<td>0.013 ± 0.002</td>
<td>0.094 ± 0.009</td>
<td>0.39 ± 0.066</td>
<td>0.032 ± 0.004</td>
<td>0.017 ± 0.004</td>
</tr>
<tr>
<td>CHCl₃ → CH₂Cl₂</td>
<td>50 mM NaN₃</td>
<td>0.42 ± 0.008</td>
<td>0.0009 ± 0.0004</td>
<td>0 ± 0</td>
<td>0.64 ± 0.093</td>
<td>0.014 ± 0.001</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>CCl₄ → CS₂</td>
<td>50 µM TCE</td>
<td>0.58 ± 0.051</td>
<td>0.028 ± 0.006</td>
<td>0.31 ± 0.069</td>
<td>1.63 ± 0.544</td>
<td>0.017 ± 0.005</td>
<td>0.026 ± 0.003</td>
</tr>
<tr>
<td>CHCl₃ → CO₂</td>
<td></td>
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<tr>
<td>CCl₄ → CO₂</td>
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<tr>
<td>CS₂ → CO₂</td>
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</tr>
</tbody>
</table>

$^a k_{CT, model} = k_1 + k_3 + k_4$

$^b k_{CF, model} = k_2 + k_5$
4.4 Simultaneous Transformation of CT and TCE

TCE (50 µM) and CT (2.6 µM) were rapidly and completely simultaneously transformed by the EV-5L and VS-5L cultures and supernatant. The primary CT transformation product measured was CF with lesser amounts of DCM and CS₂ formed, while TCE was completely respired to ETH (Figures 4.4, 4.5, and 4.7). In the presence of TCE, CT was transformed at an approximately 2-fold faster rate compared with the other treatments (Table 4.1). However, the faster CT transformation rate was not coupled to a faster CF transformation rate (Table 4.2). Instead, the CF transformation rates for both ADC+S were the same in the CT Only and the CT and 50 µM TCE treatments. Additionally, during the CT and TCE treatment the EV-5L ADC+S produced 15% (CT Only) and 27% (50 mM NaN₃) less CF while VS-5L ADC+S produced 41% (CT Only) and 53% (50 mM NaN₃) less CF. It was observed that the CT → CO₂ rate coefficient (k₄) estimated from the analytical model increased 3-fold (EV-5L ADC+S) and 4-fold (VS-5L ADC+S) during the CT and TCE treatment when compared to the CT Only treatment (Tables 4.3 and 4.4). This is suggesting that when cells are metabolizing TCE while CT is being abiotically transformed, the cells are shifting the abiotic CT product distribution. It is possible that the growing cells are exuding coenzymes such as vitamin B₁₂ capable of CT transformation, and are increasing the reducing power of the abiotic system leading to the formation of less CF and more CO₂ (Hashsham et al., 1995).

TCE Control reactors (50 µM TCE) without CT and CF saw complete, rapid respiration of multiple additions of TCE to ETH (Figures 4.6 and 4.8). Zero order TCE and cDCE dehalogenation rates (kₘₓ) were not substantially impacted by the presence of 2.6 µM CT (Figure 4.9). However, CT and the subsequent formation of CF resulted in a decrease in the VC to ETH transformation rate, which was reduced by 32% (EV-5L) and 58% (VS-5L). It should be noted that TCE and cDCE transformation occurred before the buildup of CF.
Figure 4.4: Simultaneous transformation of 2.6 µM CT and 50 µM TCE by the a) EV-5L and b) VS-5L cultures and supernatant. The presence of 50 µM TCE increased CT transformation rates and produced less CF in both cultures. Error bars represent one standard deviation.
Figure 4.5: Transformation of multiple additions of 50 µM TCE by the EV-5L culture. The initial dose was delivered at a) t = 0 d along with 2.6 µM CT. The subsequent dose was delivered at b) t = 14 d after long-term exposure to a maximum CF concentration of 1.4 µM. Figures show one replicate of triplicate batch reactors. Note the different time scales of the x axis.
Figure 4.6: Transformation of multiple additions of 50 µM TCE by the EV-5L culture without CT or CF. The two doses were delivered at a) t = 0 d and b) t = 14 d. Zero order CAH transformation rates from the second TCE addition exhibited minor reductions when compared to reactors exposed to CT and CF. Figures show one replicate of triplicate batch reactors. Note the different time scales of the x axis.
Figure 4.7: Transformation of multiple additions of 50 µM TCE by the VS-5L culture. The initial dose was delivered at a) $t = 0$ d along with 2.6 µM CT. The subsequent dose was delivered at b) $t = 14$ d after long-term exposure to a maximum CF concentration of 1.3 µM. Figures show one replicate of triplicate batch reactors. Note the different time scales of the x axis.
Figure 4.8: Transformation of multiple additions of 50 µM TCE by the VS-5L culture without CT or CF. The two doses were delivered at a) t = 0 d and b) t = 14 d. Zero order CAH transformation rates from the second TCE addition exhibited minor reductions when compared to reactors exposed to CT and CF. Figures show one replicate of triplicate batch reactors. Note the different time scales of the x axis.
Figure 4.9: Zero order transformation rates ($k_mX$) for the reductive dechlorination of 50 µM TCE by the a) EV-5L and b) VS-5L cultures. Lighter colored bars represent rates from a second addition at $t = 14$ d after the initial dose. Treatments only containing TCE (TCE Control) did not see a large decrease in rates over time. However, long-term exposure to CF (2.6 µM CT treatment) dramatically decreased transformation rates due to the inhibitory and possibly toxic effects of CF on the anaerobic dechlorinating cultures.
A subsequent addition of 50 µM TCE and 2 mM formate were added to the reactors in order to assess the impacts of long-term exposure to CF on the anaerobic dechlorinating cultures. Furthermore, the ability of growing cells to catalyze CF degradation was also explored through this additional dose of TCE. Zero order transformation rates for the chlorinated ethenes were drastically reduced by the 14-day exposure to CF$_{\text{max}}$ of 1.4 µM (EV-5L) and 1.3 µM (VS-5L) before the second addition of TCE (Figure 4.9). The EV-5L culture saw a 94% (TCE), 93% (cDCE), and 98% (VC) decrease in k$_{\text{mX}}$ rates while VS-5L saw 96% (TCE), 91% (cDCE), and 95% (VC) rate reductions. CF exhibited an inhibitory or possibly toxic effect on the dechlorinating cultures. Despite this, VC reduction to ETH was complete by VS-5L (Figure 4.7b) and nearly complete by EV-5L (Figure 4.5b) during the experimental timeframe.

In comparison, the observed reduction in k$_{\text{mX}}$ rates for a second addition of 50 µM TCE to the TCE Control reactors was not as drastic. EV-5L saw a 38% (TCE), 20% (cDCE), and 20% (VC) reduction in rates. VS-5L had decreased rates of 16% (TCE) and 19% (cDCE), while the estimated VC rate slightly increased (1%). The observed reduction in rates in the TCE Control group is likely due to endogenous decay of the cells over a 14-day period in which they were not receiving their growth substrates. The presence of growing cells metabolizing TCE from the second dose did not catalyze more rapid CF transformation in either culture. It is possible that the transformation capacity of the reducing system increased during the transformation of the second dose of TCE, but this was not reflected by an increase in CF degradation.

The ability to increase CT transformation rates and decrease the amount of CF formed while simultaneously degrading TCE and its metabolites is promising. The CT and CF transformation rates measured in this study fall within the range of pseudo-first order rates found in other systems. Reduced sulfide media containing HS$^-$ and biotite or vermiculite found that 1 µM CT was transformed at rates between 0.02-0.12 d$^{-1}$ (Kriegman-King and Reinhard, 1992). It was also found that G. metallireducens alone (4 µM CT) and biogenic magnetite alone (18.7 µM CT) catalyzed CT transformation at rates between 0.24-3.12 d$^{-1}$ and 0.24-1.68 d$^{-1}$ (McCormick et al., 2002). Pure cultures of the methanogens Methanosarcina barkeri and M. thermophila catalyzed transformation of 5-10 µM CT in the presence of Fe$^0$ at rates between 2.1-18.6 d$^{-1}$, with
faster rates in treatments containing cells and Fe⁰ (Novak et al., 1998a). Additionally, 5-10 µM CF was transformed in this system at rates of 0.21-16.2 d⁻¹, which is 1-3 orders of magnitude higher than observed with the ADC+S. The addition of vitamin B₁₂ homologs has been shown in numerous systems to dramatically increase the rate of CT degradation and decrease CF formation, shifting the pathway to the formation of CO, CO₂, and nonvolatile products (Hashsham et al., 1995; Hashsham and Freedman, 1999; Stromeyer et al., 1992; Workman et al., 1997).

Complex mixtures of contaminants complicate environmental cleanups and generally lead to less effective remediation (Bagley et al., 2000; Devlin and Müller, 1999; Justicia-Leon et al., 2014). These results suggest that the degradation of complex mixtures by D. mccartyi cultures and/or the associated abiotic conditions created by its growth deserve further exploration. While CT and CF exert inhibitory and possibly toxic effects on reductive dehalogenation (Bagley et al., 2000; He et al., 2005; Maymó-Gatell et al., 2001), the simultaneous transformation of chlorinated methanes and ethenes is desirable when designing bioremediation applications. A continuous flow column bioaugmented with the Evanite culture was capable of simultaneous transformation of PCE to VC (20%) and ETH (80%), and CT to CF (20%) and unknown products (80%) (Azizian and Semprini, in press). The inhibition of VC transformation observed in the batch tests was not observed in the column study. It is possible that the increase in H₂ concentration observed in the column study counteracted the inhibition of VC transformation. Additionally, the denitrifying organism Pseudomonas stutzeri KC degrades CT to CO₂ with little CF formation (Criddle et al., 1990a). By reducing or eliminating the formation of CF from CT degradation, it would be possible to simultaneously transform TCE and CT without exerting inhibitory or toxic effects on a reductive dechlorinating culture, thus decreasing the formation of undesirable metabolites and improving remediation efficiency.
CHAPTER 5: CHLOROFORM INHIBITION OF HYDROGEN CONSUMPTION

During the CT and CF transformation experiments, H\textsubscript{2} formation from formate and its subsequent consumption by the anaerobic mixed cultures was tracked over time. Formate is converted to H\textsubscript{2} by the following reaction: HCOO\textsuperscript{-} + H\textsubscript{2}O $\rightarrow$ HCO\textsubscript{3}\textsuperscript{-} + H\textsubscript{2}. Dissolved hydrogen is subsequently utilized by the EV-5L and VS-5L cultures for reductive dechlorination (Figure 5.1) (Marshall et al., 2014) and homoacetogenesis: 2CO\textsubscript{2} + 4H\textsubscript{2} $\rightarrow$ CH\textsubscript{3}COOH + 2H\textsubscript{2}O (Diekert and Wohlfarth, 1994; Drake et al., 2006). Previous transformation experiments and molecular analyses have confirmed the presence of acetate-producing microbes in the anaerobic dechlorinating cultures (Azizian and Semprini, in press; Marshall et al., 2014). The initial batch inhibition experiments were conducted in the absence of chlorinated ethenes in order to control for CF inhibition of CAH dehalorespiration.

A slug input of 2 mM formate (100 µmol H\textsubscript{2}/bottle) was provided as electron donor at the onset of all of the batch transformation studies. Formate was provided in excess in order to ensure that limited H\textsubscript{2} was not a confounding factor during the transformation experiments. Additionally, when excess formate was provided, enough H\textsubscript{2} was created in order to track its consumption over time throughout the experiments. Zero order maximum H\textsubscript{2} utilization rates were calculated for the EV-5L and VS-5L cultures to determine the impacts that CF had on H\textsubscript{2} consumption. In the presence of CF, H\textsubscript{2} buildup amounted to 70-100% of the expected H\textsubscript{2} to be produced (100...
µmol/bottle) from 2 mM formate. Control experiments conducted with 50 µM TCE in the absence of CF saw H₂ buildup to lower levels (50-80 µmol H₂/bottle) as the cultures were able to use it more rapidly (Figure 5.6).

Experiments conducted with 0.86 µM CT led to the formation of approximately 0.3 µM CF, which did not inhibit H₂ consumption by either the EV-5L or VS-5L culture (Figure 5.2). However, H₂ consumption was slower than in the complete absence of CT (Tables 5.2 and 5.3; Figure 5.6). After a buildup of H₂ occurred, it was rapidly consumed. When the cultures were dosed with 2.6 µM CT, 1.6 µM CF (EV-5L) and 2.2 µM CF (VS-5L) were measured as the maximum transient CF concentrations (CF<sub>max</sub>) (Figure 5.3). This led to essentially complete inhibition of H₂ consumption, which remained at high partial pressures in the batch reactor systems for 80 days (EV-5L) and 50 days (VS-5L). The consumption of H₂ occurred in the VS-5L culture sometime after 50 days of CF exposure, but was not analytically captured. Approximately 80% of the formate added accumulated as H₂. The VS-5L anaerobic dechlorinating culture and supernatant (ADC+S) was also observed to transform CF more rapidly than the EV-5L ADC+S.

It has been shown previously that low levels of CF can inhibit numerous anaerobic processes including reductive dechlorination (Bagley et al., 2000; He et al., 2005; Maymó-Gatell et al., 2001; Neumann et al., 1996) and homoacetogenesis (Liu et al., 2010; Scholten et al., 2000). It is therefore important to determine the CF threshold concentration for inhibition of H₂ consumption in the EV-5L and VS-5L cultures. It is unclear if CF is exerting a chemical inhibition on the homoacetogenic community in EV-5L and VS-5L, or if it is toxic to their cellular functions. Previous research has shown a connection between electron donor compounds, H₂ levels, and efficiency of reductive dechlorination (Azizian et al., 2010, 2008; Azizian and Semprini, in press; Behrens et al., 2008; Marshall et al., 2014).
Figure 5.2: The formation of 0.3 μM CF from 0.86 μM CT did not inhibit the utilization of 2 mM formate (100 μmol H₂/bottle) by the a) EV-5L and b) VS-5L cultures. CF was subsequently transformed. Error bars represent one standard deviation.
Figure 5.3: The formation of 1.6 μM CF (EV-5L) and 2.2 μM CF (VS-5L) from 2.6 μM CT inhibited the utilization of 2 mM formate (100 μmol H₂/bottle) by the a) EV-5L and b) VS-5L cultures. CF was slowly transformed. CF concentrations dropped below 0.6 μM in VS-5L, but H₂ consumption was not captured. Note the longer time scale compared to Figure 5.2. Error bars represent one standard deviation.
Multiple additions of 0.86 µM CT were added to the VS-5L culture in order to determine the CF threshold concentration for inhibition of H₂ consumption. As CF was produced from CT degradation, its transformation was tracked along with H₂ levels in the batch reactors (Figure 5.4). As H₂ was completely consumed, additional doses of 2 mM formate were added to the batch reactors with doses at t = 0 d, 27 d, and 56 d occurring simultaneously with an addition of 0.86 µM CT. H₂ consumption was not inhibited in the VS-5L culture by the transient buildup of less than 0.6 µM CF. When CF concentrations reached 0.6 µM from the second and third additions of 0.86 µM CT, H₂ partial pressures were transiently held static, and then rapidly declined as CF dropped below this level. The estimated zero order maximum H₂ use rates decreased with each subsequent addition of CF (Table 5.1). This is potentially due to endogenous decay of the cells when they were not receiving their growth substrates in addition to the effects of CF. It should also be noted that the rate of CF transformation decreased with each exposure.

![Figure 5.4](image-url)

Figure 5.4: Multiple additions of 0.86 µM CT produced between 0.3 – 0.68 µM CF<sub>max</sub>, which was subsequently transformed by the VS-5L culture. H₂ was produced from multiple additions of 2 mM formate to the batch reactor systems. H₂ consumption was correlated with CF concentrations less than 0.6 µM. Error bars represent one standard deviation.
Table 5.1: Estimated first order CF transformation rates ($k_{CF,obs}$) and zero order maximum H$_2$ use rates ($k_{H2,max}$) for the VS-5L ADC+S during the 0.86 µM CT multiple addition experiment. With each successive addition of 0.86 µM CT, CF transformation and H$_2$ consumption rates declined. Error estimate calculated as one standard deviation.

<table>
<thead>
<tr>
<th>CT Addition$^a$</th>
<th>[CF]$_{aq,max}$ (µM)</th>
<th>$k_{CF,obs}$ (d$^{-1}$)</th>
<th>$k_{H2,max}$ (µmol<em>bottle$^{-1}</em>$d$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.30</td>
<td>0.35 ± 0.059</td>
<td>12.97 ± 3.33</td>
</tr>
<tr>
<td>2</td>
<td>0.65</td>
<td>0.11 ± 0.015</td>
<td>8.63 ± 2.26</td>
</tr>
<tr>
<td>3</td>
<td>0.68</td>
<td>0.04 ± 0.007</td>
<td>5.05 ± 0.28</td>
</tr>
</tbody>
</table>

$^a$ Each addition represented a slug input of 0.86 µM CT and 2mM formate (100 µmol H$_2$/bottle)

To further explore this phenomenon, batch reactors were established with the EV-5L and VS-5L cultures inoculated with 2.1 µM CF, which is representative of the approximate CF$_{max}$ from the degradation of 2.6 µM CT. An initial dose of 2 mM formate (100 µmol H$_2$/bottle) was added at the start of the experiment with no subsequent additions. H$_2$ production was rapid and temporarily held static due to CF concentrations above the threshold inhibitory levels for the EV-5L (0.4 µM CF) and VS-5L (0.6 µM CF) cultures. However, H$_2$ partial pressures slowly declined above these levels, which differed from experiments conducted with 2.6 µM CT (Figure 5.3). This observed difference could be due to the longer exposure time to CF$_{max}$ when CF is biogenic versus when added directly. As CF dropped to the threshold inhibitory concentration, H$_2$ was rapidly consumed to near completion (Figure 5.5).
Figure 5.5: Transformation of 2.1 μM CF by the a) EV-5L and b) VS-5L ADC+S resulted in the reversible inhibition of H₂ use. As CF was degraded, H₂ consumption occurred to near completion in both cultures. Error bars represent one standard deviation of duplicate (EV-5L) and triplicate (VS-5L) reactors.
Inhibition of H$_2$ consumption for acetate production appears to be reversible at CF concentrations of 0.4 µM and 0.6 µM for the EV-5L and VS-5L cultures, respectively. This threshold differs between the cultures for undetermined reasons. Differences in the community composition and reduction potential of the cultures could be partly responsible for the differing abilities to tolerate exposure to CF. Experiments conducted with the acetogens *Acetobacterium woodii* and *Sporomosa ovata* found that 20 µM CF inhibited cell growth (Scholten et al., 2000). Although CF concentrations less than 20 µM were not examined, the researchers found that inhibition by CF was correlated with microorganisms that operate the Acetyl-CoA pathway. This pathway contains a corrinoid enzyme that serves as a carrier for methyl groups and is known to be inhibited by CF (Egli et al., 1988; Oremland and Capone, 1988). The Acetyl-CoA pathway has also been implicated in the transformation of CT and CF (Egli et al., 1988). Microbes in EV-5L and VS-5L could be utilizing the Acetyl-CoA pathway for homoacetogenesis, thus explaining CF inhibition of the cultures (Ferry, 1992; Thauer et al., 1989).

Experiments were conducted with 2.6 µM CT and 50 µM TCE in the EV-5L and VS-5L cultures in order to explore the possibility of simultaneous transformation and determine the inhibitory effects of CT and CF on the ADC+S (Section 4.4). H$_2$ levels were tracked throughout the course of the experiments to examine H$_2$ utilization by the cultures during chlorinated ethene (CAH) respiration in the presence and absence of CF. The following treatments were established: EV-5L & TCE (Figure 4.6); EV-5L, TCE, & CT (Figures 4.4a and 4.5); VS-5L & TCE (Figure 4.8); VS-5L, TCE, & CT (Figures 4.4b and 4.7). Formate (2 mM) was provided in excess (100 µmol H$_2$/bottle) in all treatments. The complete reduction of 50 µM TCE (4.2 µmol/bottle) to ETH requires 12.6 µmol H$_2$/bottle, which represents only 12.6% of the H$_2$ in the system. TCE was completely respired to ETH except for the second TCE addition in the EV-5L, TCE, & CT treatment, which resulted in approximately 40% VC and 60% ETH after 14 days (Figure 4.5b).

The TCE Control treatments experienced a transient buildup of H$_2$ followed by a subsequent rapid decrease as it was used by the cultures for reductive dechlorination and homoacetogenesis (Figure 5.6a). Maximum H$_2$ use rates are reported in Tables 5.2
and 5.3. The EV-5L, TCE, & CT treatment experienced a slow release of H$_2$ from formate, which remained at a high level due to the presence of CF greater than its threshold level of 0.4 µM (Figure 5.6b). The VS-5L, TCE, & CT treatment behaved differently from previous CT and CF experiments, and also exhibited H$_2$ dynamics different from the EV-5L culture under the same conditions. Despite VS-5L being exposed to a CF concentration greater than its apparent threshold inhibition level of 0.6 µM CF, H$_2$ was consumed over a 10-day period after complete TCE respiration to ETH had occurred (Figures 4.7a and 5.6a). A subsequent addition of 50 µM TCE and 2 mM formate were added to all reactors at t = 14 d. The EV-5L & TCE and VS-5L & TCE treatments experienced rapid formation of H$_2$ and subsequent consumption similar to the first addition, albeit at slower rates. The EV-5L, TCE, & CT treatment again showed a slow release of H$_2$ which then remained static at a high H$_2$ partial pressure. The VS-5L, TCE, & CT treatment once again catalyzed rapid formation of H$_2$, which was then consumed before CF dropped to 0.6 µM at t = 20 d.

The fastest zero order maximum H$_2$ use rates ($k_{\text{H}_2,\text{max}}$) for the EV-5L and VS-5L cultures occurred in the absence of CT and CF while respiring TCE and daughter products. Maximum H$_2$ consumption rates decreased due to CF exposure and endogenous decay when the cultures were not provided their growth substrates. A second addition of 50 µM TCE and 2 mM formate saw $k_{\text{H}_2,\text{max}}$ decrease by 55% (EV-5L) and 70% (VS-5L) in the TCE Control treatments. The observed reduction in $k_{\text{H}_2,\text{max}}$ in the TCE Control group is likely due to endogenous decay of the cells over a 14-day period in which they were not receiving TCE and formate. The opposite trend was observed in the VS-5L, TCE, & CT treatment, with $k_{\text{H}_2,\text{max}}$ from the second 2 mM formate addition increasing by 1.5-fold over the first addition. This occurred as CF$_{\text{max}}$ decreased from 1.3 µM to 0.86 µM during H$_2$ consumption of the first and second additions of formate, respectively. It is possible that the lower CF$_{\text{max}}$ concentration was less inhibitory to the VS-5L culture, thus allowing it to consume H$_2$ at a more rapid rate. When a culture was exposed to CF, $k_{\text{H}_2,\text{max}}$ never reached the maximum rate achieved by the TCE Control reactors. From these data and analyses, it is unclear whether CF is exerting a chemical inhibition, toxicity, or both on the H$_2$ consuming community in the anaerobic cultures. However, the increase in $k_{\text{H}_2,\text{max}}$ during the
second addition of TCE (50 µM) and formate (2 mM) in the VS-5L, TCE, & CT treatment suggests that long-term exposure to CF is not toxic to the H₂ utilizers.

Figure 5.6: Formation and consumption of a) H₂ and b) CF during the reductive dechlorination of 50 µM TCE and the simultaneous transformation of 2.6 µM CT and 50 µM TCE. H₂ consumption by the EV-5L culture was inhibited by 1.4 µM CF while 1.3 µM CF partially inhibited H₂ use by the VS-5L culture. This is shown by differences in kₘₜₐₓ in Tables 5.2 and 5.3. Error bars represent one standard deviation.
Table 5.2: Comparison of first order CF transformation rates and zero order maximum H$_2$ use rates for the EV-5L ADC+S. H$_2$ consumption was fastest in the absence of CF and reversibly inhibited above 0.4 µM CF. Error estimate calculated as one standard deviation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CT, CF, or TCE Addition$^a$</th>
<th>[CF]$_{aq,max}$ (µM)</th>
<th>k$_{CF,obs}$ (d$^{-1}$)</th>
<th>k$_{H2,max}$ (µmol*bottle$^{-1}$ *day$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.86 µM CT</td>
<td>1</td>
<td>0.3</td>
<td>0.38 ± 0.14</td>
<td>17.99 ± 1.49</td>
</tr>
<tr>
<td>2.6 µM CT</td>
<td>1</td>
<td>1.65</td>
<td>0.029 ± 0.009</td>
<td>0.126 ± 0.009</td>
</tr>
<tr>
<td>2.1 µM CF</td>
<td>1</td>
<td>2.1</td>
<td>0.074 ± 0.022</td>
<td>7.25 ± 0.86</td>
</tr>
<tr>
<td>50 µM TCE &amp; 2.6 µM CT</td>
<td>1</td>
<td>1.4</td>
<td>0.028 ± 0.002</td>
<td>-1.04 ± 0.39$^b$</td>
</tr>
<tr>
<td>50 µM TCE &amp; 2.6 µM CT</td>
<td>2$^c$</td>
<td>0.87</td>
<td>0.028 ± 0.002$^c$</td>
<td>0.55 ± 1.48</td>
</tr>
<tr>
<td>50 µM TCE$^d$</td>
<td>1</td>
<td>0$^d$</td>
<td>0$^d$</td>
<td>36.86 ± 1.96</td>
</tr>
<tr>
<td>50 µM TCE$^d$</td>
<td>2$^c$</td>
<td>0$^d$</td>
<td>0$^d$</td>
<td>16.47 ± 2.92</td>
</tr>
</tbody>
</table>

$^a$ Slug inputs of CT, CF, or TCE to the batch reactors were accompanied by an addition of 2 mM formate (100 µmol H$_2$/bottle).

$^b$ H$_2$ partial pressures slowly increased throughout the CF exposure, which is represented by a negative H$_2$ maximum use rate.

$^c$ The second addition consisted of 50 µM TCE and 2 mM formate. No additional CT or CF was added.

$^d$ TCE and H$_2$ Control experiments conducted in the absence of CT and CF.
Table 5.3: Comparison of first order CF transformation rates and zero order maximum H₂ use rates for the VS-5L ADC+S. H₂ consumption was fastest in the absence of CF and reversibly inhibited above 0.6 µM CF. H₂ was slightly inhibited in the 50 µM TCE & 2.6 µM CT treatment. Error estimate calculated as one standard deviation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CT, CF, or TCE Addition</th>
<th>[CF]_{aq, max}(µM)</th>
<th>k_{CF,obs}(d⁻¹)</th>
<th>k_{H₂,max}(µmol<em>bottle⁻¹</em>day⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.86 µM CT</td>
<td>1</td>
<td>0.3</td>
<td>0.35 ± 0.058</td>
<td>16.95 ± 1.28</td>
</tr>
<tr>
<td>2.6 µM CT</td>
<td>1</td>
<td>2.25</td>
<td>0.045 ± 0.002</td>
<td>ND b</td>
</tr>
<tr>
<td>2.1 µM CF</td>
<td>1</td>
<td>2.1</td>
<td>0.072 ± 0.07</td>
<td>4.74 ± 0.33</td>
</tr>
<tr>
<td>50 µM TCE &amp; 2.6 µM CT</td>
<td>1</td>
<td>1.3</td>
<td>0.043 ± 0.008</td>
<td>7.73 ± 1.16</td>
</tr>
<tr>
<td>50 µM TCE &amp; 2.6 µM CT</td>
<td>2 c</td>
<td>0.86</td>
<td>0.043 ± 0.008 c</td>
<td>11.34 ± 1.71</td>
</tr>
<tr>
<td>50 µM TCE d</td>
<td>1</td>
<td>0 d</td>
<td>0 d</td>
<td>49.82 ± 7.94</td>
</tr>
<tr>
<td>50 µM TCE d</td>
<td>2 c</td>
<td>0 d</td>
<td>0 d</td>
<td>15.11 ± 1.75</td>
</tr>
</tbody>
</table>

a Slug inputs of CT, CF, or TCE to the batch reactors were accompanied by an addition of 2 mM formate (100 µmol H₂/bottle).

b H₂ use rate not determined due to insufficient data.

c The second addition consisted of 50 µM TCE and 2 mM formate. No additional CT or CF was added.

d TCE and H₂ Control experiments conducted in the absence of CT and CF.
Since complete TCE respiration to ETH only requires 12.6 µmol H₂/bottle, and ETH was the only detectable metabolite in VS-5L (Figure 4.7) from 0.5 days after the first addition and from 6 days after the second TCE addition (t = 20 d), the possibility that H₂ consumption occurred solely due to CAH reductive dechlorination is unlikely. Additionally, the reductive dechlorination of 2.6 µM CT to 1.3 µM CF and 0.58 µM DCM would not be responsible for the consumption of 200 µmol H₂/bottle created from two 2 mM doses of formate (Figure 4.4b). Therefore, it is likely that the consumption of H₂ was being driven by homoacetogenesis, despite CF being higher than the apparent threshold inhibition concentration (0.6 µM CF).

It is possible that the VS-5L culture is more resilient against CF inhibition when initially stimulated with TCE and H₂. CF is a known inhibitor of methanogenesis (Bauchop, 1967b) and is used for this purpose when studying different methanogenic cultures. Previous research has found that H₂ consumption in lake or sediment environments (Conrad et al., 1989; Lovley and Klug, 1983) and in anaerobic digesters (Chen et al., 2008; Hu and Chen, 2007; Saady, 2013; Xu et al., 2010) has been partially or not inhibited by low levels of CF while methanogenesis has been completely inhibited. First order H₂ consumption rates in anoxic enrichment cultures from lake or sediment environments decreased by 60-75% (Conrad et al., 1989) and 26% (Lovley and Klug, 1983) when inhibited by 100 µM CF and 0.003% v/v CF, respectively. This points to the ability of homoacetogenic cultures to be able to tolerate low concentrations of CF, although this does not explain the differences between the EV-5L and VS-5L cultures. It also does not provide an explanation as to why VS-5L would consume H₂ after metabolizing TCE when CF concentrations are greater than 0.6 µM (Figure 5.6) but not when transforming CT and CF alone (Figure 5.3b). It is likely that the duration of CF exposure and the presence of growth substrates are important determining factors for CF inhibition of H₂ consumption.

Additionally, H₂ consumption in VS-5L could be driven by Geobacter species, which have been previously identified by molecular analysis in the EV-5L and VS-5L chemostats (Mayer-Blackwell et al., 2014). In anaerobic digester sludge inhibited with CF, Geobacter hydrogenophilus was found to grow on elevated levels of acetate and H₂, which it can oxidize for growth (Xu et al., 2010). Xu et al. (2010) also found that
the consumption of H₂ and production of acetate was possibly syntrophic and proceeded through the formation of propionate and butyrate. It was proposed that these fatty acids were used syntrophically by acetogenic *Syntrophomonas* and *Syntrophobacter* species along with homoacetogenic bacteria to form acetate. It is therefore possible that a complex bacterial community is present in the EV-5L and VS-5L cultures in addition to *D. mccartyi*, and these lower abundance species are driving the consumption of H₂ under CF inhibition conditions. The increase in $k_{H_2, max}$ during the second addition of TCE (50 µM) and formate (2 mM) in the VS-5L, TCE, & CT treatment suggests that long-term exposure to CF is not toxic to the H₂ utilizers. Instead, CF might be exerting a reversible chemical inhibition which can be minimized by providing growth substrates to the culture. Molecular characterization of the low abundance species in the anaerobic dechlorinating cultures would help to elucidate the complex dynamics of H₂ formation and subsequent consumption under different electron donor and CAH conditions. Furthermore, studies aimed at determining the mechanism driving CF inhibition for H₂ consumption would probe the role of H₂ and the complex cast of characters competing for it in the anaerobic dechlorinating cultures.
CHAPTER 6: CONCLUSIONS

CT was completely and rapidly transformed by cells and the supernatant harvested from chemostats that dehalogenate TCE to ETH. The primary transformation products measured were CF, DCM, and CS₂, with CF and CS₂ being subsequently transformed. Treatments conducted with live cells, poisoned cells, and cells growing on TCE and H₂ showed variable CT transformation rates with the fastest rates catalyzed by cells that were concurrently metabolizing TCE and its reduction products. CT (2.6 µM) was not inhibitory to TCE and cDCE dehalorespiration, but CF production inhibited the VC to ETH step. Long-term exposure to CF dramatically impacted TCE and daughter product metabolism. It is not known why enhanced CT and CF rates occurred during TCE dehalogenation, but it may be associated with the cometabolic nature of the transformations, thus more studies are warranted. Understanding the complex dynamics of co-contaminants, inhibition, and different transformation mechanisms is important to improve the efficiency of in situ remediation applications.

Low concentrations of CF appear to reversibly inhibit the consumption of H₂ by anaerobic dechlorinating cultures. In the absence of growth substrates, H₂ consumption by the anaerobic dechlorinating cultures was inhibited by CF concentrations greater than 0.4 µM (EV-5L) and 0.6 µM CF (VS-5L). When CF was transformed below these concentrations, H₂ consumption was rapid and near complete. However, the VS-5L culture displayed some resilience to CF inhibition and was able to use H₂ at CF concentrations as high as 1.3 µM after metabolizing an initial 50µM dose of TCE to ETH (Figure 4.7). It is likely that H₂ consumption in these cultures is being driven by reductive dechlorination and homoacetogenesis, which has been measured previously (Marshall et al., 2014). It is not yet possible to explain the role different microbes play in H₂ consumption and the mechanism by which CF inhibits the complex anaerobic mixed cultures. However, an increase in k₉₂,max during respiration of a second dose of 50 µM TCE in the presence of 0.86 µM CF suggests that CF is exerting a chemical inhibition rather than toxicity to the H₂ consuming species. More research that probes deeper into the molecular composition of the cultures is needed to explain how different electron donors and H₂ influence community structure.
BIBLIOGRAPHY


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APPENDIX
A.1 Sterile Anaerobic Mineral Media Transformation of Carbon Tetrachloride

Figure A.1.1: Controls with a) 2.6 \( \mu \text{M} \) and b) 8.6 \( \mu \text{M} \) CT were conducted in the sterile anaerobic reduced sulfide media. The anaerobic media did not contain any chlorinated compounds, and its preparation has been described previously (Yang and McCarty, 1998). Partial CT transformation occurred, but was substantially slower when compared with the anaerobic dechlorinating cultures and supernatant. Further analyses were not conducted. Error bars represent one standard deviation.
A.2 Observed First Order CT and CF Transformation Rates

Rate coefficients were calculated for CT and CF degradation from experimental data using a first order transformation model and a natural log-linear regression. CT and CF degradation were calculated as follows:

\[
\frac{dM_{CT}}{dt} = -(k_{CT,obs})M_{CT}
\]

\[
\ln(M_{CT}) = -(k_{CT,obs})t + \ln(M_{CT,0})
\]

\[
\frac{dM_{CF}}{dt} = -(k_{CF,obs})M_{CF}
\]

\[
\ln(M_{CF}) = -(k_{CF,obs})t + \ln(M_{CF,0})
\]

where \(M_{CT}\) and \(M_{CF}\) are the number of μmol/bottle in a batch reactor at any given time, \(k_{CT,obs}\) and \(k_{CF,obs}\) are the experimental CT and CF transformation rates, \(t\) is time, and \(M_{CT,0}\) and \(M_{CF,0}\) are the initial amounts of CT or CF introduced to the system at \(t=0\). Observed CF rates were also calculated from the maximum CF concentration formed from CT, which represented \(M_{CF,0}\) in the analysis. First order kinetics were applied based on experimental results and previous studies (Butler and Hayes, 2000; Kriegman-King and Reinhard, 1992; McCormick et al., 2002; Novak et al., 1998a; Vogel et al., 1987).
Figure A.2.1: Estimation of first order CT transformation rates ($k_{CT, obs}$) using a natural log – linear regression for the a) EV-5L and b) VS-5L cultures and supernatant. The initial aqueous CT concentration was 0.86 µM.
Figure A.2.2: Estimation of first order CT transformation rates ($k_{CT,obs}$) using a natural log – linear regression for the a) EV-5L and b) VS-5L cultures and supernatant. The initial aqueous CT concentration was 2.6 µM.
Figure A.2.3: Estimation of first order CT transformation rates ($k_{CT, obs}$) using a natural log – linear regression for the a) EV-5L and b) VS-5L cultures and supernatant. The initial aqueous CT concentration was 8.6 µM.
A.3 Carbon Tetrachloride Transformation Capacity Experiments

Experiments were conducted to examine the transformation capacity of the anaerobic cultures. Multiple additions of 0.86 µM CT were added to triplicate batch reactors containing cells and supernatant from the EV-2L (Figure A.2.1a), EV-5L (Figure A.2.1b), VS-2L (Figure A.2.2a), or VS-5L (Figure A.2.2b) chemostats as described in Chapter 3. Additions of 0.86 µM CT occurred after complete transformation of the previous dose of CT and after CF was partially transformed. Subsequent additions of formate were added when H₂ levels dropped below 10 µmol/bottle. Only CT and CF were measured during these experiments due to analytical limitations. First order CT (k_{CT,obs}) and CF (k_{CF,obs}) transformation rate constants were calculated for each CT addition.

A non-linear decrease in k_{CT,obs} and k_{CF,obs} occurred with each subsequent CT addition for all cultures (Figure A.3.3). This is due to the cometabolic and abiotic mechanisms that catalyze CT and CF transformation. CF transformation rates were 1-2 orders of magnitude less than the observed CT transformation rate. The VS cultures catalyzed faster CT transformation than the EV cultures.
Figure A.3.1: Transformation of multiple additions of 0.86 μM CT and CF by the a) EV-2L and b) EV-5L ADC+S. Error bars represent one standard deviation.
Figure A.3.2: Transformation of multiple additions of 0.86 μM CT and CF by the a) VS-2L and b) VS-5L ADC+S. Error bars represent one standard deviation.
Figure A.3.3: Decreases in a) $k_{CT,obs}$ and b) $k_{CF,obs}$ occurred when reactors were successively spiked with 0.86 µM CT after complete transformation of the previous addition. Error bars represent one standard deviation.
A.4 Transformation of Chloroform by the EV-5L and VS-5L Cultures and Supernatant

The transformation of biogenic CF was observed in the EV and VS ADC+S (Chapter 4). Experiments were conducted in order to determine the extent to which CF could be transformed when added to the EV-5L and VS-5L ADC+S in the absence of CT. An initial CF concentration of 2.1 µM was chosen because it was the approximate maximum CF concentration (CF$_{\text{max}}$) produced by the reduction of 2.6 µM CT. CF transformation was not complete, with DCM measured as the transformation product (Figures A.4.1 and A.4.2). Formate (2 mM) was added as an electron donor to the system. H$_2$ was completely consumed when CF concentrations dropped below 0.4 µM (EV-5L) and 0.6 µM (VS-5L) (Chapter 5). A subsequent addition of 2.1 µM CF was added to the system without an addition of formate. CF transformation did continue to occur, albeit slowly. This is likely due to the toxicity exerted by long exposure to CF on anaerobic microbial processes (Bagley et al., 2000; He et al., 2005) and a reduced transformation capacity in the system.

An analytical first order solution was developed to simultaneously estimate the rate of transformation for CF and its degradation products by fitting data from the batch kinetic experiments. The model was developed by isolating CF and its transformation products from within the larger CT kinetic model (Figure 3.1). CF degradation and product formation was modeled using a series of first order analytical solutions developed from the following ordinary differential equations:

\[
\frac{dM_{\text{CF}}}{dt} = -(k_2 + k_5)M_{\text{CF}} \quad (5)
\]

\[
\frac{dM_{\text{DCM}}}{dt} = k_2M_{\text{CF}} \quad (6)
\]

\[
\frac{dM_{\text{CO}_2}}{dt} = k_5M_{\text{CF}} \quad (7)
\]

Transformation rate coefficients were estimated by a non-linear least squares regression performed using the Solver Tool Pack (Microsoft Excel 2016). Model validity was confirmed by comparing the observed CF transformation rates ($k_{\text{CF,obs}}$) with those generated by the analytical solution ($k_{\text{CF,model}} = k_2 + k_5$). The formation of
CO₂ from CF has been previously confirmed (Cappelletti et al., 2012). Therefore, the kinetic model incorporates a mass balance approach and assumes that the unknown fraction is $M_{CO₂} = M_{CF,0} - (M_{CF} + M_{DCM})$ at any given time.

Observed CF (2.1 µM) transformation rates were comparable for the EV-5L and VS-5L ADC+S (Table A.4.1). However, more variability occurred during the CF transformation experiments compared with the CT studies, especially in the EV-5L ADC+S (Table A.4.2). The analytical solution overestimated the rate of CF transformation when compared to the observed CF rate. The strong inhibitory and/or toxic effects of CF seem to affect the kinetics of its transformation. Additionally, it appears that transformation of CF by the ADC+S is very sensitive to small changes in CF concentration, indicating a possible self-inhibition.

Table A.4.1: Comparison of CF first order transformation rate constants for the EV-5L and VS-5L ADC+S. Rates were calculated from experimental CF data ($k_{CF,obs}$) and from an analytical first order model ($k_{CF,model}$). Error estimate calculated as one standard deviation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$k_{CF,obs}$ (d⁻¹)</th>
<th>$k_{CF,model} (d^{-1})$⁸</th>
<th>$k_{CF,obs}$ (d⁻¹)</th>
<th>$k_{CF,model} (d^{-1})$⁸</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 µM CF</td>
<td>0.074 ± 0.023</td>
<td>0.168 ± 0.082</td>
<td>0.072 ± 0.007</td>
<td>0.107 ± 0.002</td>
</tr>
</tbody>
</table>

⁸$k_{CF,model} = k_2 + k_5$ from the first order analytical solution

Table A.4.2: First order transformation rates estimated from the analytical solutions predicting CF degradation and product formation. Comparison of CF rate constants for the EV-5L and VS-5L ADC+S. Error estimate calculated as one standard deviation.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>EV-5L</th>
<th>VS-5L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CHCl₃ → CH₂Cl₂</td>
<td>CHCl₃ → CO₂</td>
</tr>
<tr>
<td>Treatment</td>
<td>$k_2$ (d⁻¹)</td>
<td>$k_5$ (d⁻¹)</td>
</tr>
<tr>
<td>2.1 µM CF</td>
<td>0.062 ± 0.027</td>
<td>0.106 ± 0.056</td>
</tr>
</tbody>
</table>
Figure A.4.1: Transformation of 2.1 μM CF by the a) EV-5L and b) VS-5L ADC+S. A second addition of 2.1 μM CF was partially transformed after a near complete transformation of the initial addition. DCM was the measured transformation product. Duplicate and triplicate reactors were measured for the EV-5L and VS-5L cultures, respectively. Error bars represent one standard deviation.
Figure A.4.2: Analytical solution for the transformation of 2.1 µM CF by the a) EV-5L and b) VS-5L ADC+S. The first order model overestimated CF transformation. In this analysis, triplicate reactors were included for both of the ADC+S systems. Error bars represent one standard deviation.
Figure A.4.3: Estimation of first order CF transformation rates ($k_{CF,obs}$) using a natural log – linear regression for the a) EV-5L and b) VS-5L cultures and supernatant. The initial aqueous CF concentration was 2.1 µM.
Figure A.4.4: Transformation of a) 21.1 µM CF and b) 23.1 µM DCM by the VS-5L culture and supernatant. Partial CF transformation and an insubstantial amount of DCM transformation occurred during a 100-day period. Error bars represent one standard deviation.
Figure A.4.5: Estimation of first order CF transformation rates ($k_{CF,obs}$) using a natural log – linear regression for VS-5L culture and supernatant. The initial aqueous CF concentration was 21.1 µM.
A.5 Carbon Tetrachloride Transformation by the Point Mugu Anaerobic Mixed Cultures and Supernatant

The PM-2L and PM-5L anaerobic mixed cultures are PCE-respiring, chemostat-grown cultures enriched in *D. mceartyi* species. The cultures use PCE (1.12 mM) as an electron acceptor and H₂ as an electron donor, which is created from the fermentation of 4.3 mM lactate (CH₃-HCOH-COO⁻) through the following reactions:

1. Lactate + 2H₂O → Acetate (CH₃COO⁻) + HCO₃⁻ + H⁺ + 2H₂
2. Lactate → 1/3 Acetate + 2/3 Propionate (CH₃-CH₂-COO⁻) + 1/3 HCO₃⁻ + 1/3 H⁺
3. Propionate + 3H₂O → Acetate + HCO₃⁻ + H⁺ +3H₂

Additionally, PM-2L contains 1.0 mM SO₄²⁻ as a competing electron acceptor, which is completely reduced in the continuous flow reactor. A thorough investigation of the chemical and molecular dynamics of the PM-2L and PM-5L chemostat systems has been done previously (Berggren et al., 2013).

The PM-2L and PM-5L ADC+S have the capability to transform low concentrations of CT at slow rates to CF (29-40%), CS₂ (5-10%), and an unknown fraction (46-71%). CF was not subsequently transformed (Figures A.5.1 and A.5.2). The PM-2L and PM-5L ADC+S do not catalyze CT transformation rapidly due to lower biomass concentrations than the EV and VS cultures (Table A.5.1) (Berggren et al., 2013; Marshall et al., 2014; Mayer-Blackwell et al., 2014). Biomass concentration has been shown previously to be a determining factor in CT transformation rates (McCormick et al., 2002). The PM-2L and PM-5L cultures contain less biomass than the EV and VS cultures due to an influent feed containing saturated PCE (1.12 mM) compared to TCE (10 mM), thus allowing for less cell growth. CT transformation by PM-2L and PM-5L followed pseudo-first order kinetics (Figure A.5.3) with transformation rates being 1-2 orders of magnitude less than the EV and VS ADC+S. An analytical solution found that modeled transformation rates were overestimated based on the estimated observed rates (Tables A.5.2 and A.5.3).
Table A.5.1: Measurements of total suspended solids (TSS) in the continuous flow reactors containing anaerobic dehalogenating mixed cultures. The EV and VS chemostats contain higher biomass concentrations due to the higher solubility of their electron acceptor TCE (10 mM) compared to that of the PM cultures (PCE; 1.12 mM).

<table>
<thead>
<tr>
<th></th>
<th>EV-2L&lt;sup&gt;b&lt;/sup&gt;</th>
<th>EV-5L&lt;sup&gt;b&lt;/sup&gt;</th>
<th>VS-2L&lt;sup&gt;b&lt;/sup&gt;</th>
<th>VS-5L&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PM-2L&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PM-5L&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSS&lt;sup&gt;a&lt;/sup&gt; (mg/L)</td>
<td>46.1</td>
<td>35</td>
<td>49.4</td>
<td>38.5</td>
<td>26.1</td>
<td>21.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> TSS serves as a proxy measurement for biomass with approximately 50% of TSS being cellular protein

<sup>b</sup> Most recent biomass measurements reported were taken on 02/16/2016

Table A.5.2: Comparison of CT first order transformation rate constants for the PM-2L and PM-5L ADC+S. Rates were calculated from experimental CT data (k<sub>CT,obs</sub>) and from an analytical first order model (k<sub>CT,model</sub>). Error estimate calculated as one standard deviation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PM-2L</th>
<th>PM-5L</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.6 µM CT</td>
<td>k&lt;sub&gt;CT,obs&lt;/sub&gt; (d&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>k&lt;sub&gt;CT,model&lt;/sub&gt; (d&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.036 ± 0.005</td>
<td>0.073 ± 0.016</td>
<td>0.062 ± 0.022</td>
</tr>
</tbody>
</table>

<sup>a</sup> k<sub>CT,model</sub> = k<sub>1</sub> + k<sub>3</sub> + k<sub>4</sub> from the first order analytical solution

Table A.5.3: First order transformation rates estimated from the analytical solutions predicting CT degradation and product formation. Comparison of CT rate constants for the PM-2L and PM-5L ADC+S. Error estimate calculated as one standard deviation.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>CCl&lt;sub&gt;4&lt;/sub&gt; → CHCl&lt;sub&gt;3&lt;/sub&gt;</th>
<th>CCl&lt;sub&gt;4&lt;/sub&gt; → CS&lt;sub&gt;2&lt;/sub&gt;</th>
<th>CCl&lt;sub&gt;4&lt;/sub&gt; → CO&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADC+S</td>
<td>k&lt;sub&gt;1&lt;/sub&gt; (d&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>k&lt;sub&gt;3&lt;/sub&gt; (d&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>k&lt;sub&gt;4&lt;/sub&gt; (d&lt;sup&gt;-1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>PM-2L</td>
<td>0.029 ± 0.006</td>
<td>0.008 ± 0.002</td>
<td>0.036 ± 0.008</td>
</tr>
<tr>
<td>PM-5L</td>
<td>0.051 ± 0.014</td>
<td>0.011 ± 0.003</td>
<td>0.096 ± 0.033</td>
</tr>
</tbody>
</table>

<sup>a</sup> k<sub>CT,model</sub> = k<sub>1</sub> + k<sub>3</sub> + k<sub>4</sub> from the first order analytical solution
Figure A.5.1: Transformation of 0.86 µM CT by the a) PM-2L and b) PM-5L ADC+S. No appreciable CF transformation occurred in these systems. Error bars represent one standard deviation.
Figure A.5.2: Transformation of 2.6 µM CT by the a) PM-2L and b) PM-5L ADC+S. No appreciable CF transformation occurred in these systems. Error bars represent one standard deviation.
Figure A.5.3: Pseudo-first order CT transformation rates by the PM-2L and PM-5L ADC+S. As \([\text{CT}_0]_{\text{aq}}\) increased, \(k_{\text{CT,obs}}\) decreased in a non-linear manner. Error bars represent one standard deviation.

The PM-2L and PM-5L ADC+S receive lactate as a fermenting electron donor in the continuous flow reactors, and were dosed with 2 mM lactate at the start of the batch transformation studies. Based on the fermentation of lactate from the equations above, 2 mM lactate should yield 267 µmol H\(_2\)/bottle, making it a more effective electron donor than formate where 2 mM formate yields 100 µmol H\(_2\)/bottle. However, it appears that the complete fermentation of lactate to H\(_2\) is inhibited by the presence of CF in the batch reactor systems. When batch reactors were inoculated with either the PM-2L or PM-5L ADC+S, 2.6 µM CT, and 2 mM lactate, only 7.5% (20 µmol H\(_2\)/bottle) of the possible H\(_2\) (267 µmol H\(_2\)/bottle) formed, based on the stoichiometry of lactate fermentation (Figure A.5.4). If the fermentation of propionate to H\(_2\) is inhibited, 67 µmol H\(_2\)/bottle should still form from the fermentation of lactate to acetate and H\(_2\). The low levels of H\(_2\) formed show that multiple lactate fermentation reactions are inhibited by the presence of CF.

This was explored further when the PM-2L and PM-5L ADC+S were dosed with 0.86 µM CT, 2 mM lactate, and 2 mM formate. The lower CF concentrations
formed from the transformation of 0.86 µM CT should not be as inhibitory to the cultures. It was found that the addition of 2 mM formate and 2 mM lactate did allow for H₂ levels to accumulate to a higher level when compared to batch reactors that were dosed only with 2 mM lactate. However, H₂ levels only reached maxima of 68 µmol H₂/bottle (PM-2L) and 96 µmol H₂/bottle (PM-5L). The remaining possible H₂ not formed was either consumed by a competing electron acceptor reaction and not measured, or did not occur due to the inhibition of CF. More research is needed to elucidate the complex dynamics of CF inhibition of fermentation reactions in the anaerobic dechlorinating cultures.

Figure A.5.4: H₂ was added to the PM-2L and PM-5L reactors as lactate (2 mM) and formate (2 mM). The PM-2L and PM-5L reactors spiked with 0.86 µM CT received 2 mM formate and 2 mM lactate, while the 2.6 µM CT treatment received only 2 mM lactate. H₂ consumption by the cultures was inhibited by the presence of CF (Chapter 5). Error bars represent one standard deviation.