BIOSTIMULATION OF TRICHLOROETHENE DECHLORINATION BY ORGANOHALIDE RESPIRING BACTERIA WITH ENZYMATICALLY PRODUCED ORGANOCHLORIDES

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BIOSTIMULATION OF TRICHLOROETHENE DECHLORINATION BY ORGANOHALIDE RESPIRING BACTERIA WITH CHLOROPEROXIDASE PRODUCED ORGANOCHLORIDES

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DEDICATION

This dissertation is dedicated to my dearest grandma, who has raised me and filled my life with all her love. This is the last promise I made to her and I hope she would like it.

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Abstract: Anaerobic microcosms were set up for trichloroethylene (TCE) dechlorination with solvent extractable or water extractable organic matter (OM) reacted with the enzyme chloroperoxidase (CPO). CPO-reacted water-soluble OM was more suitable for bio-stimulating TCE dechlorination than CPO-reacted solvent-extracted OM. Microcosms were set up with additional variables such as carbon source and soil type. Results showed that inhibition (vs. stimulation) of CPO-reacted-OM also occurs. This inhibition was influenced by the amended electron donor: Methanol>Acetate>Molasses. CPO-reacted-OM clearly was an inhibitor in reactors seeded with contaminated aquifer or stream sediment. Overall, the results indicate CPO-reacted-OM can develop a dechlorinating microbial community but may be a competitive inhibitor with TCE. Microbial analyses (16S rRNA gene amplicon sequencing and qPCR) were conducted to determine the interaction between TCE, CPO reacted OM, and the microbial communities. 16S rRNA gene sequencing and qPCR assays showed consistent results for *Dehalobacter*. Inoculation material such as contaminated aquifer and stream sediments supported the growth of different bacteria during TCE dechlorination (such as *Methanosarcina*) compared to the *Dehalobacter* and *Clostridium* which dominated the growth in microcosms with other soil amendments. Microcosms with *Dehalobacter* growth during TCE dechlorination degraded TCE faster as well. Toxicity was conducted to determine the effect of natural organochlorines on bacterial communities. Under anaerobic conditions, increasing the amount of CPO reacted OM 5-fold caused the methane production to decrease from 79.78% to 20.79% of the methane production observed in the control. Aerobic toxicity tests indicated that adding any amount of CPO reacted OM or the OM control would cause the oxygen consumption to decrease more than half of the control with no amended OM added. These results show that the CPO reacted OM inhibits methanogens but not heterotrophic aerobes. Thus, either CPO-OM is toxic to only certain microbes or the toxicity is based on a redox-type of mechanism. The results of this dissertation point to a number of complexities with regards to the natural dechlorinating niche and the possibility to use natural organochlorides as biostimulants in the bioremediation of TCE.

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CHAPTER I

INTRODUCTION

Chlorinated solvents are a large family of organic solvents containing chlorine, such as carbon tetrachloride (CT), perchloroethene (PCE), trichloroethene (TCE), and vinyl chloride (VC). These solvents have been widely used for commercial and industrial purposes, from machinery cleaning, paint thinners, pesticides to other chemical manufacturing. Unfortunately, due to their large volumes of production and use, chlorinated solvents are a pervasive groundwater contaminant and many pose serious health threats due to their toxic and sometimes carcinogenic effects (Henschler,1994; Volpe et al.,2007).

Most *in situ* bioremediation of chlorinated ethenes, as well as several other organohalide pollutants, depends on the activity of organohalide respiring bacteria (OHRB) such as the *Dehalococcoides mccartyi* (Dhc) species (Maymo-Gatell et al., 1997) and some other dehalogenators. Bioremediation at contaminated sites is often incomplete, time consuming and have operational limitations, so there is a critical need to find more efficient methods to enhance the dechlorination of these pollutants. Published studies have demonstrated that OHRB grow while dechlorinating naturally occurring organochlorides, expanding our understanding of organohalide respiring physiology (Krzmarzick et al., 2012). By exploiting this physiology, a synthesized mixture of "natural organochlorides" was produced using a commercially available chloroperoxidase (CPO) enzyme, isolated from a fungus. I investigated the ability of this

organochloride mixture to stimulate the degradation of trichloroethene (TCE) and the microorganisms involved in the process under different anaerobic conditions in microcosm studies. I tested the toxicity of this organochloride mixture using standardized toxicity tests.

Results from this study indicated the ability of organochlorides to stimulate the TCE degradation and the effect on the completeness, effectiveness and bacterial communities involved in the process. Also, it revealed sophisticated relationship between TCE, high concentration of organochlorides and dechlorinators' community. Furthermore, the toxicity result clarified the toxic effect of CPO reacted organic matters on methanogens and aerobic microbes, which gave a better understanding when applying this technique to *in situ* bioremediation and future research.

This study discussed the TCE degradation process from different aspects, delving into the interaction in-between and reasons that could affect the process, while some were still left unclear, which may give some ideas for future study. For example, for the best result of degradation efficiency, the appropriate amount of organochlorides needed still needs to be determine. Based on the microbial analysis data, a lot more bacteria may not have been identified in the microcosms that can be potential dechlorinators that contribute to the degradation process. Also, the implications of the toxicity of CPO reacted organic matter on methanogens will be well worth studying.

CHAPTER II

REVIEW OF LITERATURE

2.1 Contamination of Chlorinated Ethenes

Chlorinated ethenes vary in number of chlorine substituents, from most chlorinated, tetrachloroethene (PCE), to monochlorinated vinyl chloride (VC) (Bradley, 2000). PCE, trichloroethene (TCE) and other chlorinated solvents are widespread groundwater and soil pollutants due to inappropriate disposal methods and extensive application in chemicals production, metal degreasing and dry cleaning (Doucette et al., 2007; Riley, 1992;) A major problem associated with the contamination of groundwater systems and soils by these compounds is the formation of dense non-aqueous phase liquids (DNAPLs). PCE and TCE form DNAPLs that sink through permeable groundwater aquifers until a non-permeable zone is reached (Matteucci, 2015). These contaminants are difficult to remediate. The entrapped DNAPL mass tends to dissolve into the flowing groundwater, serving as a long-term source of groundwater contamination.

TCE is believed to be carcinogenic and mutagenic (USEPA, 1999), and considered as a primary pollutant with a 5 µg/L maximum contamination level (MCL) allowed in drinking water (ASTDR, 1997; Spitz and Moreno, 1996). Dichloroethene (DCE) occurs in groundwater mainly from *in situ* microbial dechlorination of TCE.and consists of three congeners. Trans-DCE, cis-DCE and 1,1-DCE have their MCLs of 100, 70 and 7 µg/L respectively. Vinyl chloride (VC) is

the dechlorinated degradation product of all congeners of DCE, which then may be dechlorinated to the nontoxic product ethene, as shown in Figure 2.1. VC is the only known carcinogen and is generally considered to be the greatest threat to human health of all chlorinated solvents. It is an EPA priority pollutant and has a drinking water MCL of 2 µg/L (Hartmans, 1995). The toxicity of these chlorinated compounds to humans and potential risks to the environment led to an intense interest in the transformations of these compounds in the environment and in engineering bioremediation processes. Fortunately, anaerobic dechlorinating microorganisms have been identified in a variety of bacteria genera. Among these, several isolates of the genus *Dehalococcoides* can completely reductively dechlorinate chlorinated ethenes to the nontoxic end-product ethene (Seshadri, 2005).

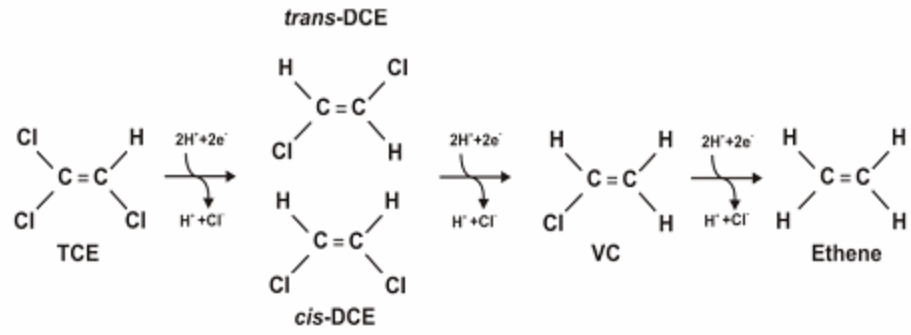


Figure 2. 1 Dechlorination of trichloroethene

2.2 Microbial degradation of chloroethenes

Studies have revealed that three types of metabolic processes are generally involved in the biological degradation of chlorinated ethenes. McCarty (1994) has provided an overview of the reductive dechlorination process for chlorinated solvents. This is an anaerobic process in which chlorinated ethenes are used as terminal electron acceptors as a component of microbial metabolism. Similarly, Alvarez-Cohen and McCarty (1991) and Hanson and Brusseau (1994)

have reported another biodegradation method known as co-metabolism. In this process chlorinated ethenes are anaerobically degraded as a result of fortuitous biochemical interactions, which yield no benefit to bacteria. The third method is direct oxidation, an aerobic or anaerobic process in which sparsely chlorinated ethenes are used as electron donors (Bradley and Chapelle, 1996; McCarty and Semprini, 1994). Figure 2.2 is showing these three different biological degradation pathways of chlorinated compounds.

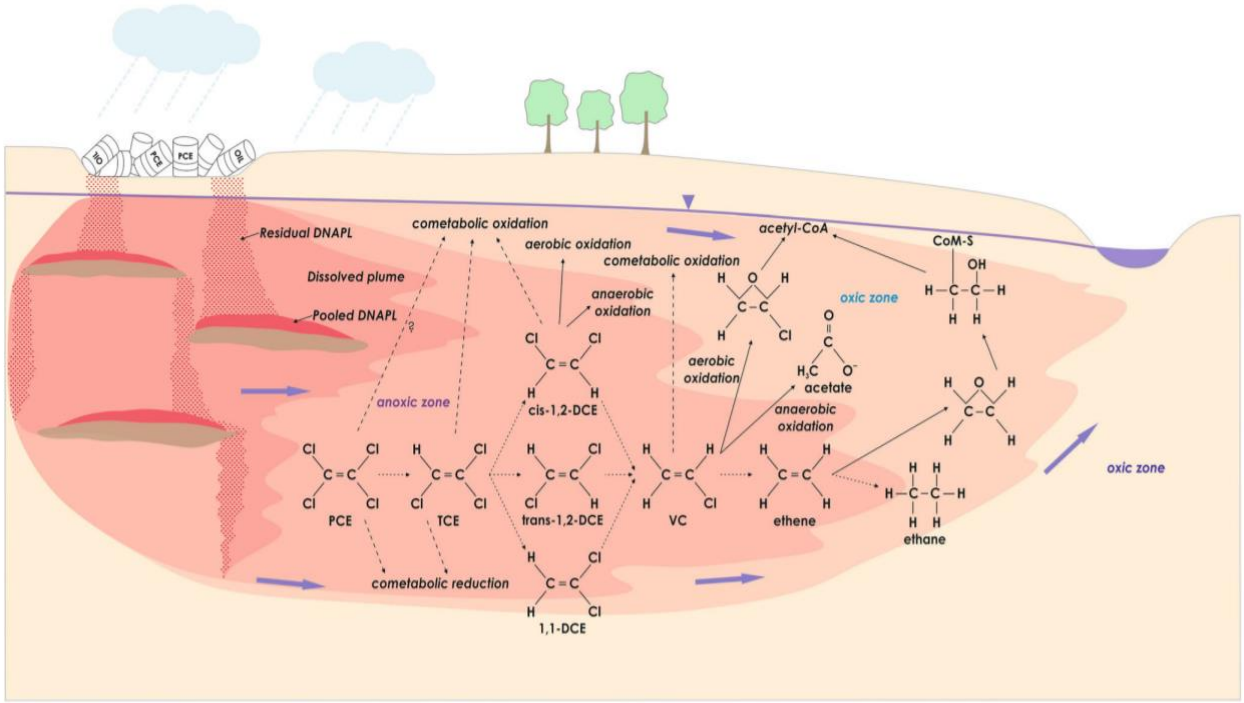


Figure 2. 2 Conceptual model of a chloroethene-contaminated aquifer with the relevant biological degradation pathways (modified after Mattes et al. 2010).

Dotted arrows reductive reactions, solid arrows oxidative reactions, dashed arrows co-metabolic reactions (Dolinová, et al., 2017)

Under anaerobic conditions, the process of microorganisms catalyzing the reductive dechlorination of chlorinated compounds energetically is known as “organohalide respiration” (Cutter et al., 2001). During organohalide respiration, chloroethenes are used as electron acceptors and energy generated from exergonic dechlorination reactions is used for

microorganism growth. The direct electron donor to the organohalide respiring bacteria is usually hydrogen (H_2), which develops biologically *in situ* from many different hydrogen-releasing substrates. Single-compound substrates such as acetate, benzoate, butyrate, methanol, ethanol, glucose, and lactate are hydrogen releasing compounds. In bioremediation technologies, complex substrates such as emulsified vegetable oils, organic mulches, and molasses are often applied to provide the *in situ* bacterial communities with electron donor, as these substrates are readily metabolized into the smaller hydrogen releasing substrates (Dolinová, et al., 2017). De Bruin et al. has reported a complete sequential reductive dechlorination of PCE to ethane, using lactate as the electron donor, in a continuous-flow, fixed bed column filled with anaerobic river sediments and anaerobic granular sludge (De Bruin et al. 1992). After 2 weeks of operation, PCE was reduced to the less-chlorinated compounds TCE, cis-1,2-DCE, and VC in the reactors seeded with river sediments. At day 105, PCE was dechlorinated stepwise via TCE, cis-1,2-DCE, and VC to ethene, then ethene was reduced to ethane. While in the column filled with ground anaerobic granular sludge, after 2 months of operation, PCE was only dechlorinated to TCE and cis-1,2-DCE, and at the end of the experiment, cis-1,2-DCE was the only end-product. This experiment demonstrated that complete dechlorination of ethenes was dependent on the bacterial community, and mirrored observations in the field that showed a stall at cis-1,2 DCE (or VC) at several contaminated sites.

In general, the higher the number of halogen substituents on a given molecule, the higher is the oxidation state, and, therefore, greater is the ease with which it is reduced (Vogel, 1994). In particular, the reductive dehalogenation reaction is a process in which the halogen compound is reduced and a chlorine atom is replaced by one of hydrogen. It mainly occurs in compounds with a high number of halogen substituents, which are totally unaffected by aerobic microorganisms, such as PCE and TCE (Matteucci, 2015), while DCE and vinyl chloride are accumulated, which favor aerobic environment for oxidative biodegradation of less chlorinated ethenes. Aeppli et al.

deployed the compound-specific carbon stable isotope analysis (CSIA) to monitor the *in situ* transformation of PCE. Results came out that the investigated zones with favorable conditions for the *Dehalococcoides* spp. dechlorinated chlorinated ethenes completely, while cis-DCE and VC had a potential to accumulate in the contaminated aquifer (Aeppli et al. 2010). Thus, the presence of the genus *Dehalococcoides* appeared important for complete dechlorination.

In the process of organohalide respiration, the addition of electron donor may not only stimulate the activity of dehalogenating microorganisms but also stimulate the activity of competing microbial populations, such as methanogens, acetogens and sulfate and nitrate reducers. Aulenta et al. (2005) conducted a study of the ability of different electron donors like hydrogen, methanol, butyrate, and yeast extract to sustain long-term (500 days) reductive dechlorination of PCE. This study showed the excellent ability of H_2 to stimulate complete dechlorination of PCE to ethene in about 80 days. In the presence of methanol, butyrate and yeast extract as electron donors, dechlorination rates were limited by the fermentation of the organic substrates. As the experiment sustained, after 100 days, the H_2-fed reactor had a declined dechlorination rate, which might be caused by the increased competition between dechlorinators and methanogens, or the accumulation of toxics and inhibitory metabolisms. After 500 days of operation, more than 65% of the added PCE was dechlorinated to ethene in the H_2-fed reactor, versus 36%, 22%, and <1% in the methanol-fed, butyrate-fed, and control reactors, respectively (Aulenta et al. 2005).

Competitive inhibition can occur between the various bacterial populations degrading chloroethenes, with PCE and TCE, for example, inhibiting degradation of VC by *Dehalococcoides (Dhc.) mccartyi* st. 195 and VC inhibiting degradation of PCE and TCE by *Dehalobacter* (Dhb.) *restrictus* when the strains are grown together. Lai and Becker (2013) used qPCR to measure the abundance of both bacteria. The study showed that initially *Dhb. restrictus* was predominantly responsible for dechlorination of PCE to DCE, which supported the growth of

Dhc. mccartyi 195. As *Dhc. Mccartyi* st. 195 accrued biomass, it increasingly outcompeted *Dhb. restrictus* for PCE and TCE. The major dechlorination product was VC. The production of VC by *Dhc. mccartyi* 195 significantly slows TCE transformation by *Dhb. restrictus*, and the higher concentrations of TCE that result from VC inhibition will slow the rate of dechlorination of VC by *Dhc. mccartyi* 195, resulting in more VC accumulation (Lai and Becker 2013). Likewise, higher chlorinated ethenes inhibit reductive dechlorination of less-chlorinated ethenes, while the less-chlorinated ethenes slightly inhibit dechlorination of more chlorinated ethenes (Yu et al. 2005). A study conducted by Yu et al. used a competitive inhibition model to stimulate the dechlorination process of PCE in a single reactor. Their study showed that PCE inhibited reductive dechlorination of TCE but not cis-DCE, while TCE strongly inhibited dechlorination of cis-DCE and VC. Also, cis-DCE inhibited VC transformation to ethylene.

During aerobic metabolic oxidative degradation, chlorinated compounds are used as growth substrates (electron donors); the bacteria using them as a source of both carbon and energy. Opposite to anaerobic degradation, oxidative chloroethene degradation is more efficient with less chlorinated substituents. Aerobic metabolic degradation has been reported for TCE, cis-DCE and VC (Dolinová, et al., 2017). Dey and Roy reported an isolated strain belonging to the genus *Bacillus*, *Bacillus sp.* 2479, that was the first bacterium capable of degrading TCE as the sole carbon source. They speculated the possible mechanism of TCE degradation may be that the bacterium produces toluene dioxygenase-like enzymes, which normally catalyzes the oxidation of toluene, and the complete meta- cleavage degradative pathway may not be necessary to completely detoxify the TCE (Dey and Roy, 2009). Mukherjee and Roy first reported *Stenotrophomonas maltophilia* st. PM102 that grew on TCE as the sole carbon source. Their experiment results showed that the isolate PM102 was capable of degrading 90% TCE at pH 7 after 48 hours of growth and 77% TCE at pH 5 after 72 hours of growth (Mukherjee and Roy, 2012). Giddings et al. reported a novel bacterium, *Polaromonas* sp. JS666, which was able to

aerobically oxidize cis-DCE as sole carbon and energy source. In their study, subsurface sediments or groundwater from six cis-DCE contaminated sites were used to construct microcosms, each one was able to degrade DCE while pH remained neutral. Even when JS666 was challenged with an alternate carbon source, or in the presence of competitive/predatory microorganisms, there was a measure of success for chlorinated ethene degradation, which proved that JS666 could be a promising candidate to *in situ* dechlorinate accumulated DCE under aerobic condition (Giddings et al., 2010).

Aerobic metabolic biodegradation of single chloroethene compounds is more efficient than on chloroethene mixtures. Zhao et al. (2010) noted fastest degradation for cis-DCE using an enrichment culture, with the degradation rate decreasing in the presence of a second chloroethene or VC. The rate of VC degradation, on the other hand, remained unchanged in the presence of PCE, TCE, cis-DCE, trans-DCE and 1,1-dichloroethene at equimolar concentrations, but decreased slightly in the presence of high concentrations of cis-DCE.

Many methanotrophic, toluene- and phenol-degrading bacteria can degrade chloroethenes co-metabolically, with no carbon or energy benefit during the degradation process. Bacteria capable of degrading chloroethenes in this way use alternative growth substrates. The chloroethenes are degraded by the enzymes originally produced for degradation of the bacterial growth substrate (Semprini 1997). Research about TCE transformation rate and capacity of a mixed methanotrophic culture was measured by Alvarez-Cohen and McCarty (1991). TCE initially had a limited transformation rate and capacity of resting cells. Adding alternative energy source (formate) resulted in the increased transformation rate of TCE. Significant declines of methane conversion rates were observed for both resting cells and formate-fed cells when exposed to TCE, suggesting the toxic effect of TCE and its transformation products. A study on co-oxidation of DCEs by butane monooxygenase (BMO) in the butane-utilizing bacterium *Pseudomonas*

butanovora were conducted by Doughty et al. (2005). This study tested different organic acids as exogenous reductant sources and determined if DCEs could serve as surrogate inducers of BMO gene expression. Results came out showing that lactic acid supported greater rates of oxidation of all three DCEs than other organic acids tested. The impact of oxidation of 1,1-DCE and trans-DCE caused on BMO activity loss was more significant then cis-DCE. Oxidation of similar amounts of each DCE also caused different negative effects on lactic acid-fed respiration. Despite 1,1-DCE being consumed 10 times faster than trans-DCE, respiration declined at similar rates, suggesting that the oxidation products of trans-DCE was more toxic to respiration than 1,1-DCE. In general, the cometabolic process is slower than metabolism of the growth substrates (Fetzner 1998; Suttinun et al. 2013). When the growth substrate is depleted, co-metabolic degradation of chloroethenes ceases.

2.3 Biostimulation of chlorinated ethenes

Biostimulation of chloroethene compounds has drawn a lot of attention in the past 40 years. Prior to 1980, chlorinated solvents such as tetrachloroethene (PCE), trichloroethene (TCE), 1,1,1-trichloroethane (1,1,1-TCA) and carbon tetrachloride (CT) were recalcitrant to biodegradation (Bradly, 2003). In the early 1980s, research first proved that common chloroethene compounds like PCE, TCE could be biodegraded by microorganisms that naturally present in soil and groundwater (ESTCP, 2005). Due to the lack of molecular techniques and microbial identification at that time, little was known about the bacteria involving in the bioremediation process and their biodegradation mechanisms. In the late 1980s, the persistence of these compounds in the subsurface for decades became clear (Bradley, 2003). During the 1980s, research about biotransformation products of accumulated chloroethene compounds were widely reported and attributed to microbial reductive dechlorination (Parsons et al., 1984; Vogel, 1994). During this

time, more and more naturally occurring organochlorines were identified, but chlorine-carbon bonds were still considered fundamentally anthropogenic. By 1992, Gribble's review on naturally occurring organohalogen compounds summarized that more than 1500 different halogenated chemicals from both biotic and abiotic sources were produced and discharged into our biosphere by plants, bacteria and other natural processes, and growing monthly. Furthermore, the quantities of some of these naturally occurring halogenated compounds far exceeds the anthropogenic level and have been existed for thousands of years (Gribble, 1992 & 1994). This gave researchers a direction to investigate the natural organochlorine-degrading microbial process.

2.4 Naturally occurring organochloride

It was reported that in 1968, more than 30 naturally occurring organohalogen compounds were documented. By 2012 (Gribble 2012) more than 5000 natural organohalogen compounds were discovered and identified.

In marine environments, natural occurring organohalogens are produced by a variety of species, and in certain sponges, up to 12% of the sponge dry weight can be accounted for by organobromines including bromoindoles, bromophenols and bromopyrroles (Gribble 1999; Turon et al., 2000; Ahn et al., 2003). In these systems some natural organohalogens have been shown to bioaccumulate in higher organisms in a manner like the bioaccumulation of anthropogenic compounds (Teuten et al., 2005; Vetter and Gribble, 2007). A mixed indigenous bacterial culture from a sea sponge, found to contain *Dehalococcoides*-like bacteria as well as other putative halorespirers, was shown to reduce several synthetic brominated phenols (Ahn et al., 2003). Because brominated phenolic compounds can be naturally produced by sea sponges (Gribble, 1999), this study associated natural halorespiring communities in marine systems to naturally occurring organohalogens. Another study found that mixed cultures containing *Dehalococcoides*-

like microorganisms from uncontaminated sediment in the North Sea could degrade TCE to trans- and cis-DCE (Kittelmann and Friedrich, 2008). Based on evidence existed for the natural production of tetrachloroethene by marine algae (Abrahamsson et al., 1995), it could also be considered as a putative natural substrate for *Dehalococcoides*-like organisms in marine systems.

Organochlorines account for the major chlorine species in weathering plant materials (Myneni, 2002). The fraction of chlorine present as aromatic or aliphatic organochlorines increases as fresh plant material becomes senescent and begins to humify (Myneni, 2002). Studies on the degradation of naturally occurring organochlorines have been demonstrated that in terrestrial systems the chloroperoxidase (CPO) enzymes, found in a variety of plants and fungi, can chlorinate natural organic matter with both aliphatic and aromatic—including phenolic—moieties (Reina et al., 2004). An example of CPO chlorinated organic compound is shown in Figure 2.3. These enzymes also chlorinate aliphatic and aromatic structures during the breakdown of large molecular weight lignin molecules (Ortiz-Bermúdez et al., 2003). Research has shown that undefined mixture of natural organochlorines produced with CPO enzymes stimulates the growth of *Dhc*-like bacteria compared to organic amendment controls (Krzmarzick et al., 2012). During the growth of *Dhc*-like bacteria, chloride was found to be concomitantly released, suggesting a reductive dechlorinating process (Krzmarzick et al., 2012)

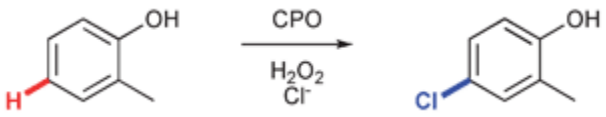


Figure 2. 3 An example of chlorination reaction catalyzed by chloroperoxidase (CPO)

2.5 Organohalide Respiring Bacteria (OHRB)

OHRB mainly belong to three bacterial phyla (*Chloroflexi*, *Firmicutes*, and *Proteobacteria*), and are capable of utilizing various halogenated organic substrates as their terminal electron acceptors via their reductive dehalogenation system. Since the description of *Desulfomonile tiedjei* as the first isolated OHRB (DeWeerd et al. 1990), numerous bacterial strains capable of OHRB have been obtained in axenic culture, providing indispensable insights into their phylogenetic, physiological and biochemical traits. Members of the genus *Dehalococcoides* comprise the biggest groups of isolates to date (19 isolates), followed by strains of *Desulfitobacterium* (17 isolates). The known isolates can be divided into facultative and obligate groups based on whether OHR is their only energy-gaining metabolism (Maphosa et al. 2010). The members of the facultative OHRB are characterized by a more versatile metabolism. In general, they have the ability to grow on a wide range of electron acceptors, and include proteobacterial OHRB such as *Geobacter*, *Desulfuromonas*, *Anaeromyxobacter*, *Desulfomonile*, *Desulfovibrio*, *Desulfoluna*, *Sulfurospirillum*, *Comamonas*, *Shewanella* as well as *Desulfitobacterium* from the phylum *Firmicutes*.

Dehalococcoides, *Dehalogenimonas*, and a few other isolates form the obligate OHRB class *Dehalococcoidia*. Among this group of currently isolated OHRB, *Dehalococcoides mccartyi* (*Dhc*) is one of the most studied genus and species because it was the first to be isolated that has the ability of fully dechlorinate PCE to the nontoxic end product ethene (Löffler et al., 2013; Maymo-Gatell et al., 1997). *Dehalococcoides* is also able to dechlorinate aromatic pollutants and other aliphatic pollutants (Loffler et al., 2013; Yan et al., 2006; Bedard et al., 2006; Kube et al., 2005).

Despite their specialized niche of OHR, *Dehalocccoides*-like organisms appear widespread in both contaminated and uncontaminated environments (Hendrickson et al, 2002; Krzmarzick et al.,

2012; Krzmarzick et al., 2013). In uncontaminated environments, the OHR *Chloroflexi* are somewhat correlated with the fraction of total organic carbon (TOC) present as organochlorines (Krzmarzick et al., 2012). In a study by Krzmarzick et al., organohalide respiring *Chloroflexi* were found to grow while enzymatically (chloroperoxidase) produced organochlorines were dechlorinated, thus strongly supporting the hypothesis that OHRB occupy a niche in terrestrial soils using natural organochlorines as terminal electron acceptors (Krzmarzick et al., 2012, Adrian et al., 2007; Bunge et al., 2008, Hiraishi et al., 2008; Kittelmann and Friedrich et al., 2008a; Kittelmann and Friedrich et al., 2008b).

2.6 *Dehalobacter*

Dehalobacter was first proposed as a new genus in 1998 (Holliger et al. 1998), with PER-K23 as the strain of the new species *Dehalobacter restrictus*. The strain *Dehalobacter restrictus* strain PER-K23 was first isolated from a PCE dechlorinating enrichment culture with Rhine river sediments and anaerobic granular sludge to dechlorinate PCE (Holliger et al. 1993). PER-K23 catalyzed the dechlorination of PCE via TCE to cis-1,2 DCE, paralleled by chloride production and PER-K23 growth. The *Dehalobacter* spp. are known as obligate OHRB, which grow in the organohalide respiration, utilizing only H_2 as an electron donor and PCE and TCE as electron acceptors.

Dehalobacter restrictus has been getting more attention in recent years, since it played an important role in bioremediation of an increasing number of organohalides. Lim et al. (2014) found *Dehalobacter* growing in their study of dechlorination of natural organochlorines. Grostern et al. studied the organisms involved in the dechlorination of 1,2-dichloroethane (1,2-DCA) and 1,1,2-trichloroethane (1,1,2-TCA) to ethene from a mixed anaerobic subculture enriched from a

multilayered aquifer at a chlorinated solvent disposal facility (Grostern et al., 2006). Denaturing gradient gel electrophoresis (DGGE) analysis and qPCR results showed that both *Dehalobacter* and *Dehalococcoides* grew in the dechlorination of 1,2-DCA. *Dehalobacter* only grew in the dechlorination of 1,1,2-TCA to VC but not the process of VC to ethene, while *Dehalococcoides* only grew in the dechlorination of VC to ethene. Their findings indicated that in mixed cultures, multiple dechlorinators can be competitive or complementary.

2.7 Reductive dehalogenase genes

Reductive dehalogenase (rdh) genes code for the enzymes that catalyze organohalide respiration reactions (Hug et al., 2013). Only four rdh genes from *Dehalococcoides* strains (pceA, bvcA, tceA, vcrA) have been specifically studied, and thus, the majority of rdh genes identified have unknown substrate specificities (Waller et al., 2005). In addition to the multitude of rdh genes that exist, the few well-characterized rdh enzymes studied have been found to have wide substrate ranges (Fung et al., 2007). It is proven that the rdh TceA from *Dehaloccocoides mccartyi* sp. 195 not only contributes to the dechlorination of TCE to ethene, but is also found to dechlorinate a wide range of chlorinated and brominated alkanes and alkenes (Magnuson et al., 2000). Very evolutionarily divergent rdhs are also found to contain the ability to dechlorinate the same pollutant (Lohner et al., 2013). Much research has found that the obligately OHRB such as the *Dehalococcoidia* and *Dehalobacter* usually contain numerous rdh genes, as many as 39, but the facultative OHRB usually have a single or at most a few rdhs (Hug et al., 2013; Nonaka et al., 2006; Futagami et al., 2008; Hölscher et al., 2004; Kube et al., 2005; Richardson et al., 2013; Seshadri et al., 2005; McMurdie et al., 2009). Rdh genes also have been found to be strictly induced from organohalides, though when rdh are induced, they are often broadly transcribed (Wagner et al., 2013; Waller et al., 2005). Several rdh genes are also simultaneously transcribed

during the dechlorination of a single chlorinated substrate and multiple chlorinated substrates can induce transcription of the same rdh gene (Waller et al., 2005). These characteristics imply that *Dehalococcoides* species may have evolved to halorespire a rich diversity of halogenated compounds simultaneously.

The known rdh genes share many characteristics. All rdh loci are composed of two genes that are believed to be simultaneously transcribed (Kube et al., 2005). The larger A gene is believed to encode the active subunit, while the B gene is believed to encode a small hydrophobic protein that acts as a membrane anchor (Kube et al., 2005). Conserved amino acid regions for putative rdh genes include the Tat signal peptide, which is not unique to rdh genes, two regions necessary for an iron-sulfur cluster, and a short region in the accompanying B genes (Krajmalnik-Brown et al., 2004). In addition, nearly every rdh gene has a two-component regulatory system or MarR-type transcriptional regulators, indicating strict regulation of the genes (Kube et al., 2005).

CHAPTER III

COMPARISON OF TCE DECHLORINATION BY OHRB WITH ENZYME-AMENDED SOLVENT EXTRACTABLE AND WATER EXTRACTABLE ORGANIC MATTERS

3.1 Introduction

The hypothesis that *Dehalococcoides*-like organisms use natural organochlorines as their electron acceptors has been discussed in recent literature (Adrian et al., 2007; Bunge et al., 2008; Hiraishi 2008; Kittelmann and Friedrich, 2008a; Kittelmann and Friedrich 2008b; Tas et al., 2011). Research concerning this hypothesis, however, has been indirect and has comprised of the dechlorination of anthropogenic compounds that are also naturally produced. Laboratory cultures of *Dehalococcoides* strains 195 and CBDB1 have been shown to grow on 2,3-dichlorophenol and 2,3,4-trichlorophenol and are also capable of dechlorinating several other chlorophenols (Adrian et al., 2007). Additionally, 2,3-dichlorophenol has been shown to induce transcription of several rdh genes in *D. mccartyi* 195, and the same rdh used to dechlorinate PCE to TCE is thought to be responsible for its dechlorination (Fung et al., 2007).

Studies on the methods of synthesizing organochlorides and enzyme involved in the chlorination reaction have been widely developed. The enzymatic ability of commercially available chloroperoxidase (EC 1.11.1.10, CPO) was confirmed to catalyze the chlorination reaction (Asplund et al., 1993). The reaction is strongly dependent on pH range, which has to be kept

strictly within 3.0-3.5, and it won't proceed in the absence of hydrogen peroxide (H_2O_2). Despite the strict conditions to proceed the chlorination reaction, the organic matter (OM) extraction process from soil and plants is also important to the seeding treatment. Research conducted by Wan Mohd Zamri et al. (2021) found that the water extractable fraction (HiF) dominated in all soil extract treatments, with the percentage accounting for more than 54% of their dissolved organic matter (DOM) as dissolved organic carbon (DOC). Humic substances (HS) were the second most abundant fraction, ranging from about 23–32%. All treatments of soil extracts were mainly in acidic conditions. Meanwhile, the hydrophobic neutral fraction (HoN) was considered very low (0–6%) of the total DOM. Also operating the extraction process at autoclave temperature (105℃-121℃) increased the DOM fraction in DOC compared to room temperature.

Based on the literature mentioned above, experiments were designed to compare the biostimulation of the reductive dechlorination of TCE with amendments of CPO-treated solvent extractable and water extractable organic matters.

3.2 Methods

In preliminary experiments, two types of organic matters (OM) were applied in the process of TCE dechlorination, one was solvent extractable (solvent extracted) OM and the other was water extractable (water soluble) OM. The TCE dechlorination biostimulation was tested on these two types of OM separately.

TCE was amended to four sets of microcosms – the first one was given OM reacted with CPO to produce "natural organochlorides", the second one was given non-reacted (without any enzymatic reaction) OM (controls for stimulation which may occur via the OM), the third one was not given

any OM co-amendment (non-stimulated condition control), and the last one was an autoclaved control (to control for TCE loss due to abiotic fate and transport).

Soil and Sediments Collection

Oak forest soil used for OM extraction was collected locally in Payne County, Oklahoma. This material is rich with decaying detritus and collected only from the top 1 inch of the soil horizon. For microbial seeding material, stream sediments were collected from a slow running stream at Ray Harrell Nature Park (Broken Arrow, OK) 1 foot away from the stream edge and 4 inches beneath the surface. (Lim, 2014).

Extraction of Organic Matter

Solvent Extractable Organic Matter Extraction

The solvent extractable OM was extracted from the oak forest soil into solvents. The soil was evenly separated into centrifuge tubes with dichloromethane added into each 50 ml falcon tubes. These tubes were vortexed and incubated at 35°C overnight. Then put into the Cole Parmer Sonic Oscillator to sonicate for 15 minutes with the heat function on. The liquid phase was transferred to a flask and this extraction process was then repeated sequentially with Hexane and Acetone on each batch of soil. All of the solvents transferred were blown down to dryness. The dried OM was reconstituted in buffer (see below) and was ready for the enzymatic chlorination process.

Water Extractable Organic Matter Extraction

To prepare the organic matter (OM) for the synthesis of organochlorines, the water extractable organic matters was extracted from the oak forest soil into water. The soil was evenly separated

into 50 ml falcon tubes and DI water added. These tubes were then put into the Cole Parmer Sonic Oscillator to sonicate for 1 hour with the heat function on.

To filter the aqueous organic matter solution, the solution was filtered using a vacuum filtration apparatus. The OM was first extracted through course fiberglass membranes, and then through 1.5 µm pore-size Millipore membranes. The remaining solution, free of particulates, was then ready for the enzymatic chlorination process.

Synthesis of organochlorines

To synthesize the organochlorines, chloroperoxidase (CPO) enzyme (Sigma-Aldrich) was used to chlorinate the organic matter extracts. Both solvent extractable and water extractable OM extractions were buffered with 0.1 M KH_2PO_4 and 20 mM KCl was added for free chloride. The solvent extractable extract was directly reconstituted in the buffered solution, while the salts were directly added to the aqueous extract. Each extracted organic matter was then split evenly by volume into two flasks - one was for the control amendment, while the other was chlorinated for the "natural organochlorines" amendment. The pH of each flask was adjusted to 3.0 and maintained at 3.0 to 3.5 throughout the process. For the chlorination process, 10 µL of CPO enzyme (for every 100 ml OM extract) was added to only the "natural organochlorines" flask. Both control and natural organochlorines flasks were then amended with 100 µL of a 0.1 M solution of hydrogen peroxide every 30 minutes for 1.5 hours. Both reactors will be gently swirled by hand after each addition. This process was repeated twice a day for two days.

Anaerobic Mineral Media

The anaerobic mineral media used in the microcosms were designed for reductively dechlorinating bacteria. For 1 L media, 1 g NaCl, 500 mg $MgCl_2 \cdot 6H_2O$, 200 mg KH_2PO_4, 300 mg NH_4Cl, 15 mg $CaCl_2 \cdot 2H_2O$, 15 mM NaHCO3, 0.2 mM L-Cysteine, 48 mM $Na_2S \cdot 9H_2O$, 1 ml trace element A and 1 ml trace element B were added. The media was autoclaved and moved into an anaerobic glove box (97% N_2, 3% H_2) while near boiling to avoid re-oxygenation. Prior to dispensing into the microcosm bottles, the pH was adjusted to 7.0-7.5 with H_2PO_3 or NaOH.

Microcosm setup

Batch microcosms in this research were used to test for the degradation of TCE. Table 1 showed a summary of all microcosms used for this experiment. Each microcosm treatment was operated in triplicate. To determine the organohalide-respiring bacteria populations that were stimulated with organochlorines, two reactor conditions were used. All microcosms were amended with 2 g of sediment, 80 mL of anaerobic mineral media, and 0.1 mM of TCE. One triplicate set of microcosms was amended with 20 mL of the CPO-produced organochlorines; a second triplicate set was amended with 20 mL of control extract. The unstimulated control and autoclaved control were amended with 20 mL of DI water.

Table 3. 1 Batch microcosms setup for both solvent extractable and water extractable OM

Soil	Pollutant	Amendment	Description
Oak soil	TCE	CPO amended OM Extract	All microcosms are set up at pH 7, methanol as carbon source in triplicates.
Oak soil	TCE	Control OM Extract	
Oak soil	TCE	-	
Oak soil	TCE	Autoclaved	

In this experiment, TCE were amended to four sets of microcosms - one which was given OM reacted with CPO, one which was given non-reacted OM, one which was not given any OM co-amendment, and autoclaved control. The first two microcosms are designated to determine the effect of CPO amended OM on TCE degradation, the 2nd and 3rd microcosms are for determining if OM could stimulate the dechlorination process, the last two reactors are to make sure if any abiotic reactions occur during the experiment.

Sampling Method

For all the microcosms, samples were taken every 2-3 days for TCE analysis. To avoid excess TCE evaporation from opening the bottles, a 1 ml glass syringe was used to collect the slurry in the serum bottles for GC analysis. Bottles were hand -shaken before taking the slurry samples to make sure the soil and solution were well mixed, then 1 ml slurry was taken using the glass syringe and transferred to a 27 ml headspace glass vial (Wheaton). Then 100 µL headspace from each vial was injected into GC-ECD or GC-FID using a 100µL gastight syringe (Hamilton).

Gas chromatography -Electron capture detector (GC-ECD)

TCE concentrations were measured via gas chromatography using Agilent Technologies 7890B gas chromatography system with a micro- electron capture detector (GC-ECD). An Agilent J&W DB-1 column (29 m x 320 µm x 1 µm) was used. The column flowrate (He) was 1.6 mL/min. The inlet of the GC was set for a split injection mode. The injector temperature was set at 250˚C, the inlet pressure at 6.7 psi and septum purge flow at 3 mL/min. The µECD detector was set at 290 ˚C with a makeup flow (ArMe) of 60 ml/min. The protocol for GC-ECD was a 15 ˚C/min ramp up from 50 ˚C to 150 ˚C and hold for 1 minute. The total run time was 7.66 min.

For analysis of standards and samples, manual injections of 100 µL headspace were used. TCE and its daughter products cis-DCE, trans-DCE and 1,1-DCE were analyzed on the GC-ECD. Vinyl chloride and ethylene were analyzed on a GC with a flame ionization detector using external calibration curves. However, only peaks for TCE and cis-DCE were observed in the experiments.

3.3 Results and Discussion

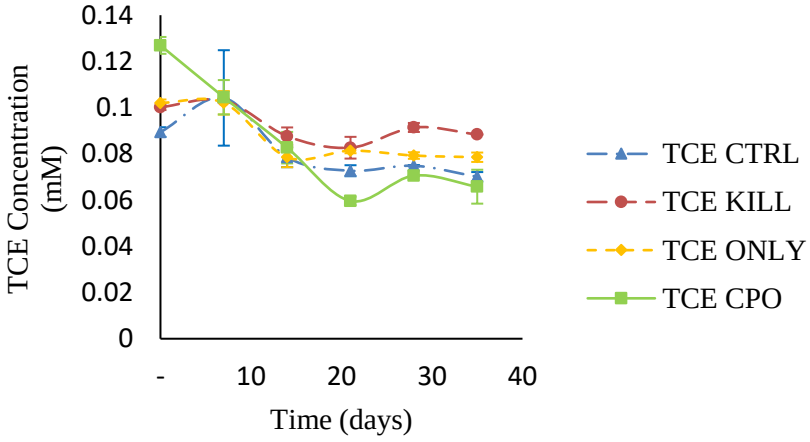


Figure 3. 1 The concentrations of TCE over time in microcosms amended with CPO produced organochlorides from solvent extractable organic matter (TCE CPO), microcosms amended with a solvent extractable OM extract control (TCE CTRL), microcosms without a co-amendment (TCE only), and autoclaved control (TCE kill)

The dechlorination data in Figure 3.1 showed results from the experiment in which solvent extractable organic matter was used (OM from hexane and acetone soluble fractions). Reactors amended with CPO-produced organochlorindes lost 41% TCE within 20 days then stalled, compared to the OM amended controls where 30 % of TCE was reduced within 20 days. This decrease was statistically significant (student T-test), indicating some biological degradation due to the CPO-reacted OM. The decrease in the control was similar to that shown in the autoclaved

and unstimulated control, indicating an abiotic loss of TCE, and no significant effect from the OM control or live micro-organisms over the killed-controlled (autoclaved). Thus, though there appeared to be some stimulation of TCE dechlorination from the CPO-reacted OM, the cessation of the degradation after 20 days indicates a relatively poor stimulatory affect. This stall might be caused by many different aspects. For example, (1) the solvent extractable CPO-reacted OM didn't support adequate growth of halorespirers to continue TCE dechlorination forward; (2) inhibitory effect due to solvent extractable CPO-reacted OM; (3) limited ability of hydrophic CPO-OM to stimulate TCE-degrading genes. These possible causes were not investigated further in this study, but may be of interest in further work to analyze mechanistically the organochloride degrading niche. The possibility of inadequate electron donor, carbon source, or nutrients is unlikely due to excessive electron donor, carbon, and nutrient addition.

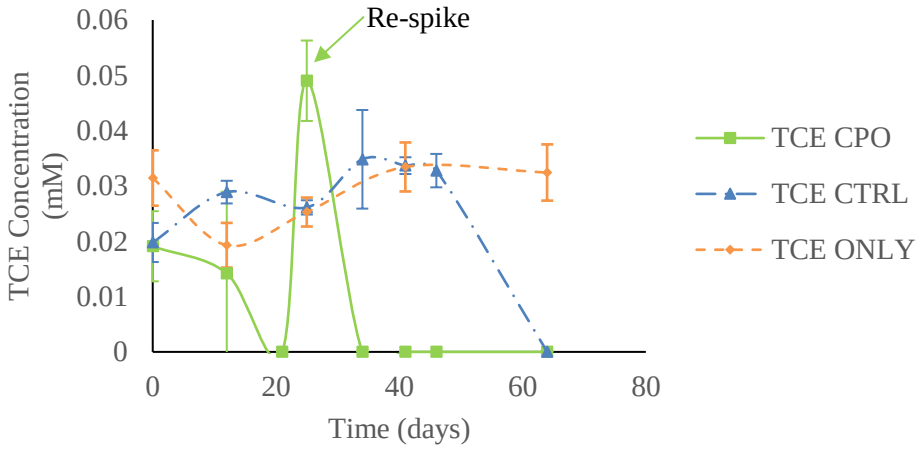


Figure 3. 2 The concentrations of TCE over time in microcosms amended with CPO produced organochlorides with water extractable OM (TCE CPO), microcosms amended with a water extractable OM control (TCE CTRL), and microcosms without a co-amendment (TCE ONLY)

In the second experiment shown in Figure 3.2, the CPO-produced organochlorides were sourced from water extractable OM (water soluble OM extract). After a lag time of 12 days, TCE degraded completely within another 9 days in the microcosms amended with synthesized organochlorides. Then a second dosage of TCE was re-spiked in these microcosms and was fully depleted with 9 days with no observed lag time, indicating that the microbial acclimation was enhanced. In the microcosms that containing the control water extractable OM, the dechlorination reaction didn't occur until day 46 and finally TCE was gone by day 64. This indicates the water extractable OM is also capable of stimulating TCE dechlorination, albeit with a longer lag time and degradation rate compared to water extractable CPO-reacted OM. So, both microcosms that containing water extractable amended OM have degraded all TCE dosage, while neither of microcosms containing the solvent extractable OM have fully degraded TCE.

Results obtained from these experiments gave an idea for the following research plan that water extractable OM based microcosm are more suitable for organohalide respirers to degrade TCE, and the organic matter amended with CPO enzyme made a significant difference on TCE degradation rates and lag time, which means that the enrichment of organic matter with synthesized organochlorides did improve the stimulation of TCE degradation.

CHAPTER IV

BIO-STIMULATION OF TCE DECHLORINATION BY OHRB

4.1 Introduction

Based on the results from previous chapter, it's proved that water extractable OM based microcosm are more suitable for organohalide respirers to degrade TCE, and the organic matter amended with CPO enzyme made a significant difference on TCE degradation rates and lag time, which means that the enrichment of organic matter with synthesized organochlorides did improve the stimulation of TCE degradation.

In this chapter, the objective is to determine the change in dechlorination rates, lag time and completeness of TCE under different conditions due to enzymatically amended natural organochlorides against the control organic matter extract. Microcosms are similar to those in the previous experiment except with the additional variables of some environmental factors in the microcosms. I tested independently carbon source/electron donor amendment and soil types compared to the standard condition above. This determined the robustness of the biostimulation approach. It is expected that different conditions may increase or inhibit the dechlorination rate, lag time, completeness and even microbial community component to various degrees, but biostimulation will be evident in some conditions.

4.2 Method

In this chapter, the research plan consisted of two sets of experiments, the first one was stimulating TCE dechlorination with different conditions, such as soil type and carbon source, to determine the change in dechlorination rates, lag time and completeness of TCE under different conditions due to enzymatically amended natural organochlorides and the controls. The second one was using fresh leaves as OM extraction matrix, to reduce the organo-Cl/organic carbon percentage in the OM extracts, and determine the effect of organochloride on TCE dechlorination processes.

Soil and Sediments Collection

Soil for OM extraction and sediments soil are the same as Chapter III, section 3.2 Soil and Sediments Collection.

For microbial seeding material, four different types of soil were used, they are oak forest soil, grass land soil, stream sediments and contaminated aquifer. Oak forest soil and grass land soil were collected from an oak forest and grass land located in Stillwater, OK, where has no known anthropogenic contamination of chlorinated compounds. Both soil samples were troweled from topsoil (2'-10' below the surface), roots and any decaying detritus were removed, only left the soil. Stream sediments was collected from a slow running stream at Ray Harrell Nature Park (Broken Arrow, OK) 1 foot away from the stream edge and 4 inches beneath the surface. (Lim, 2014). Contaminated groundwater aquifer material was collected from a site in North Carolina exhibiting in situ bioremediation of PCE to ethene. This material was collected and sent to the lab thanks to engineers at Environmental Resources Management (ERM). Fresh leaves were

trimmed from post-oak (*Quercus stellata*) trees in Payne County, OK, during the early summer months.

Extraction of Organic Matter

For both soil and fresh leaves extraction, the process was the same as Chapter III, section 3.2 Water Extractable Organic Matter Extraction.

Synthesis of organochlorines

The chlorination process is the same as described in Chapter III, section3.2. After the reaction, the reactors were heated in a 105 ˚C incubator till the inner temperature reached 80 ˚C for 30 minutes to deactivate the enzyme, then let them cool down to room temperature, and stored in 4 ˚C fridge for further use.

Ultraviolet test (UV)

UV test was applied to the chlorinated OM during the process of chlorination, both control and CPO group. The addition of chlorine to the organic compounds would change the UV absorbance, in that case, the difference of UV absorbance between control and CPO group would indicate the success of chlorination reaction.

UV test was performed by Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer. Wavelength range was 200 nm to 600 nm. Samples for UV test were taken from both control and CPO group whenever CPO was added into the reactor. 200 µL volume of sample were transferred into the microplate, and tested every 12 hours after the chlorination reaction started (T1- 12hr., T2-24hr., T3-36hr., T4-48hr., T5- enzyme deactivated), the samples were run in triplicates.

Anaerobic Mineral Media

Same recipe used as Chapter III, section 3.2 *Anaerobic Mineral Media*.

Titanium Citrate

Titanium (Ti(III)) Citrate is a reductant serving as an oxygen scavenger, eliminating any traces of oxygen in a culture medium, and prevents the growth of facultative anaerobes (Zehnder, 1976). Ti(III) citrate is nontoxic, whereas sulfide and cysteine may be toxic to some bacteria. Ti(III) citrate though may not be preferred if sulfide is providing a necessary source of sulfur for the culture.

In this experiment, Ti(III) citrate is prepared by adding 15% titanium chloride solution into 1 mM sodium citrate buffering solution, then neutralize the solution pH to 7.0, all under anaerobic conditions.

Microcosm bottles setup

When the media was cooled down and ready to be dispensed into the microcosm bottles, the following compounds were added into the media, 15 mM $NaHCO_3$, 0.2 mM L-Cysteine, 48 mM $Na_2S{\cdot}9H_2O$, then adjusted the pH to 7.0/7.5 with H_2PO_3 and NaOH.

Batch microcosms in this research was used to test for the degradation of TCE. Table 4.1 showed a summary of all microcosms used for this research. Each microcosm situation was operated in triplicate. To determine the organohalide-respiring populations that were stimulated with organochlorines, two reactor conditions were used. One triplicate set of microcosms was amended with the CPO-produced organochlorines; a second triplicate set was amended with the

control extract. Changing factors included carbon source and soil type, microcosms amended with either factor were compared within their own group to qualify the effect to the TCE degradation and microbial communities.

Table 4. 1 Summary of batch microcosms setup

<table>
<tr><th colspan="6">Microcosm setup</th></tr>
<tr><th>No. of Microcosm</th><th>carbon source</th><th>soil</th><th>pH</th><th>Redox</th><th>Amendments</th></tr>
<tr><td>A</td><td>methanol</td><td rowspan="3">oak forest soil</td><td rowspan="3">7.0</td><td rowspan="6">Methanogenic</td><td rowspan="6">Each microcosm will be amended with two conditions separately, CPO-OM extract and Control OM extract.

For example, Microcosm A (Methanol, oak, 7.0, methanogenic) will have CPO-OM amendment and Control-OM amendment conditions, and each condition will be operated in triplicates.

One autoclaved microcosm (methanol, oak, 7.0, methanogenic) is operated as a background control.</td></tr>
<tr><td>B</td><td>acetate</td></tr>
<tr><td>C</td><td>molasses</td></tr>
<tr><td>D</td><td rowspan="3">methanol</td><td>grass land soil</td><td rowspan="3">7.0</td></tr>
<tr><td>E</td><td>contaminated aquifer</td></tr>
<tr><td>F</td><td>stream sediment</td></tr>
</table>

Microcosm reactors were set up in 160 mL serum bottles capped with Viton septa (Sigma-Aldrich) and sealed with aluminum crimps. All the serum bottles were moved into an anaerobic glove box with a 3% H_2 -97% N_2 headspace, and added 80 ml anaerobic mineral media, 20 ml CPO amended OM extract or control OM extract, 2 g of soil, 10 mM potassium acetate, and 1 ml of 100×vitamin solution to provide cobalamin, an essential cofactor for rdhs. TCE was added at 100 μM as a methanol stock.

Sampling Method

The sampling method for both gas chromatography (GC-ECD, GC-FID used in this section is the same as that in Chapter III. Both GC-ECD and GC-FID used 100 μl headspace samples from the sampling vials.

Data Gathering

For all the microcosms, samples were taken every 2-3 days for TCE analysis and natural organochloride analysis. The dechlorination of TCE and its daughter products (dichloroethene, vinyl chloride and ethene) were monitored with gas chromatography (GC) equipped with an electron capture detector (ECD) or flame ionization detector (FID). When observing the dechlorination occured, samples were taken every day until all the TCE was degraded.

Agilent 7890 GC-ECD was used to measure TCE and cis/trans-dichloroethylene, GC method was the same setting as described in Chapter III.

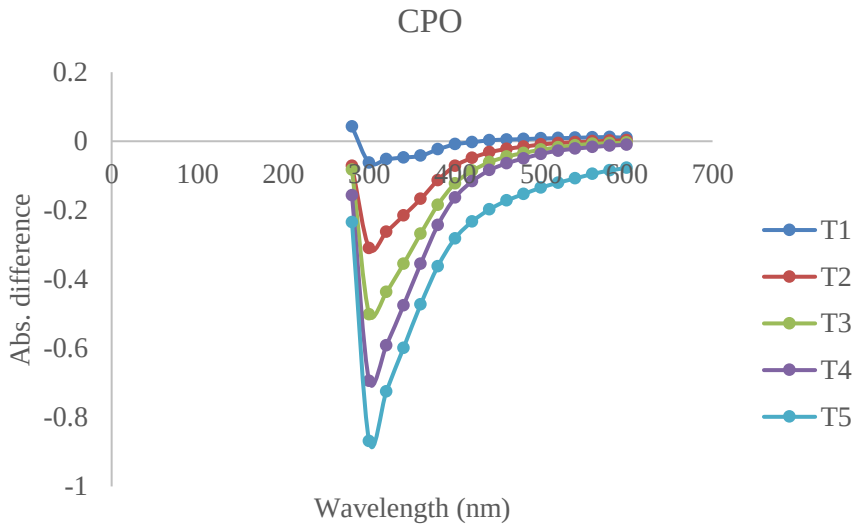
Gas Chromatography- Flame Ionization Detector (GC-FID)

Concentrations of vinyl chloride and ethylene were measured via gas chromatography equipped with flame ionization detector by Agilent Technologies 6890 GC-FID with a Supelco Glass Column 60/80 carbopack column, the column flowrate (Helium) was a constant flow of 60.0 mL/min. The injector temperature was set at 250˚C, H2 flow was 30.0 mL/min, air flow was 450.0 mL/min. The oven was kept at 90˚C isothermally for a total runtime of 5 minutes.

4.3 Results and Discussion

Ultraviolent absorption test in this experiment measured the absorbance of free chloride in the solution within 280nm-600nm wavelength range. As the chlorination reaction proceed, more free chloride ion would be binding to the extracted organic matters, which led to the loss of free chloride, also the loss of UV absorbance.

(a)



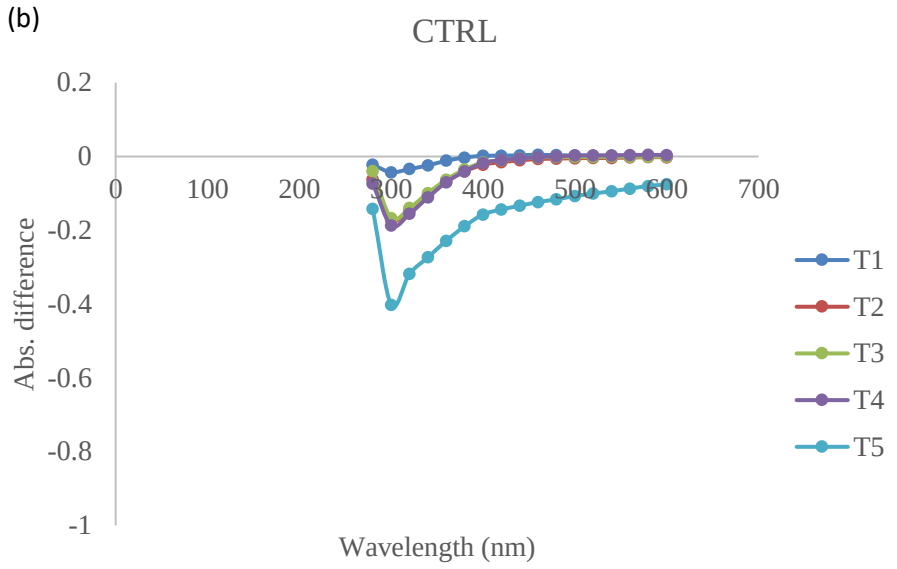


Figure 4. 1 UV test for chlorination reaction from (a) CPO amended OM and (b) non-reaction OM (T1-12 hr., T2-24hr., T3-36hr., T4-48hr., T5- enzyme deactivated)

From the UV absorbance results of chlorination reaction, significant difference between the CPO amended organic matters and the non-reaction OMs was observed. T_0 was right before adding any compounds into the OM extract, T_1 was after the first dose of CPO+ H_2O_2 were added into OM extract/ only H_2O_2 was added for control reactor, T_2 was after the second dose was added, T_3 was after the third dose was added, and T_4 was after the last dose, T_5 is after the reacted OM extracts were heated to 80℃ to stop any enzymatic reactions. The absorbance difference of each sample at each time point was calculated by wavelength measured at T_i minus the wavelength measured at T_0. At the end of the reaction (T5), the CPO reactor had 2 times of the absorbance loss than the control reactor, which proved that the chlorination reaction did happen comparing to the control.

Below shows the degradation of TCE based on different conditions. *Dehalobacter* qPCR assay results (discussed in detail in the next chapter) are also shown where such data was gathered.

Microcosm A: methanol as carbon source, oak forest soil as seed material

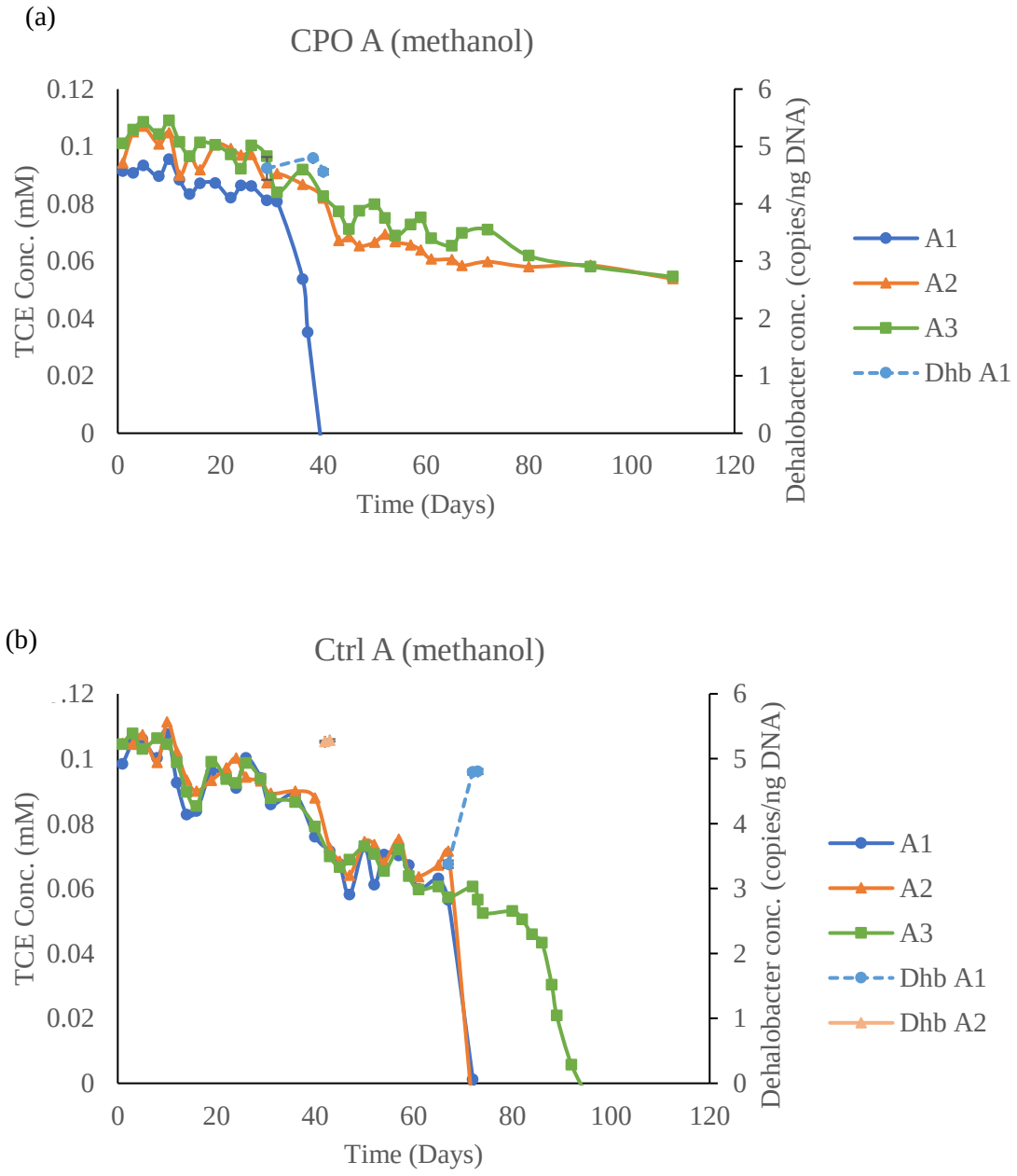


Figure 4. 2 TCE degradation curves with (a) CPO amended OM with methanol as carbon source and (b) control OM with methanol as carbon source

Microcosm B: Acetate as carbon source, oak forest soil as seed material

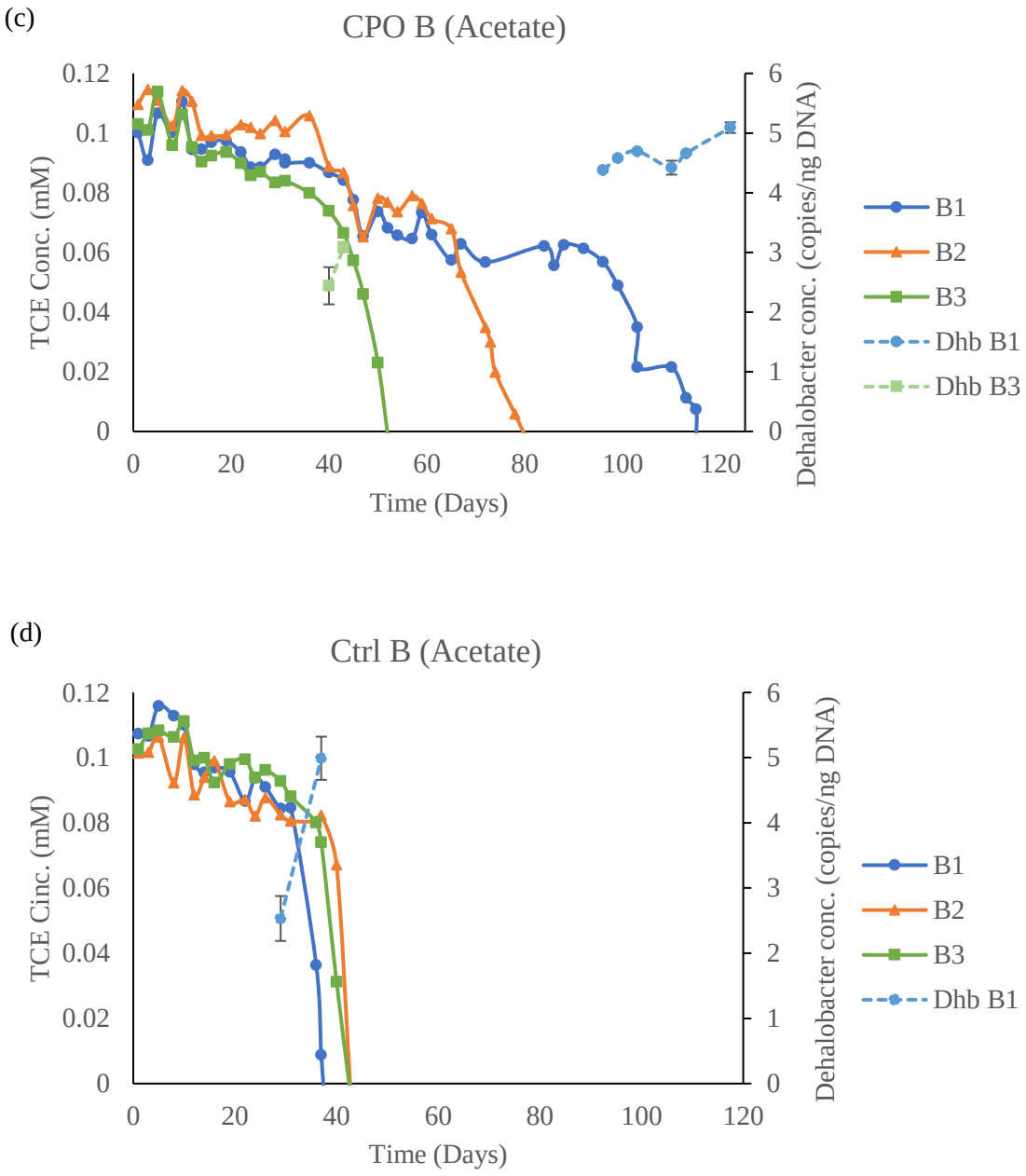


Figure 4. 3 TCE degradation curve with (a) CPO amended OM with acetate as carbon source and (b) control OM with acetate as carbon source

Microcosm C: Molasses as carbon source, oak forest soil as seed material

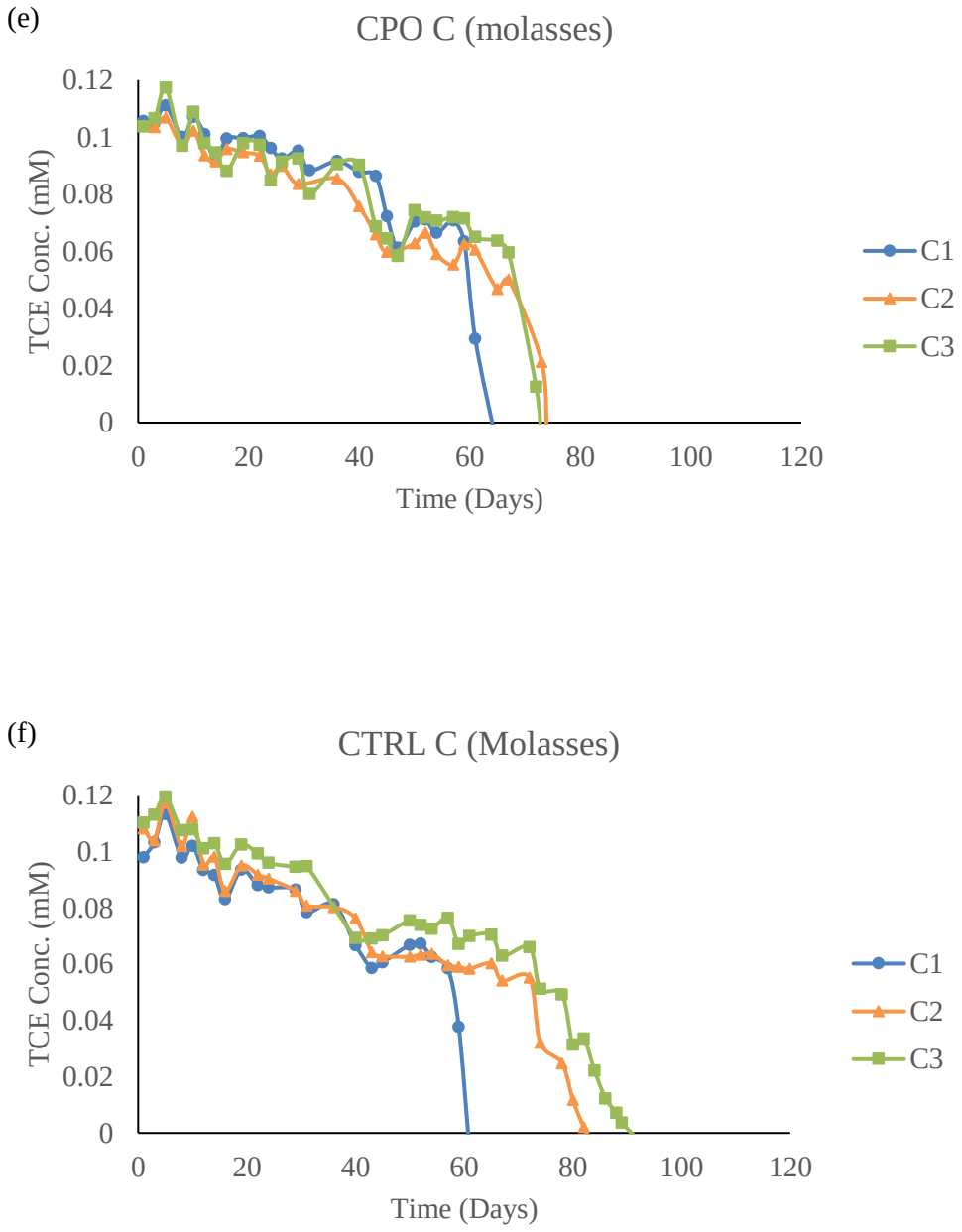


Figure 4. 4 TCE degradation curve with (e) CPO amended OM with molasses as carbon source. (f) control OM with molasses as carbon source

Microcosm D: Methanol as carbon source, grassland soil

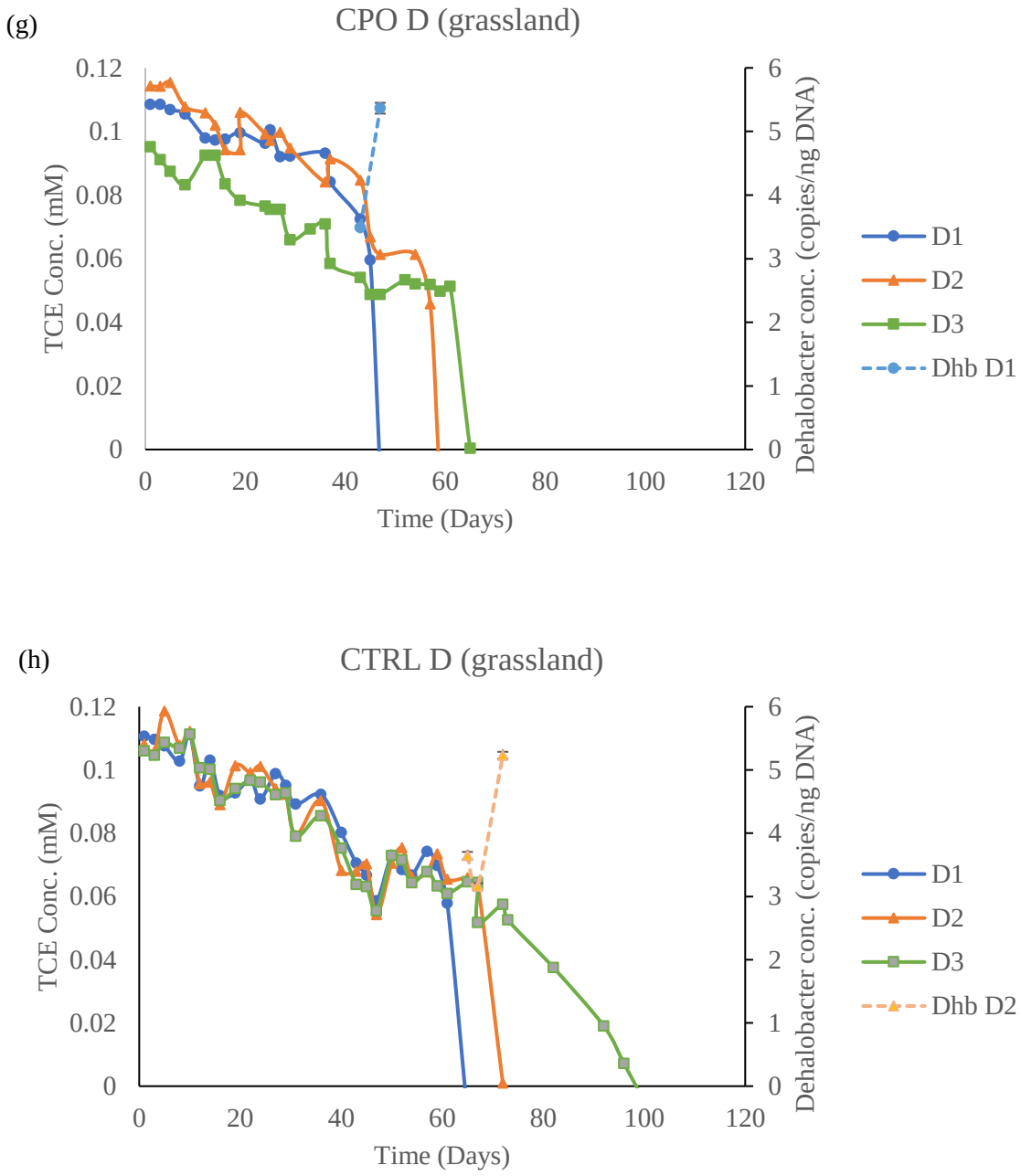


Figure 4. 5 TCE degradation curve with (g) CPO amended OM with grassland soil as seed material (h) control OM with grassland soil as seed material

Microcosm E: Methanol as carbon source, contaminated aquifer

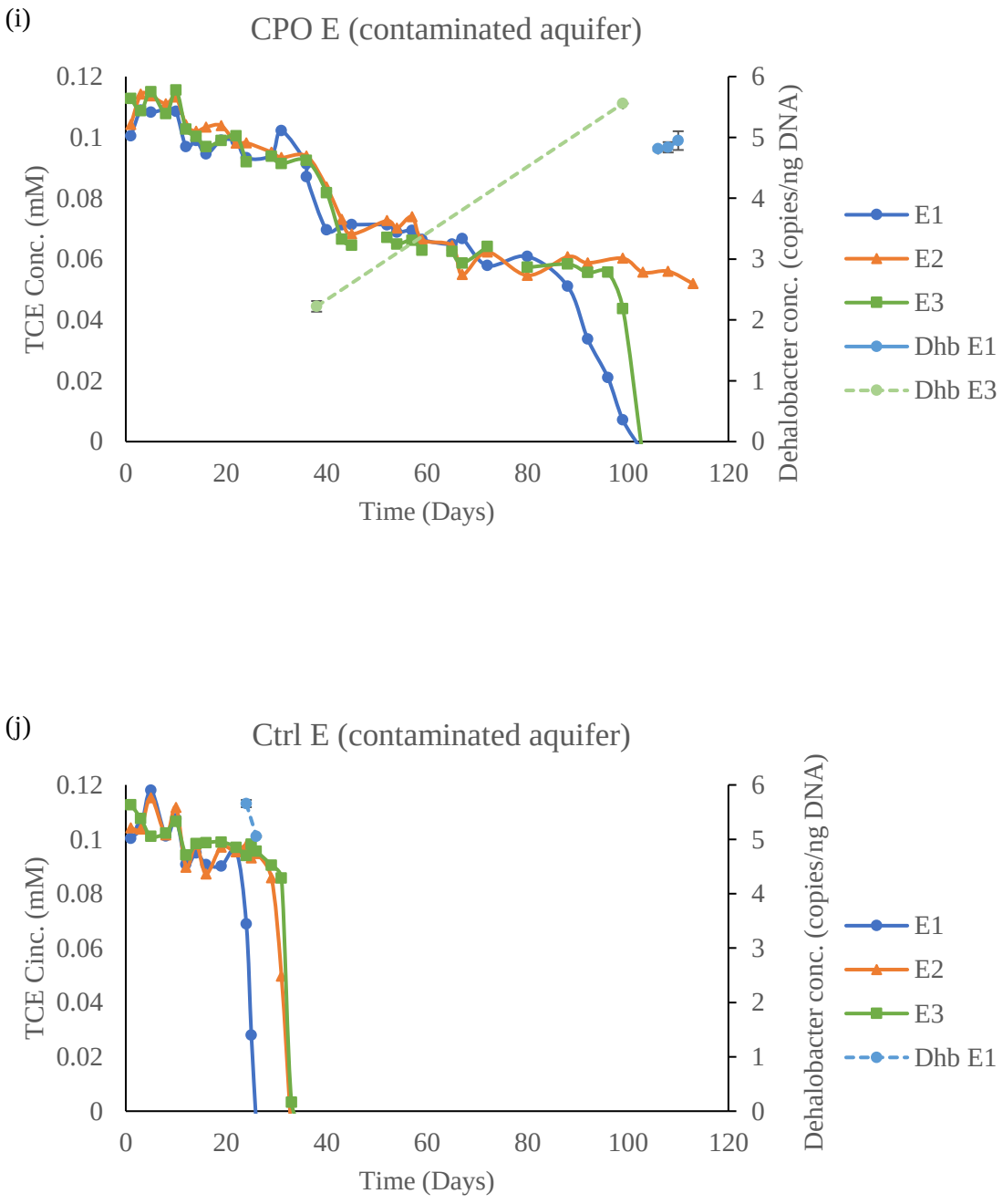


Figure 4. 6 TCE degradation curve with (i) CPO amended OM with contaminated aquifer as seed material (j) control OM with contaminated aquifer as seed material

Microcosm F: Methanol as carbon source, stream sediment

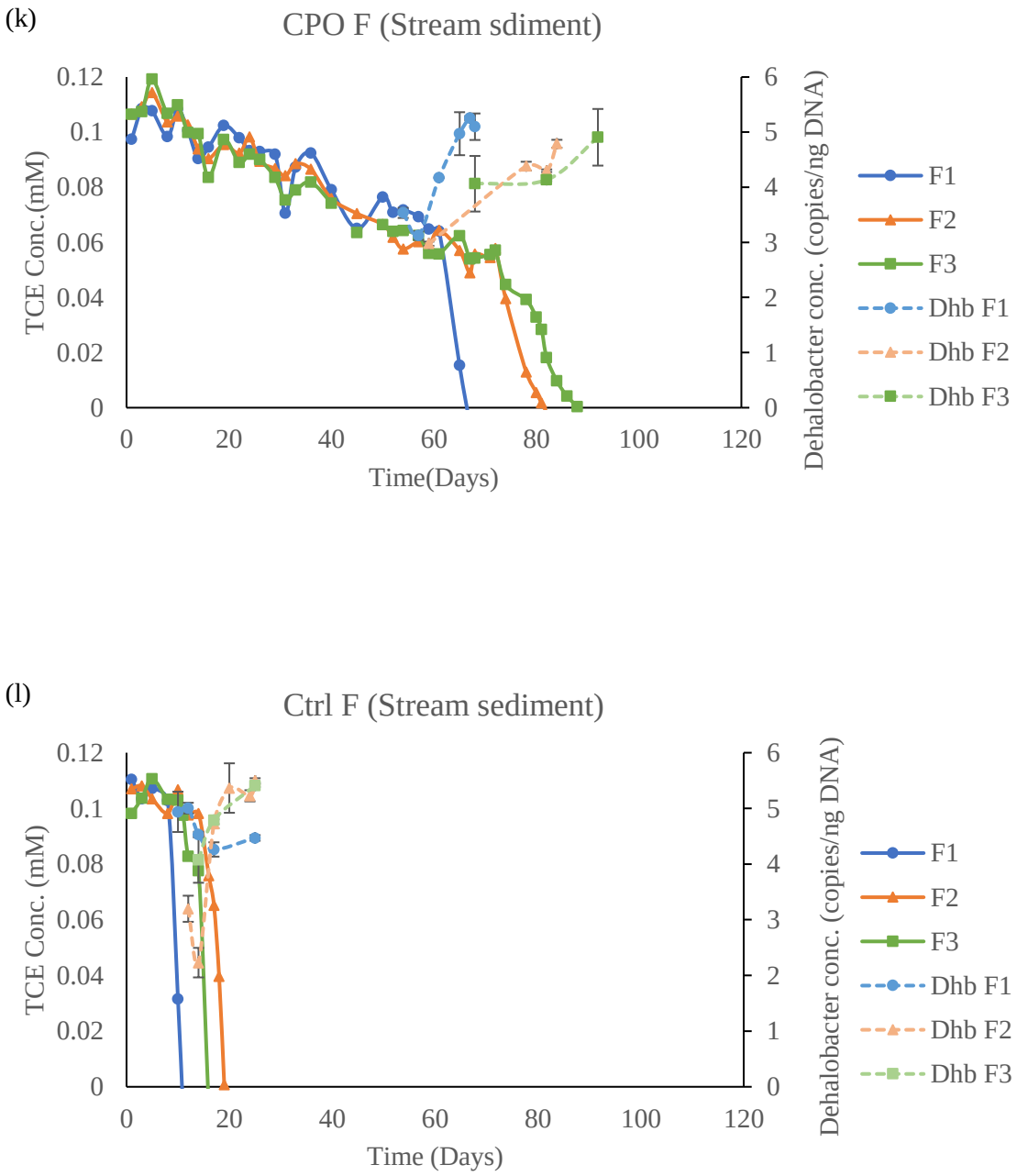


Figure 4. 7 TCE degradation curve with (k) CPO amended OM with stream sediment as seed material (l) control OM with stream sediment as seed material

Table 4.2 summerized some key data from the graphs above to campare the results between reactors.

Table 4. 2 Lag time and degradation rates of TCE degradation in each microcosm.

NO.	Microcosm	Lag Time	Degradation rate	Notes
A. Methanol Oak soil	CPO	31 to >108 days	0.013 mM/day	Two microcosms didn't degrade TCE completely after 108 days
	CTRL	67 - 86 days	0.014 mM/day	One microcosm didn't degrade after 86 days
B. Acetate Oak soil	CPO	43~99 days	0.0068~0.0023 mM/day	Three microcosms showed degradation after 43 days, 67 days, and 103 days separately
	CTRL	31 - 36 days	0.016 mM/day	
C. Molasses Oak soil	CPO	57 - 67 days	0.008 mM/day	
	CTRL	57 - 78 days	0.019~0.004 mM/day	Two microcosms started degradating after more than 50% loss of TCE
D. Methanol Grassland Soil	CPO	45 - 61 days	0.013~0.030 mM/day	
	CTRL	59 - 73 daysz	0.002~0.014 mM/day	
E. Methanol Contaminated Aquifer	CPO	92 - 96 days	0.0062 mM/day	One microcosms didn't degrade completely after 103 days
	CTRL	24 - 31 days	0.048 mM/day	
F. Methanol Stream Sediment	CPO	61 - 72 days	0.007 mM/day	
	CTRL	11 - 12 days	0.05 mM/day	

Table 4.2 summerized some key data from the graphs above to campare the results between reactors. TCE degradation was observe under all conditions but with various range of degradation rate and lag tims. The degradation of TCE in the reactors were less reproducible than in the microcosms in Chapter 3. Thus, this data is shown here as individual microcosms, as necessary. In selected microcosms in which dechlorination occurred, DNA extracts and qPCR for *Dehalobacter* is also shown, which appeared to be the most likely dechlorinator based on 16S rRNA gene amplicon sequencing (see Chapter 5) and qPCR. A summary of the results in shown in Table 4.2 for all conditions tested.

Under some conditions, like reactors of CPO A and CPO B, the lag time varied from 31~108 and 43~99 days as listed below, and some indicidual microcosms didn't show the degradation at all. Comparing the lag time and reaction rate between CPO and Contrl in each group, it clearly showd that the Controls all have shorter lag time and higher degradation rate, in group A, B and D, lag time varied about 10~30 days between CPO and Control reactors. Group C had the same lag time and controls had slightly lower degradation rate. While the differences were not significant, group E and F had some some obvious difference. TCE degradation with group E, the stream sediments and Group F, the contaminated aquifer has shown a significant difference between and CPO amended reactors and control OM reactors, while the control microcsoms reacted 60 and 50 days eariler than CPO amended microcosms respectively and the reaction rate is about 8 times faster.

Cis-Dichloroethylene (cis-DCE) as degradation product was detected in all the TCE degraded reactors and stalled at this point, no further products (vinyl chloride and ethylene) were detected in any reactors. In that case, CPO-produced organochlorides inhibited TCE dechlorination in microcosms seeded with aquifer material undergoing *in situ* chlorinated ethene remediation, and seeded with sediments, but didn't have a siginificant effect on other microcosms. At this point, the best guess was that CPO produced organochlorides can develop a dechlorinating community,

but may be a competitive inhibitor with TCE, similar to that seen in many co-contaminated sites. Thus, soils with sufficient communities might not get stimulated, but CPO produced organochlorides might help develop dechlorinating communities in soils in which dechlorinating communities are under-developed. The microbial analysis, including qPCR and metagenomics sequencing would help to explain it.

Longer lag time in repeated experimental conditions with uncontaminated soil supported this- a higher dosage of CPO produced organochlorides was amended and thus take longer to dechlorinate, and thus inhibit TCE dechlorination longer. To confirm it, the following experiments were operated using OM extracted from fresh leaves which may contain more inorganic chloride than organic chloride (Myneni, Satish CB., 2002). All the other setup procedure remained the same.

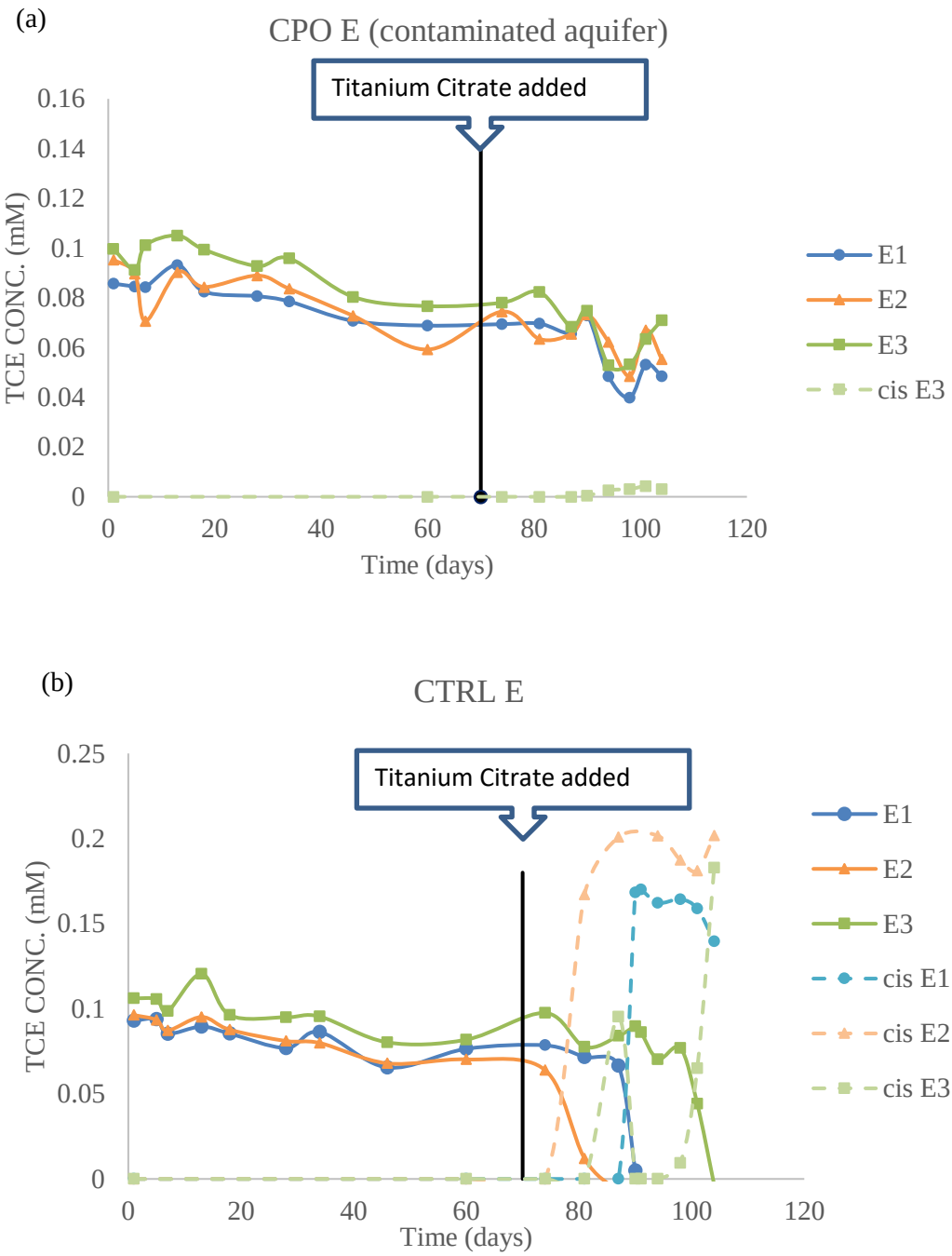


Figure 4. 8 TCE degradation curve with (a) CPO amended OM (fresh leaves) with contaminated aquifer as seed material (b) control OM (fresh leaves) with contaminated aquifer as seed material

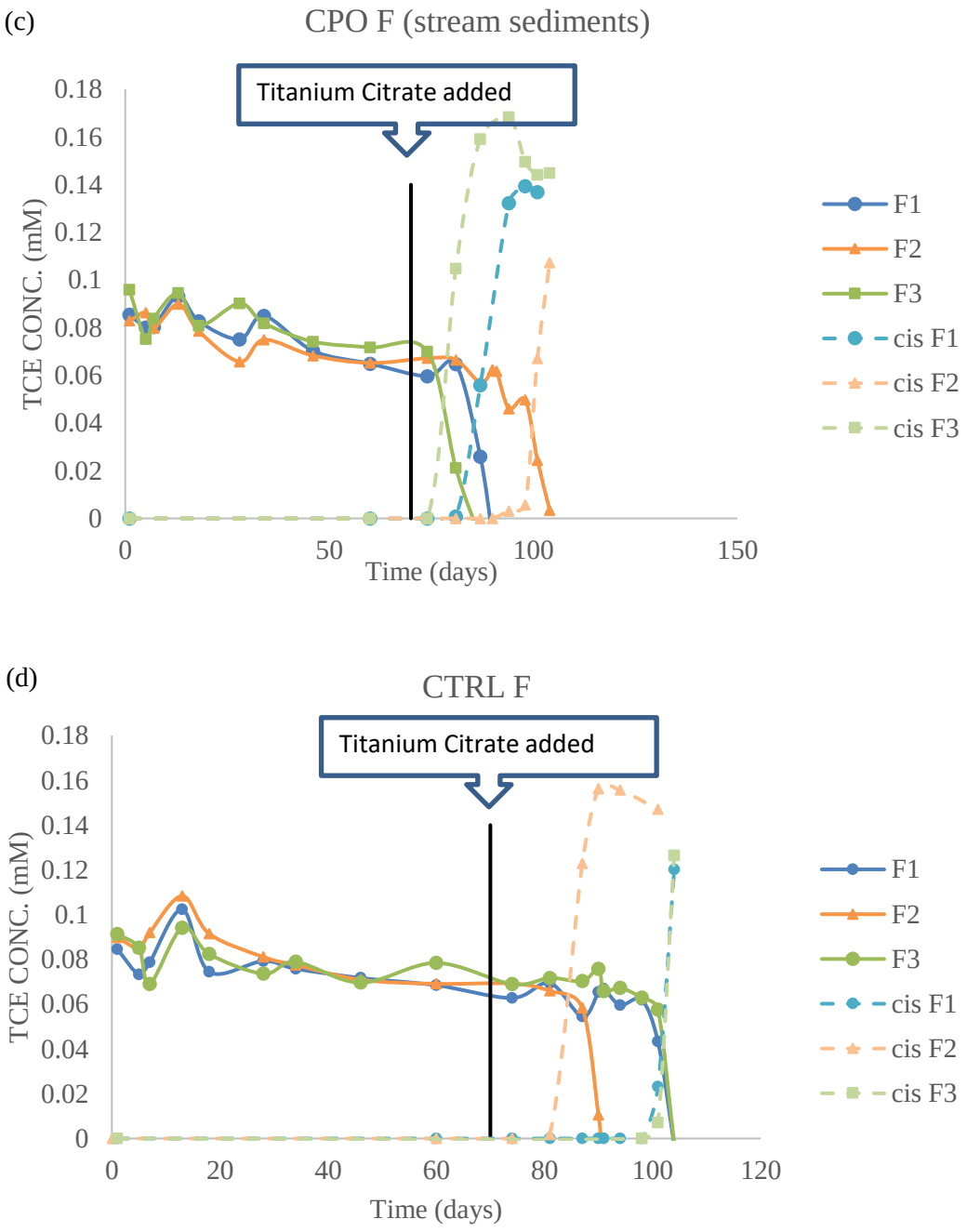


Figure 4. 9 TCE degradation curve with (c) CPO amended OM (fresh leaves) with stream sediments as seed material (d) control OM (fresh leaves) with stream sediments as seed material

The degradation process didn't begin until extra titanium citrate was added into the reactors, this indicated the importance of highly reduced conditions being needed. The reaction was able to occur within a couple of days. The CPO E microcosm didn't degrade TCE even after extra titanium citrate was added, while other three microcosms all degraded TC to cis-DCE and stalled.

Based all the results obtained by now, it is clear that CPO reacted OM may actually inhibit. This inhibitory effect showed up stronger based on electron donor (acetate > mollasses > methanol), microbial community (grassland > contaminated aquifer > oak forest soil; CPO was even perhaps a stimulant with grassland communities over the CTRL; but was a clear inhibitor in contaminated aquifer and oak soils, and stream sediment). And, a possible factor in the redox, which the final two figures showed that reactors with fresh leaves extracted OM didn't start dechlorinating in several conditions until a shot of strong reductant indicating that redox may also be a factor that affecting the reaction.

CHAPTER V

MICROBIAL ANALYSIS OF ORGANOHALIDE RESPIRING BACTERIA IN TCE BIODEGRADATION

5.1 Introduction

In situ bioremediation of chlorinated pollutants depends on the activity of organohalide respiring bacteria. *Dehalococcoides mccartyi* (Dhc) are one of the most studied species among currently isolated bacteria; it is currently unique in its ability to fully dechlorinate chlorinated ethenes to the nontoxic end product ethene (Löffler et al., 2013; Maymo-Gatell et al., 1997). Dhc are also able to dechlorinate a wide range of other aliphatic and aromatic pollutants (Löffler et al., 2013; Yan et al., 2006; Bedard et al., 2007). Several obligately organohalide respiring bacteria closely related to the *Dhc* have also been isolated, such as *Dehalogenimonas* and *Dehalobium chlorocoercia* strain DF-1 (Yan et al., 2009; Bowman et al., 2013; May et al., 2008; Wu et al., 2002) forming the class *Dehalococcoidia* (Löffler et al., 2013). Strains of *Clostridium, Dehalobacter, Desulfitobacterium Anaeromyxobacter, Desulfomonile, Desulfuromonas, Geobacter,* and *Sulfurospirillum* can also reductively dechlorinate some halogenated hydrocarbons (Shelton et al., 1984; Holliger et al., 1993; Krumholz et al., 1996; Dennie et al., 1998; Chang et al., 2000; Sung et al., 2006; Luijten et al., 2003; Suyama et al.,2003; Sanford et al., 2002; Yoshida et al., 2009; Nonaka et al., 2006) and even more bacteria can dechlorinate

pollutants cometabolically (Lohner and Spormann, 2013; Nzila, 2013). Though studied perhaps less intensely than the *Dehalococcoidia*, these other groups of organohalide respiring bacteria may have important roles for bioremediation applications. Stimulating the growth of *Dhc* and other dechlorinators to higher populations at contaminated sites will likely lead to faster and more complete bioremediation and open up more sites to in situ bioremediation approaches where the growth of these bacteria may be otherwise unfavorable.

In this chapter, the goal is to look into the bacteria that involving in the TCE degradation, their thrive and decay, and how they are affected by different conditions. Also, the microbial analysis will help to determine the biochemical interaction between TCE, natural organochlorines and dechlorinators' communities.

5.2 Methods

16S rRNA gene amplicon sequencing

Samples from the microcosms in Chapter 4 were saved frozen. Selected samples were chosen for a 16S rRNA gene amplicon sequencing analysis, and further samples where chosen for qPCR analysis. PowerSoil DNA isolation kit (Qiagen) was used to extract the DNA from slurry samples, all the pellet and slurry left in the centrifuge tube were transferred into the bead tubes provided in the kit, then follow the kit protocol to finish the isolation process (Walters,2016).

Samples from the following reactors were subjected to the 16S rRNA gene amplicon sequencing: CPO-OM amended D1, E3, F1, and Ctrl-OM amended D2, E1, and F2. "D" microcosms were seeded with grassland soil, E was seeded with contaminated aquifer material, and F microcosms were seeded with stream sediment, so these samples could show differences between soil inoculums. Each of these six microcosms were analyzed just prior and after TCE was

dechlorinated. Amplicon sequencing was carried out at Molecular Research DNA (MR DNA) in Shallowater TX. DNA extracts were submitted, and upon arrival MR DNA performed 16S rRNA gene amplification, sequencing, and bioinformatical quality control and analysis. The 16S rRNA genes were amplified with primers 515F (GTG YCA GCM GCC GCG GTA A) and 806R (GGA CTA CNV GGG TWT CTA AT) which are based on the Updated Earth Microbiome Project (EMP) 16S v4 (Walters et al., 2016). The amplicons were sequenced on an Ion S5 XL (Thermo Fisher) and approximately 20,000 reads were performed per sample. Barcodes and primers were removed from the reads, reads with ambiguous base calls were removed, and sequences high homopolymer runs exceeding 6 bp were removed. The sequences were denoised and the operational taxonomical units (OTUs) were defined by clustering at 3% divergence followed by removal of singleton sequences and chimeras. The OTUs were then classified using BLASTn and compiled into counts and percentages.

The percentage read data was then analyzed in excel to identify the OTUs with the largest (by percentage) increases and decreases in each microcosm during TCE dechlorination. The OTU consensus sequences were used to also build phylogenetic trees using MEGA. Sequences from the amplicon sequencing and from closely related sequences on NCBI's GenBank (via nBlast) were used to build phylogenetic trees. Alignment was built using MUSCLE, tree topography was determined using Neighbor-Joining method, and the evolutionary distances were identified via Maximum Composite Likelihood Method (Saitou and Nei 1987; Tamura et al 2004; and Kumar et al 2018).

Quantitative-Polymerase Chain Reaction (qPCR) Method

qPCR was applied to quantify the *Dehalobacter spp.* which was identified in most samples to have largely increased during TCE dechlorination to cis-DCE. *Dehalobacter* qPCR assays were carried out as described in Smith et al. (2015) with primers 447F and 647R.

5.3 Results and Discussion

Below showed partially the bacteria identified by 16S rRNA gene amplicon sequencing. The shown organisms in the tables were ranked by their growth/decline percentage (only top five were shown) and high percentages (>2%) in each group.

Table 5. 1 CPO D1. Grassland soil as seeding material, methanol as carbon substrate

OTU name	Taxonomy	OTU percentage		
		Before TCE dechlorination	After TCE dechlorination	Increased percentage
OTU_13	uncultured *Dehalobacter sp.*	0.0048	1.1193	22995%
OTU_4041	uncultured *Dehalobacter sp.*	0.0024	0.3508	14375%
OTU_3942	uncultured *Dehalobacter sp.*	0.0024	0.1180	4769%
OTU_4804	uncultured *Clostridium sp.*	0.0024	0.0191	690%
OTU_5947	uncultured *Sedimentibacter sp.*	0.0024	0.0191	690%
OTU_1456	uncultured *Clostridium sp.*	2.3844	1.7698	-26%
OTU_3534	*Burkholderia sp.* z29_2	2.4038	1.8495	-23%
OTU_1012	uncultured *Clostridium sp.*	3.2422	2.7360	-16%
OTU_2488	*Methanosarcina* sp. af020341.1	1.3328	1.1384	-15%
OTU_2	uncultured *Clostridium sp.*	1.3328	1.1511	-14%

Table 5. 2 CPO D1. High percentage (>2%) OTUs in microcosm containing Grassland soil as seeding material, methanol as carbon substrate

OTU name	Taxonomy	OTU percentage		
		Before TCE dechlorination	After TCE dechlorination	Increased percentage
OTU_185	uncultured *clostridium sp.*	5.3552	7.7583	45%
OTU_19	*gracilibacter* thermotolerans	2.1324	2.4203	14%
OTU_5785	*methanosarcina sp.* str. fr af020341.1	4.4029	4.2698	-3%
OTU_4727	uncultured *acetivibrio sp.*	2.4692	2.2545	-9%
OTU_2208	uncultured *clostridium sp.*	8.0280	7.0344	-12%
OTU_1012	uncultured *clostridium sp.*	3.2422	2.7360	-16%
OTU_3534	*burkholderia sp.* z29_2	2.4038	1.8495	-23%
OTU_1456	uncultured *clostridium sp.*	2.3844	1.7698	-26%

Table 5. 3 Control D2. Grassland soil as seeding material, methanol as carbon substrate

OTU name	Taxonomy	OTU percentage		
		Before TCE dechlorination	After TCE dechlorination	Increased percentage
OTU_107	*Clostridium* termitidis str. dsm 5396 x71854.1	0.0067	1.4398	21355%
OTU_13	uncultured *Dehalobacter sp.*	0.0134	1.1934	8792%
OTU_4041	uncultured *Dehalobacter sp.*	0.0067	0.3503	5119%
OTU_1270	uncultured *Ruminococcus sp.*	0.0067	0.0722	975%
OTU_2982	uncultured *Acidobacterium sp.*	0.0067	0.0581	766%
OTU_27	uncultured *Clostridium sp.*	1.4227	0.3309	-77%
OTU_3689	uncultured *Sporomusa sp.*	2.5502	0.9716	-62%
OTU_6437	*Sporomusa sp.* an4	3.5031	1.4768	-58%
OTU_2915	uncultured *Acetivibrio sp.*	1.2818	0.6742	-47%
OTU_2208	uncultured *Clostridium sp.*	1.6845	0.9030	-46%

Table 5. 4 Control D2. High percentage (>2%) OTUs in microcosm containing Grassland soil as seeding material, methanol as carbon substrate

OTU name	Taxonomy	OTU percentage		
		Before TCE dechlorination	After TCE dechlorination	Increased percentage
OTU_2356	uncultured *ruminococcus sp.*	4.362	6.676	53%
OTU_19	*gracilibacter* thermotolerans	4.604	3.615	-21%

Table 5. 5 CPO E3. Contaminated Aquifer as seeding material, methanol as carbon substrate

OTU name	Taxonomy	OTU percentage		
		Before TCE dechlorination	After TCE dechlorination	Increased percentage
OTU_3166	uncultured *Dehalobacter sp.*	0.0089	0.9011	10023%
OTU_13	uncultured *Dehalobacter sp.*	0.0267	1.3909	5108%
OTU_5487	uncultured *Clostridium sp.*	0.0030	0.0816	2651%
OTU_4041	uncultured *Dehalobacter sp.*	0.0178	0.4082	2193%
OTU_4000	uncultured *Dehalobacter sp.*	0.0030	0.0628	2016%
OTU_3534	*Burkholderia sp.* z29_2	6.3947	3.8683	-40%
OTU_1430	uncultured *Clostridium sp.*	6.1187	3.9844	-35%
OTU_1929	uncultured *Burkholderia sp.*	2.7953	1.8807	-33%
OTU_9	uncultured *Alkalibacter sp.*	13.0623	10.0914	-23%
OTU_2085	uncultured *Clostridium sp.*	2.0623	1.9687	-5%

Table 5. 6 CPO E3. High percentage (>2%) OTUs in microcosm containing Contaminated Aquifer as seeding material, methanol as carbon substrate

OTU name	Taxonomy	OTU percentage		
		Before TCE dechlorination	After TCE dechlorination	Increased percentage
OTU_1012	uncultured *clostridium sp.*	2.6439	5.0802	92%
OTU_2208	uncultured *clostridium sp.*	5.8160	10.5655	82%
OTU_1456	uncultured *clostridium sp.*	1.8012	3.0079	67%
OTU_47	uncultured *dysgonomonas sp.*	4.7211	4.9766	5%
OTU_2085	uncultured *clostridium sp.*	2.0623	1.9687	-5%
OTU_9	uncultured *alkalibacter sp.*	13.0623	10.0914	-23%
OTU_1929	uncultured *burkholderia sp.*	2.7953	1.8807	-33%
OTU_1430	uncultured *clostridium sp.*	6.1187	3.9844	-35%
OTU_3534	*burkholderia sp.* z29_2	6.3947	3.8683	-40%

Table 5. 7 Control E1. Contaminated Aquifer as seeding material, methanol as carbon substrate

OTU name	Taxonomy	OTU percentage		
		Before TCE dechlorination	After TCE dechlorination	Increased percentage
OTU_321	*Escherichia coli* o157:h7 str. ss17	0.0084	1.1500	13592%
OTU_2083	*Escherichia coli*	0.0042	0.1737	4036%
OTU_258	*Sporomusa* termitida	0.0588	1.4316	2335%
OTU_168	uncultured *Clostridium sp.*	0.0336	0.7041	1996%
OTU_3451	uncultured *Anaerovorax sp.*	0.0084	0.0610	627%
OTU_6530	*Clostridium* xylanolyticum str. atcc 4963 x71855.1	2.3433	0.1924	-92%
OTU_3138	*Burkholderia* cenocepacia	2.8262	1.2063	-57%
OTU_5698	*Burkholderia* cenocepacia	12.1908	5.8484	-52%
OTU_3	*Bacteroides* xylanolyticus	6.8072	3.4123	-50%
OTU_1165	*Clostridium sp.* k11	5.5768	3.2199	-42%

Table 5. 8 Control E1. High percentage (>2%) OTUs in microcosm containing Contaminated Aquifer as seeding material, methanol as carbon substrate

OTU name	Taxonomy	OTU percentage		
		Before TCE dechlorination	After TCE dechlorination	Increased percentage
OTU_6	uncultured *anaerovorax sp.*	2.8598	4.5341	59%
OTU_1217	uncultured *anaerovorax sp.*	3.4897	5.3931	55%
OTU_5304	uncultured *anaerovorax sp.*	7.3279	10.9035	49%
OTU_40	*lysinibacillus* sphaericus jg_7b am903104.1	2.3811	2.1450	-10%
OTU_1165	*clostridium sp.* k11	5.5768	3.2199	-42%
OTU_3	bacteroides xylanolyticus	6.8072	3.4123	-50%
OTU_5698	*burkholderia* cenocepacia	12.1908	5.8484	-52%
OTU_3138	*burkholderia* cenocepacia	2.8262	1.2063	-57%

Table 5. 9 CPO F1. Stream sediment as seeding material, methanol as carbon substrate

OTU name	Taxonomy	OTU percentage		
		Before TCE dechlorination	After TCE dechlorination	Increased percentage
OTU_6466	*Methanosarcina* acetivorans str. c2a ae010299.1	0.0045	0.0911	1935%
OTU_1651	uncultured *Methanosarcina sp.*	0.0984	1.2802	1201%
OTU_1817	*Methanosarcina* acetivorans str. c2a ae010299.1	0.0492	0.5809	1080%
OTU_1681	uncultured *Methanoculleus sp.*	0.0022	0.0255	1040%
OTU_4234	*Methanosarcina* mazei	0.0022	0.0219	877%
OTU_20	*Clostridium sp.* nml 04a032	4.1703	3.1414	-25%
OTU_5980	*Pseudomonas* aeruginosa	2.2239	1.7829	-20%
OTU_297	*Pseudomonas* aeruginosa	5.7096	4.8678	-15%
OTU_1	*Pseudomonas* resinovorans str. c87	3.9130	3.4382	-12%
OTU_4005	*Pseudomonas* resinovorans str. c87 fj950593.1	9.2243	8.5118	-8%

Table 5. 10 CPO F1. High percentage (>2%) OTUs in microcosm containing Stream sediment as seeding material, methanol as carbon substrate

OTU name	Taxonomy	OTU percentage		
		Before TCE dechlorination	After TCE dechlorination	Increased percentage
OTU_8	uncultured *methanosarcina sp.*	2.7608	5.3759	95%
OTU_55	uncultured *anaeromyxobacter sp.*	2.8212	2.7153	-4%
OTU_4005	*pseudomonas* resinovorans str. c87 fj950593.1	9.2243	8.5118	-8%
OTU_3534	*burkholderia sp.* z29_2	3.5618	3.2652	-8%
OTU_1929	uncultured burkholderia sp.	1.9285	1.7191	-11%
OTU_1	*pseudomonas* resinovorans str. c87 fj950593.1	3.9130	3.4382	-12%
OTU_297	*pseudomonas* aeruginosa	5.7096	4.8678	-15%
OTU_20	*clostridium sp.* nml 04a032	4.1703	3.1414	-25%
OTU_1430	uncultured *clostridium sp.*	1.5683	1.1764	-25%

Table 5. 11 Control F2. Stream sediment as seeding material, methanol as carbon substrate

OTU name	Taxonomy	OTU percentage		
		Before TCE dechlorination	After TCE dechlorination	Increased percentage
OTU_207	*Clostridium* nitrophenolicum str. 1d am261414.1	0.0018	0.7224	40381%
OTU_58	uncultured *Acetivibrio sp.*	0.0178	4.1793	23320%
OTU_2209	*Methanosarcina* siciliae str. c2j u89773.1	0.0036	0.8123	22659%
OTU_106	*Lutispora* thermophila	0.0036	0.7472	20835%
OTU_3166	uncultured *Dehalobacter sp.*	0.0089	1.8509	20644%
OTU_79	*Desulfotomaculum* nigrificans str. cw_01 ay742958.1	1.9291	0.3441	-82%
OTU_5304	uncultured *Anaerovorax sp.*	3.5387	0.8185	-77%
OTU_1217	uncultured *Anaerovorax sp.*	1.8506	0.4217	-77%
OTU_18	*Gracilibacter* thermotolerans	6.3869	2.4958	-61%
OTU_3138	*Burkholderia* cenocepacia	5.3750	2.3532	-56%

Table 5. 12 Control F2. High percentage (>2%) OTUs in microcosm containing Stream sediment as seeding material, methanol as carbon substrate

OTU name	Taxonomy	OTU percentage		
		Before TCE dechlorination	After TCE dechlorination	Increased percentage
OTU_8	uncultured *methanosarcina sp.*	0.107	7.438	6847%
OTU_1165	*clostridium sp.* k11	2.595	1.342	-48%
OTU_3	*bacteroides* xylanolyticus	3.114	1.606	-48%
OTU_5698	*burkholderia* cenocepacia	24.314	12.064	-50%
OTU_3138	*burkholderia* cenocepacia	5.375	2.353	-56%
OTU_18	*gracilibacter* thermotolerans	6.387	2.496	-61%
OTU_5304	uncultured *anaerovorax sp.*	3.539	0.819	-77%

From the tables above, it's easily found that species appeared in the top 5 growth ranking in CPO&CTRL D, CPO E, and CTRL F belong to *Dehalobacter sp.*; *Methanosarcina sp.* occupied 4 out of 5 top growing species in CPO F reactor only. The OTU percentage of decreasing strains were not as significant as the growing ones. Looking into the microcosms that identified a high percentage (>2%), *Anaerovorax sp., Methanosarcina sp., Ruminococcus sp.* are all putative

fermenters that existed in anaerobic soil environment, while *clostridium sp.* was found having a high percentage in the community and has been reported some specific strain had the ability to enhance the degrade PCE to cis-DCE (YC Chang et al.,2000).

Table 5. 13 *Dehalobacter sp.* concentration in different microcosms at before/after degradation

Group NO.	Microcosm	Triplicate	*Dehalobacter sp.* concentration (copies/ng DNA)		Days	Difference
			Before	After		
A. Methanol/ Oak soil	CPO-OM	1	4.617	4.793	29-38	0.175
	CTRL-OM	1	3.376	4.803	67-73	1.428
		2	5.261	5.283	42-43	0.021
B. Acetate/ Oak soil	CPO-OM	1	4.378	5.091	96-122	0.713
		3	2.442	3.091	40-43	0.649
	CTRL-OM	1	2.533	4.991	29-37	2.458
C. Molasses/ Oak soil	CPO-OM	1	3.489	5.366	43-47	1.877
	CTRL-OM	2	3.159	5.232	67-72	2.073
E. Methanol/ Grassland soil	CPO-OM	1	4.812	4.948	106-110	0.135
		3	2.223	5.558	38-99	3.335
	CTRL-OM	1	5.658	5.055	24-26	-0.603
F. Methanol/ Contaminated aquifer	CPO-OM	1	4.172	5.254	61-67	1.082
		2	4.383	4.789	78-84	0.406
		3	4.134	4.906	82-92	0.772
	CTRL-OM	1	5.005	4.263	12-17	-0.743
		2	3.197	4.724	12-17	1.526
		3	4.080	4.791	14-17	0.711
E. (fresh leaves OM extract) Methanol/ Grassland soil	CPO-OM	1	3.411	3.215	94-101	-0.196
		2	3.446	3.706	94-101	0.260
	CTRL-OM	1	3.843	4.768	94-98	0.925
		2	4.767	5.022	94-101	0.255
		3	3.288	4.286	94-101	0.999
F. (fresh leaves OM extract) Methanol/ Contaminated aquifer	CPO-OM	1	4.264	5.101	81-94	0.837
		2	4.696	2.571	81-94	-2.125
		3	4.868	3.792	81-97	-1.075
	CTRL-OM	1	4.214	4.127	81-98	-0.087
		2	4.220	4.004	81-94	-0.216
		3	3.969	3.820	81-94	-0.149

So, delving in the microbial activity of *Dehalobacter* would be a good way to better understand the degradation process. Real time PCR （qPCR） was applied to quantify the bacteria population in group A, B, D, E, F, including both CPO and control reactors. qPCR results of *Dehalobacter* were shown in Figure 4.2- Figure 4.7 (dash line curves). Notable growth of *Dehalobacter* was observed in reactors Control A, CPO and Control B, CPO and Control D, CPO E and F, the growth of *Dehalobacter* during the degradation period is about 100~1000 times as the beginning of the dechlorination reaction. While in Control E, the population of *Dehalobacter* was relatively high, but no growth was observed during the reaction, also in Control F, a small increase of population growth was observed. The qPCR results came out consistent with the OTU results, reactors detected high in OTU percentage in *Dehalobacter sp.* tested high bacteria population growth, and others didn't detect the bacteria growing for all reactors except CPO F. For reactor CPO F, the OTU test didn't detect obvious increase of *Dehalobacter*, while the qPCR quantified an evident increase of *Dehalobacter* population, one possible reason is *Methanosarcina* has dominated the microbial community and had a much larger population than *Dehalobacter* in the CPO F reactor.

Combining these results with the results from Chapter IV, it's possible that seeding material like contaminated aquifer and stream sediments induced different bacteria (such as *Methanosarcina*) growing other than *Dehalobacter, Clostridium, Acetivibrio,* which degraded TCE faster; or CPO amended OM has developed the dechlorinating microbial community, but didn't dominant the dechlorination process.

Below are the phylogenetic trees showing the all the increasing OTU sequences (before and after the dechlorination) detected in each reactor and the phylogenic relationship in between.

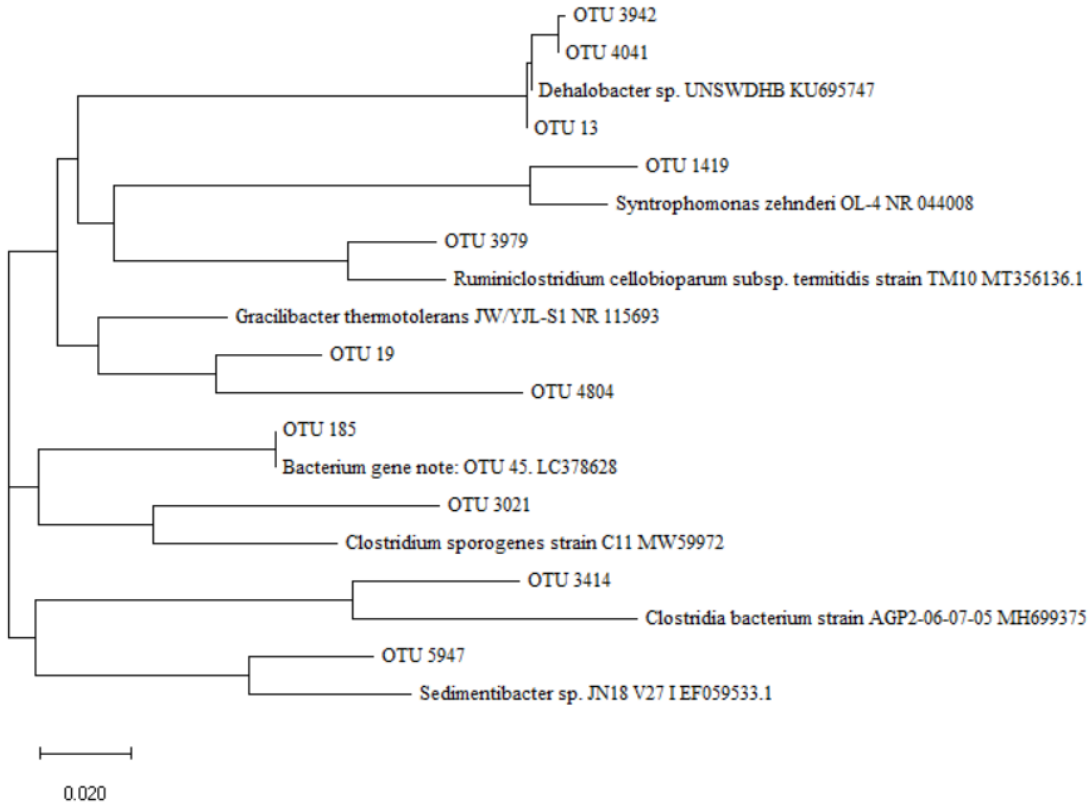


Figure 5. 1 Phylogenetic tree of growing bacteria involved in the dechlorination in CPO D reactor

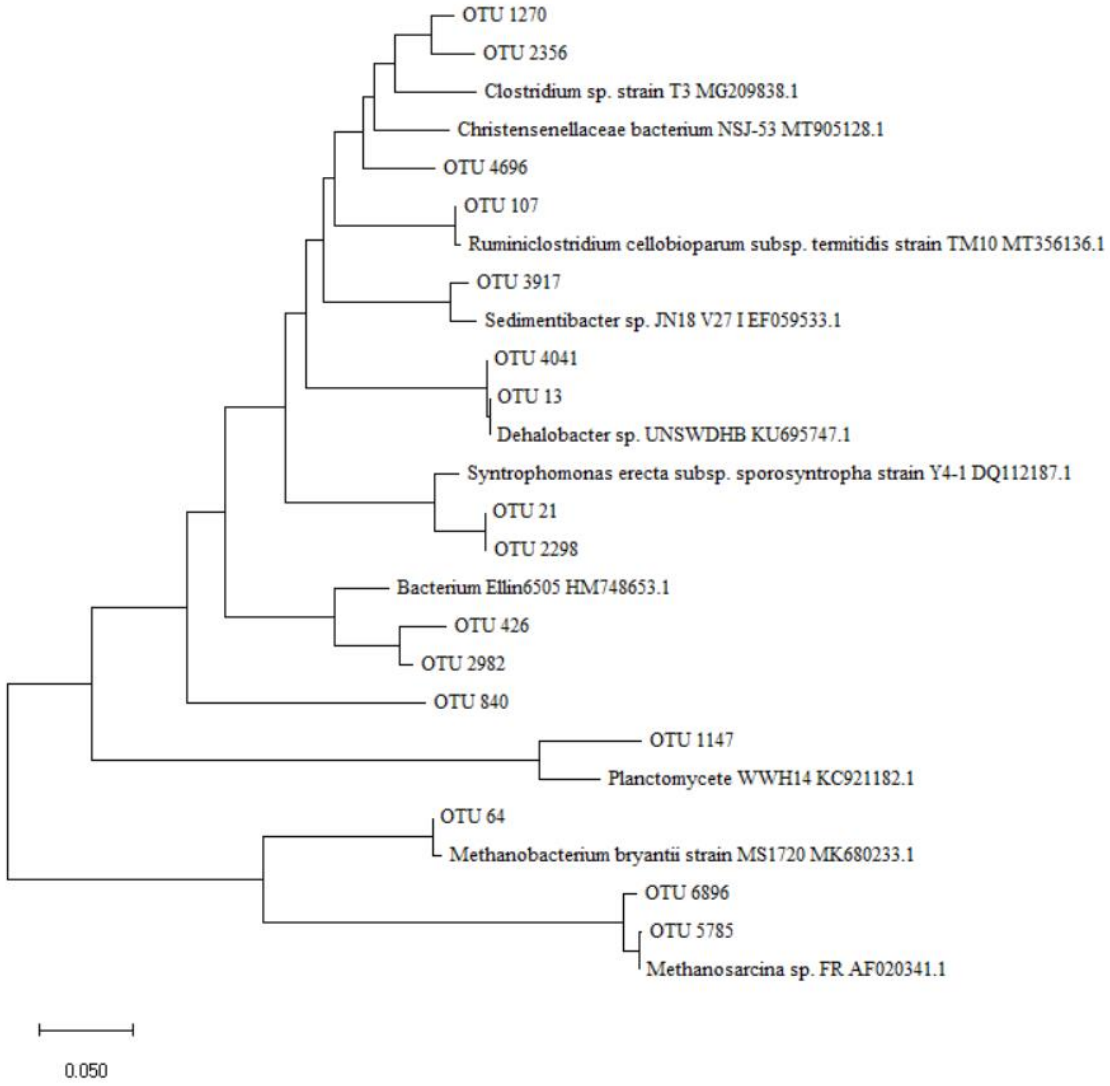


Figure 5. 2 Phylogenetic tree of growing bacteria involved in the dechlorination in Control D reactor

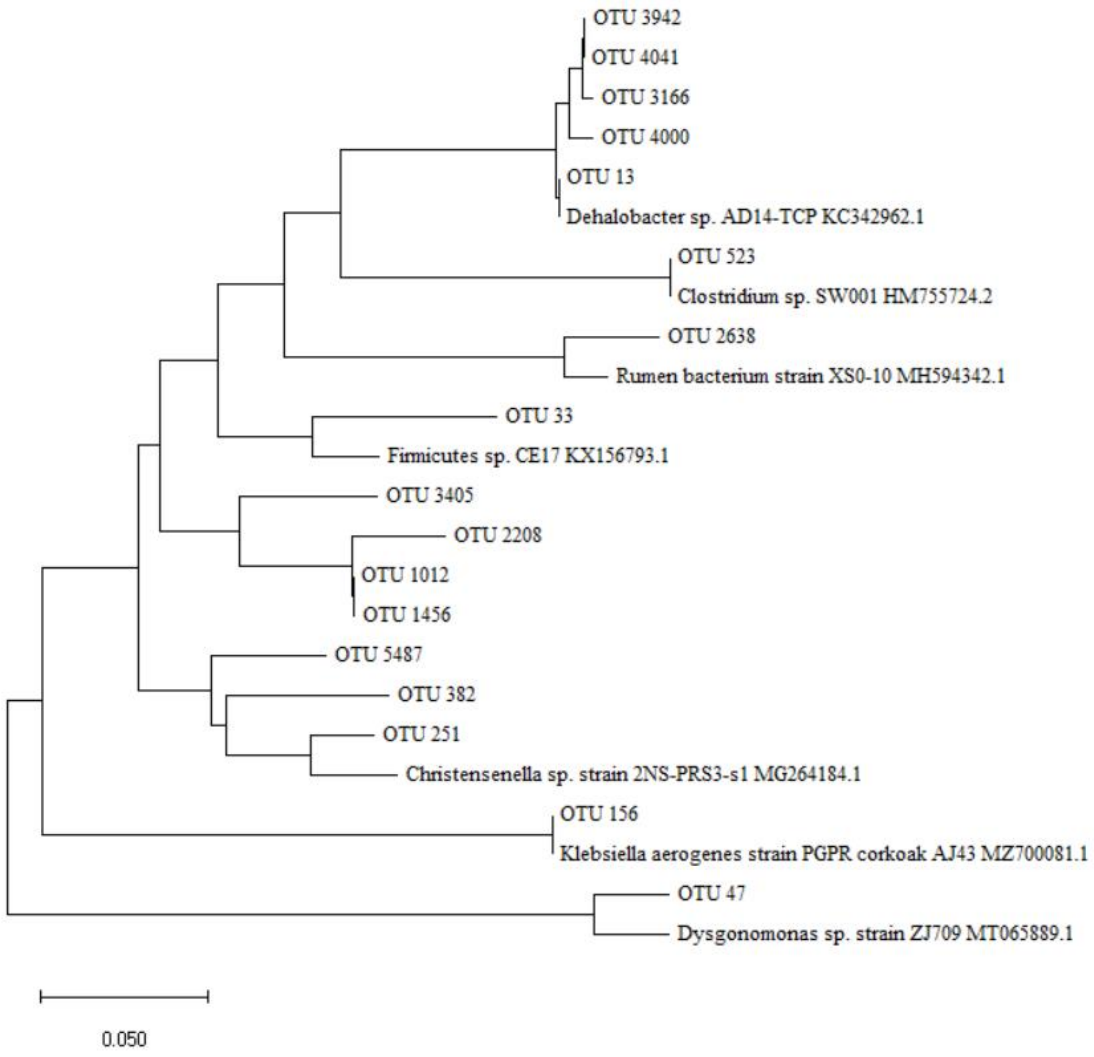


Figure 5. 3 Phylogenetic tree of growing bacteria involved in the dechlorination in CPO E reactor

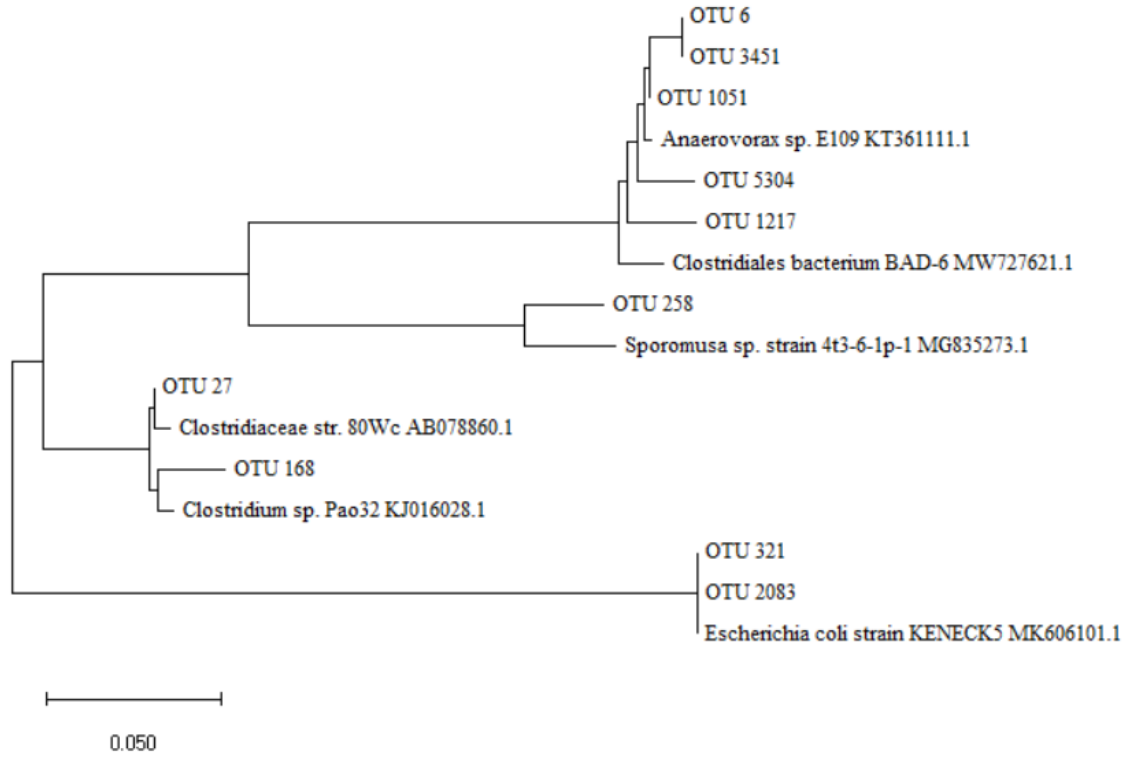


Figure 5. 4 Phylogenetic tree of growing bacteria involved in the dechlorination in Control E reactor

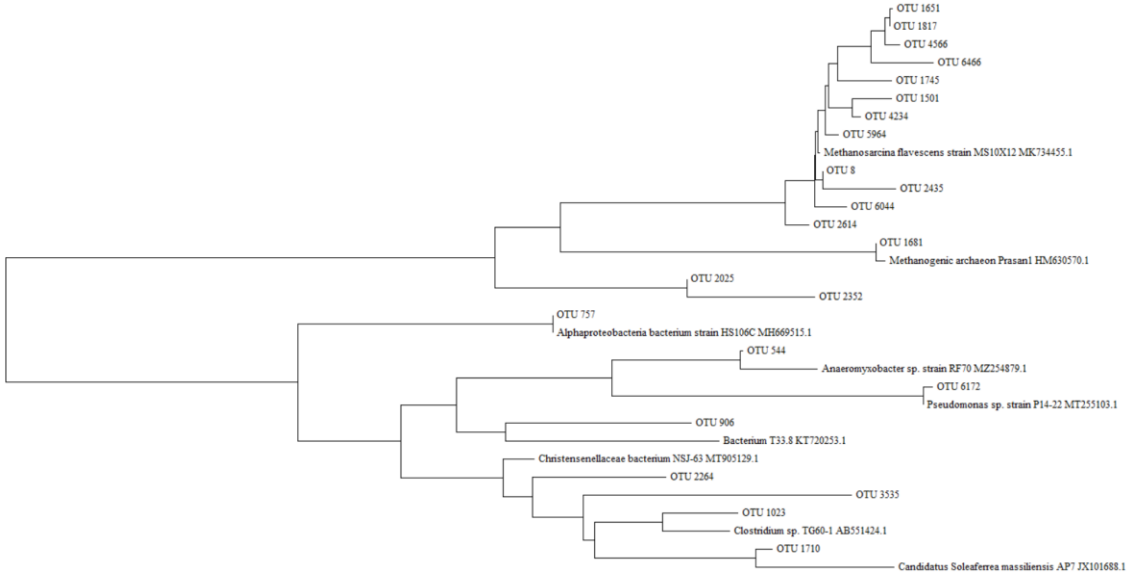


Figure 5. 5 Phylogenetic tree of growing bacteria involved in the dechlorination in CPO F reactor

CHAPTER VI

TOXICITY TESTS

6.1 Introduction

Natural geogenic and biogenic processes can produce notoriously toxic organohalogens such as polychlorinated dibenzo-p-dioxins (PCDDs) and polybrominated diphenyl ethers (PBDEs), with some of the latter being even more toxic than their anthropogenic counterparts (Wiseman et al., 2011). PCDDs were found in sediments dated at 1–10 million years of age from the Yellow Sea, the East China Sea and the Pacific Ocean (Hashimoto et al., 1995) indicating the ancient origin of natural organohalogens. Moreover, volatile halomethanes emitted to the atmosphere from the oceans and forest soils are important ozone-depleting metabolites (Hossaini et al., 2015). Although most natural organohalogens such as antibiotics are produced in trace quantities, others such as halomethanes are naturally produced in massive quantities. For example, of the 700- 820 Gg/y global production of chloroform, 90% or more is of natural sources (Field, 2016).

In this chapter, the microbial and ecological toxicity characteristics of the natural organochlorines used will be tested under aerobic and anaerobic conditions, so that the effect of natural organochlorines on bacterial communities can be assessed. This objective further explores the biogeochemical interactions between the bacterial community and natural organochlorines, which may be important for bioremediation.

6.2 Methods

Toxicity tests on the CPO-reacted OM (water extracted) were carried out in several ways. First was an anaerobic toxicity test, by observing how the biogas (CH_4) production changes with different amount of enzymatically produced organochlorines (CPO) added in the digester. The second test was aerobic toxicity test, which measured the O_2 reduction in the activated sludge reactor to test the inhibitory effect to the cell growth. Finally, CPO-OM and control OM was submitted to a commercial toxicity lab (Cove Environmental, Stillwater OK) for tests on saltwater and freshwater fish species in a collaboration Justin Scott (currently a PhD candidate, Integrative Biology).

Anaerobic toxicity test

Anaerobic digester sludge from the Stillwater wastewater treatment plant was used in this test. After collection, sludge was stored at 4 ˚C until further use. The sludge was transferred into the anaerobic glovebag, fed with ~1 mM of acetate to acclimate, and activate the microorganisms one day before the test.

After the microorganisms were activated, five different concentrations of CPO produced organochlorines were dispensed into 160 mL serum bottles. Each condition was prepared and tested in triplicate, see Table 3 below. Microcosms were amended with 100 mM of acetate and incubated at 37 ^{o}C.

Table 6. 1 Reactor Setup for Anaerobic and aerobic Toxicity Test

Bottle ID	inhibitory substance	substance volume (mL)	Each treatment was prepared in triplicate, and each anaerobic digester was filled with 20 mL of mineral media and 1 ml of active anaerobic digester sludge. A set of control microcosms were set-up as well which contained no CPO-OM or CTRL-OM amendment.
100% CPO-OM	CPO-OM CTRL-OM	5 0	
80% CPO-OM	CPO-OM CTRL-OM	4 1	
60% CPO-OM	CPO-OM CTRL-OM	3 2	
40% CPO-OM	CPO-OM CTRL-OM	2 3	
20% CPO-OM	CPO-OM CTRL-OM	1 4	
0% CPO-OM	CPO-OM CTRL-OM	0 5	

Data collecting

Several times a day for the first 5 days, and once a day for a few days thereafter, the methane produced in the headspace was measured. For each microcosm, the production rate was then calculated (zero-order kinetics) and normalized to VSS concentration. Between microcosms in a set, the average and standard deviation was determined.

Methane production was measured using Agilent 6890 GC-FID, the instrument setting was the same as measuring VC and ethylene, and had an isothermic method at 100^{o}C. The sampling schedule is based the reaction rate. The first 2 days, each bottle was measured every 6 hours, then

every 12 hours in the third day, then sampled once a day after that, until the end of the test. Each bottle was sampled and measured twice at each time point to ensure accuracy, and the average was calculated and used for standard curve and toxicity analysis. The concentration of methane production was calculated through the standard curve, and the average slope of the methane production curve by time from triplicates was used as Methane generation (% CH_4 day^{-1} g VSS^{-1}) for the methanogenesis toxicity analysis (% CH_4 refers to the % CH_4 in the 135ml headspace, by using ideal gas law, 1% CH_4 day^{-1} g VSS^{-1} corresponds to 8.83×10^{-4}g CH_4 day^{-1} g VSS^{-1}).

Aerobic Toxicity Test

In the aerobic toxicity test, conditions were similar to the above with the exception that activated sludge was used as the inoculum, the microcosms were aerobic, and the depletion of oxygen was measured to indicate microbial activity rate. Again, each microcosm contained 20 mL of a mineral media (250 mg/L of K_2HPO_4; 10 mg/L $CaCl_2$-$2H_2O$; 100 mg/L of $MgSO_4$-$6H_2O$, 280 mg/L of NH_4Cl, 4000 mg/L of $NaHCO_3$; 100 mg/L of yeast extract, and 1 mg/L of trance element solution. The trace element solution was 2000 mg/L $FeCl_2$-$4H_2O$, 50 mg/L each of $ZnCl_2$, $MnCl_2$-4H2O, $NiCl_2$-$6H_2O$, H_3BO_3, and $(NH_4)6Mo_7O_{24}$-$4H_2O$, 90 mg/L $AlCl_3$-$6H_2O$, 2000 mg/L of $CoCl_2$-$6H_2O$, 30 mg/L of $CuCl_2$-$2H_2O$, 100 mg/L $NaSeO_3$-$5H_2O$, and 1 mL of 36% HCl. The amount of CPO and CTRL was varied as shown above in Table 6.1. The bottles were capped in the atmosphere.

The O_2 was analyzed with a gas chromatograph with a thermal conductivity detector (Agilent 8890), using helium as a carrier gas and a isothermic temperatures of 50℃. 100 µL headspace injections were used to measure O_2. All measurements were done in duplicate. At the end of a run, the oven was increased to 150 C for ten minutes to release any water vapor. Three injections

of air were used at the beginning of each of run as a standard. Measurements for O_2 were taking twice a day, and then daily for a week. At the end of the experiment, the pH and TSS was measured for each bottle. The rates of O_2 consumption were normalized to TSS an analyzed for each microcosm bottle. The averages and standards deviations of triplicate microcosms were then determined in excel.

Saltwater and Freshwater Toxicity Tests

The CPO-reacted OM and the Control OM was also submitted to Cove Environmental LLC (Stillwater, OK) for acute toxicity assays on fleas, freshwater fish (embryos), and saltwater fish (embryos). Because of the high K and Cl amounts in the extracts, the OM was processed through a series of extractions in Dichloromethane and solid phase extraction columns (eluted with methanol) to separate the OM into solvents, while the ions largely remained in the aqueous phase. The extracted OMs were then roto-evaporated and subjected to bubbling overnight to evaporate the volatiles. Some carry-over water phase remained, but after three days of bubbling, a negative GC-FID analysis of the headspace determined the solvents were fully evaporated. The extract was then re-diluted to its original volume. An analysis of the major ions indicated enough salts were removed to proceed, and the tests balanced out the salt content to a suitable level for the species tested. The tests used up to 12.5% total volume of the CPO or the CTRL extract. The TOC of the CPO extract was 409 mg/L and for the CTRL extract was 288 mg/L.

6.3 Results and Discussion

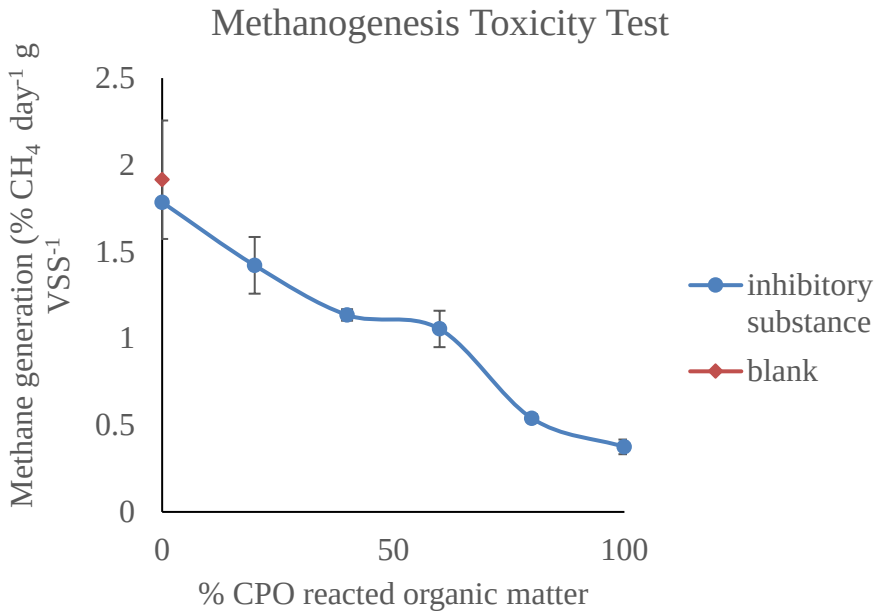


Figure 6. 1 Methane generation based on different percentage of CPO reacted organic matter

Comparing the blank sample (diamond plot) and the ones with CPO amended organic matters (round plot), there is no significant difference of the methane generation between the blank sample and the control (no CPO-amended) organic matters, which indicated that the no CPO added organic matters are not toxic to the methanogens presented in the anaerobic digester sludge. From the figure above, it's obvious that as the amount of CPO reacted organic matter increased, there was a significant impact on the methane production from the methanogens presented. As the added amount of CPO reacted organic matter increased from 0 to 20%, 40%, 60%, 80% and 100%, the methane production decreased by 20.22%, 35.96 %, 41.01%, 69.66%, and 79.21%, which proved the toxicity of CPO reacted organic matter comparing to the organic matter without CPO amendment.

Aerobic Toxicity Test

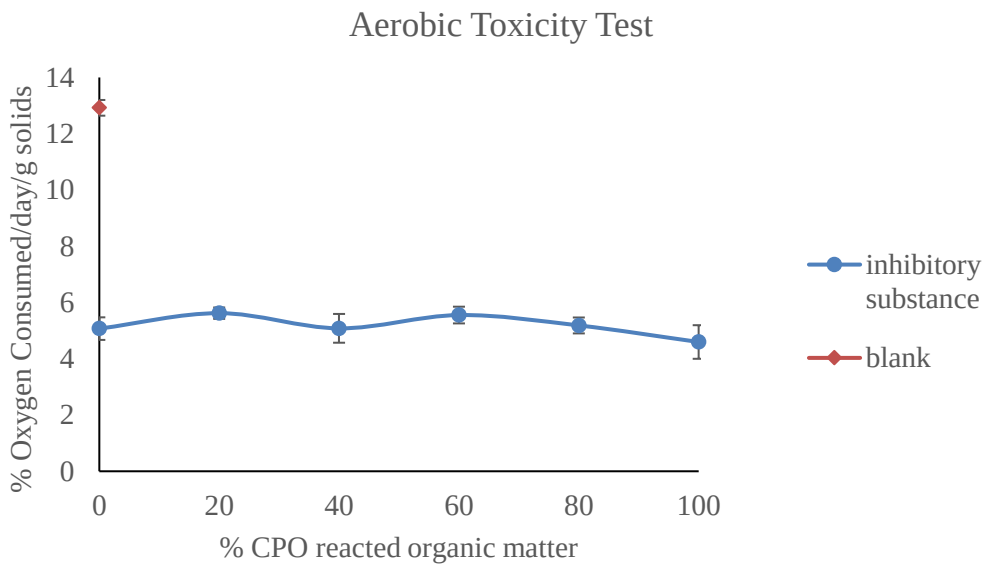


Figure 6. 2 percentage of oxygen consumed in the aerobic toxicity test based on different percentage of CPO reacted organic matter

The blank sample (diamond plot) which has no inhibitory substance (neither CPO reacted organic matters or control organic matters) has 12.92% oxygen consumed per day by each gram of solids. After adding the amended organic matters, the percentage of oxygen consumed has decreased more than half of the blank sample, staying at around 5% oxygen consumed per day by each gram of solids. This finding obviously indicated that both CPO amended organic matters and the control are toxic to the soil microbes. One possible reason might be the high salinity in the organic matter solutions strongly affect the aerobic microbes, so both CPO reacted and no-reaction OM were showing the toxic effect.

Fish Toxicity Tests

Several concentrations of CPO and Control extracts were tested (up to 50 ppm as TOC) for acute toxicity. In all experiments, both the CPO and the Control extracts were not found to exhibit acute toxicity. The number of viable embryos were statistically the same as lab-controls of clean water. Studies in the future may include chronic toxicity tests.

All told, the toxicity of CPO reacted OM in the anaerobic tests but lack of toxicity in the aerobic and fish tests comparing to the control indicates that either CPO-reacted OM is toxic to only certain microbes or the toxicity is based on a redox-type of mechanism. These results are encouraging, in that any future use of CPO-based OM in the field will be more feasible if it is found to be non-toxic.

CHAPTER VII

CONCLUSIONS AND FUTURE WORK

Results from this study indicated the ability of organochlorides to stimulate TCE degradation and the effect on the completeness, effectiveness and bacterial communities involved in the process. Also, it revealed the sophisticated relationships between TCE, concentrations of organochlorides and the dechlorinating communities. Furthermore, the toxicity result clarified the toxic effect of CPO reacted organic matters on methanogens and aerobic microbes, which gave a better understanding of when to apply this technique to *in situ* bioremediation and future research. Detailed conclusions from each experiment are listed below.

1. CPO-reacted water-soluble OM was capable of enhancing halorespirers and improving biodegradation, i.e., proved it was more suitable for bio-stimulating TCE dechlorination than CPO-reacted solvent-extracted OM.

2. Inhibition of CPO reacted OM compared to Control OM was found to be influenced by electron donor, Methanol>Acetate > Molasses

3. CPO reacted OM clearly was an inhibitor in microcosms seeded with contaminated aquifer or stream sediments, indicating CPO reacted OM is likely a competitive inhibitor with TCE in certain microbial communities.

4. Microbial analysis showed that CPO reacted OM can support a dechlorinating community to degrade TCE.

5. Inoculum material such as contaminated aquifer and stream sediments stimulate the growth of different bacteria during TCE dechlorination (such as *Methanosarcina*), compared to soils where *Dehalobacter* and *Clostridium* grew and dechlorinated TCE faster.

6. CPO-OM was found to inhibit methanogenesis but not aerobic heterotrophs compared to control OM, indicated that either CPO-OM is toxic to only certain microbes or the toxicity is based on a redox-type of mechanism.

This study found that stimulating the TCE degradation process with CPO reacted OM is complex – both inhibition and stimulation activities were found. Soil microbial communities and electron donor amendment affected whether inhibition or stimulation occurred, however, the mechanisms of this is not completely understood. Presumably the concentration of CPO reacted OM played a large role between experiments as well, and likely also explain why CPO reacted OM becomes an inhibitor or whether it stimulates. For the best result of degradation efficiency, the appropriate amount of organochlorides needs to be determined. A more comprehensive method to quantify and control the chlorination process would help to better evaluate its ability of stimulating degradation processes. Based on the microbial analysis data, a lot more bacteria were still not identified in the microcosms and those can be potential dechlorinators that contribute to the degradation process. Also, the toxicity effects on methanogens may have benefits (fewer competitors for electron donors) or may also contribute to inhibition since dechlorinators often scavenge cobalamin from methanogens. Answering these further questions will help elucidate if and how CPO-reacted OM (or other natural organochlorides) can be used as a biostimulant in contaminated environments.

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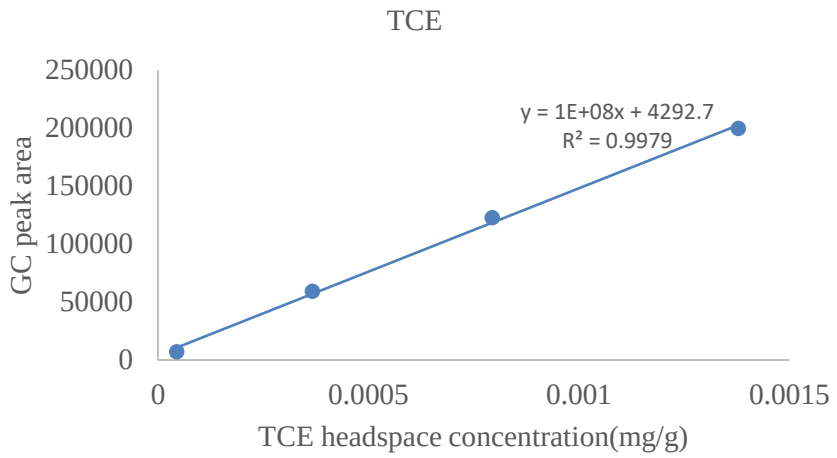
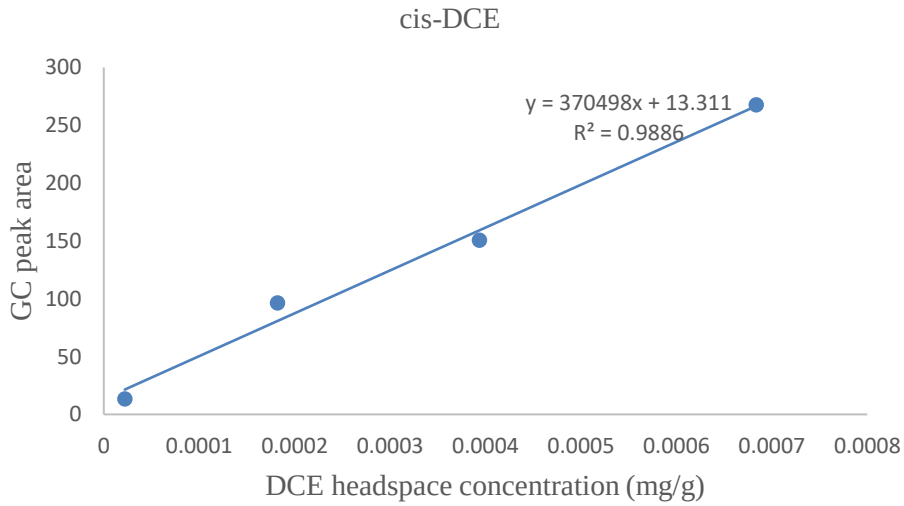
APPENDICES

Gas chromatography standards





Microcosm CPO D1. Grassland soil as seeding material, methanol as carbon substrate

OTU name	Taxonomy	OTU percentage		
		Before TCE dechlorination	After TCE dechlorination	Increased percentage
OTU_13	uncultured *Dehalobacter sp.*	0.0048	1.1193	22995%
OTU_4041	uncultured *Dehalobacter sp.*	0.0024	0.3508	14375%
OTU_3942	uncultured *Dehalobacter sp.*	0.0024	0.1180	4769%
OTU_4804	uncultured *Clostridium sp.*	0.0024	0.0191	690%
OTU_5947	uncultured *Sedimentibacter sp.*	0.0024	0.0191	690%
OTU_3021	uncultured *Ruminococcus sp.*	0.0024	0.0191	690%
OTU_1419	uncultured *Pelospora sp.*	0.0048	0.0351	624%
OTU_3979	uncultured A*cetivibrio sp.*	0.0024	0.0159	558%
OTU_3414	*Ruminococcus* bromii dq882649.1	0.0024	0.0159	558%
OTU_185	uncultured *Clostridium sp.*	5.3552	7.7583	45%
OTU_19	*Gracilibacter* thermotolerans	2.1324	2.4203	14%
OTU_1456	uncultured *Clostridium sp.*	2.3844	1.7698	-26%
OTU_3534	*Burkholderia sp.* z29_2	2.4038	1.8495	-23%
OTU_1012	uncultured *Clostridium sp.*	3.2422	2.7360	-16%
OTU_2488	*Methanosarcina* sp. af020341.1	1.3328	1.1384	-15%
OTU_2	uncultured *Clostridium sp.*	1.3328	1.1511	-14%
OTU_92	*Gracilibacter* thermotolerans	1.7738	1.5274	-14%
OTU_2208	uncultured *Clostridium sp.*	8.0280	7.0344	-12%
OTU_4727	uncultured *Acetivibrio sp.*	2.4692	2.2545	-9%
OTU_5785	*Methanosarcina sp*. af020341.1	4.4029	4.2698	-3%

Microcosm Control D2. Grassland soil as seeding material, methanol as carbon substrate

OTU name	Taxonomy	OTU percentage		
		Before TCE dechlorination	After TCE dechlorination	Increased percentage
OTU_107	*Clostridium* termitidis str. dsm 5396 x71854.1	0.0067	1.4398	21355%
OTU_13	uncultured *Dehalobacter sp.*	0.0134	1.1934	8792%
OTU_4041	uncultured *Dehalobacter sp.*	0.0067	0.3503	5119%
OTU_1270	uncultured *Ruminococcus sp.*	0.0067	0.0722	975%
OTU_2982	uncultured *Acidobacterium sp.*	0.0067	0.0581	766%

OTU_4696	uncultured *Clostridium sp.*	0.0134	0.0986	634%
OTU_840	uncultured *Conexibacter sp.*	0.0067	0.0458	582%
OTU_6896	*Methanosarcina* lacustris str. mm ay260430.1	0.0268	0.1725	543%
OTU_1147	uncultured *Zavarzinella sp.*	0.0067	0.0405	503%
OTU_5785	*Methanosarcina sp.* str. fr af020341.1	0.5235	1.9961	281%
OTU_426	uncultured *Acidobacterium sp.*	1.2281	2.6403	115%
OTU_64	*Methanobacterium sp.* m2 dq517520.1	1.4764	2.7301	85%
OTU_21	uncultured *Syntrophomonas sp.*	2.4428	4.0889	67%
OTU_2356	uncultured *Ruminococcus sp.*	4.3621	6.6764	53%
OTU_2298	uncultured *Syntrophomonas sp.*	3.7246	3.9728	7%
OTU_3917	*Sedimentibacter sp.* jn18_v27_i	1.2885	1.2990	1%
OTU_27	uncultured *Clostridium sp.*	1.4227	0.3309	-77%
OTU_3689	uncultured *Sporomusa sp.*	2.5502	0.9716	-62%
OTU_6437	*Sporomusa sp.* an4	3.5031	1.4768	-58%
OTU_2915	uncultured *Acetivibrio sp.*	1.2818	0.6742	-47%
OTU_2208	uncultured *Clostridium sp.*	1.6845	0.9030	-46%
OTU_803	uncultured *Acetivibrio sp.*	2.0603	1.1283	-45%
OTU_3698	uncultured *Verrucomicrobium sp.*	2.2482	1.2726	-43%
OTU_227	candidatus soleaferrea massiliensis ap7	1.0268	0.5914	-42%
OTU_1430	uncultured *Clostridium sp.*	1.8858	1.1582	-39%
OTU_6559	uncultured *Anaerobranca sp.*	2.0737	1.4170	-32%
OTU_19	*Gracilibacter* thermotolerans	4.6037	3.6154	-21%
OTU_6874	uncultured *Gracilibacter sp.*	1.2281	1.0015	-18%
OTU_59	uncultured *Acetivibrio sp.*	1.3422	1.3219	-2%

Microcosm CPO E3. Contaminated Aquifer as seeding material, methanol as carbon substrate

OTU name	Taxonomy	OTU percentage		
		Before TCE dechlorination	After TCE dechlorination	Increased percentage
OTU_3166	uncultured *Dehalobacter sp.*	0.0089	0.9011	10023%
OTU_13	uncultured *Dehalobacter sp.*	0.0267	1.3909	5108%
OTU_5487	uncultured *Clostridium sp.*	0.0030	0.0816	2651%
OTU_4041	uncultured *Dehalobacter sp.*	0.0178	0.4082	2193%
OTU_4000	uncultured *Dehalobacter sp.*	0.0030	0.0628	2016%
OTU_3942	uncultured *Dehalobacter sp.*	0.0089	0.1476	1558%

OTU_251	uncultured *Clostridium sp.*	0.0089	0.1130	1170%
OTU_3405	*Clostridium sp.* enrichment culture clone cs5	0.0030	0.0314	958%
OTU_523	*Pelosinus sp.* enrichment culture clone 014	0.0030	0.0283	852%
OTU_156	cu915186.1 and dynamics adapted microbiota during degradation tunisian zarzatine enriched seawater polluted tunisian crude oil clone	0.0059	0.0408	588%
OTU_2638	uncultured *Clostridium sp.*	0.0059	0.0408	588%
OTU_382	uncultured *Ruminococcus sp.*	0.0030	0.0188	535%
OTU_1012	uncultured *Clostridium sp.*	2.6439	5.0802	92%
OTU_2208	uncultured *Clostridium sp.*	5.8160	10.5655	82%
OTU_1456	uncultured *Clostridium sp.*	1.8012	3.0079	67%
OTU_33	uncultured *Anaerobranca sp*.	1.4718	1.9404	32%
OTU_47	uncultured *Dysgonomonas sp.*	4.7211	4.9766	5%
OTU_3534	*Burkholderia sp.* z29_2	6.3947	3.8683	-40%
OTU_1430	uncultured *Clostridium sp.*	6.1187	3.9844	-35%
OTU_1929	uncultured *Burkholderia sp.*	2.7953	1.8807	-33%
OTU_9	uncultured *Alkalibacter sp.*	13.0623	10.0914	-23%
OTU_2085	uncultured *Clostridium sp.*	2.0623	1.9687	-5%

Microcosm Control E1. Contaminated Aquifer as seeding material, methanol as carbon substrate

OTU name	Taxonomy	OTU percentage		
		Before TCE dechlorination	After TCE dechlorination	Increased percentage
OTU_321	*Escherichia coli* o157:h7 str. ss17	0.0084	1.1500	13592%
OTU_2083	*Escherichia coli*	0.0042	0.1737	4036%
OTU_258	*Sporomusa* termitida	0.0588	1.4316	2335%
OTU_168	uncultured *Clostridium sp.*	0.0336	0.7041	1996%
OTU_3451	uncultured *Anaerovorax sp.*	0.0084	0.0610	627%
OTU_1051	uncultured *Anaerovorax sp.*	1.6336	2.8960	77%

OTU_6	uncultured *Anaerovorax sp.*	2.8598	4.5341	59%
OTU_1217	uncultured *Anaerovorax sp.*	3.4897	5.3931	55%
OTU_5304	uncultured *Anaerovorax sp.*	7.3279	10.9035	49%
OTU_27	uncultured *Clostridium sp.*	1.4740	1.8728	27%
OTU_6530	*Clostridium* xylanolyticum str. atcc 4963 x71855.1	2.3433	0.1924	-92%
OTU_3138	*Burkholderia* cenocepacia	2.8262	1.2063	-57%
OTU_5698	*Burkholderia* cenocepacia	12.1908	5.8484	-52%
OTU_3	*Bacteroides* xylanolyticus	6.8072	3.4123	-50%
OTU_1165	*Clostridium sp.* k11	5.5768	3.2199	-42%

Microcosm CPO F1. Stream sediment as seeding material, methanol as carbon substrate

OTU name	Taxonomy	OTU percentage		
		Before TCE dechlorination	After TCE dechlorination	Increased percentage
OTU_6466	*Methanosarcina* acetivorans str. c2a ae010299.1	0.0045	0.0911	1935%
OTU_1651	uncultured *Methanosarcina sp.*	0.0984	1.2802	1201%
OTU_1817	*Methanosarcina* acetivorans str. c2a ae010299.1	0.0492	0.5809	1080%
OTU_1681	uncultured *Methanoculleus sp.*	0.0022	0.0255	1040%
OTU_4234	*Methanosarcina* mazei	0.0022	0.0219	877%
OTU_4566	*Methanosarcina sp.* af020341.1	0.0022	0.0200	795%
OTU_1501	*Methanosarcina* mazei	0.0022	0.0200	795%
OTU_906	*Symbiobacterium sp.* ka13	0.0045	0.0364	714%
OTU_757	producing pigmented colonies aerosol and soil remote glaciated areas (antarctica alps andes) snow s surface depth 10 cm clone vallot_b5 eu429484.1	0.0022	0.0182	714%
OTU_2435	*Methanosarcina* mazei	0.0022	0.0182	714%
OTU_1745	*Methanosarcina* siciliae str. c2j u89773.1	0.0022	0.0182	714%
OTU_2025	Candidatus *methanomassiliicoccus* intestinalis issoire_mx1	0.0022	0.0182	714%
OTU_6172	*Pseudomonas sp.* chol7	0.0022	0.0182	714%
OTU_5964	uncultured *Methanosarcina sp.*	0.1812	1.4113	679%

OTU_6044	*Methanosarcina sp.* met5bhj	0.0358	0.2622	633%
OTU_544	*Anaeromyxobacter sp.* nc_009675.1	0.0022	0.0164	633%
OTU_1023	uncultured *Acetivibrio sp.*	0.0067	0.0473	605%
OTU_2352	candidatus *Methanomassiliicoccus* intestinalis issoire_mx1	0.0045	0.0310	592%
OTU_2614	uncultured *Methanosarcina sp.*	0.0201	0.1366	578%
OTU_1710	candidatus soleaferrea massiliensis ap7	0.0022	0.0146	551%
OTU_2264	uncultured *Clostridium sp.*	0.0022	0.0146	551%
OTU_3535	*Clostridium* lundense	0.0022	0.0146	551%
OTU_8	uncultured *Methanosarcina sp.*	2.7608	5.3759	95%
OTU_20	*Clostridium sp.* nml 04a032	4.1703	3.1414	-25%
OTU_5980	*Pseudomonas* aeruginosa	2.2239	1.7829	-20%
OTU_297	*Pseudomonas* aeruginosa	5.7096	4.8678	-15%
OTU_1	*Pseudomonas* resinovorans str. c87	3.9130	3.4382	-12%
OTU_4005	*Pseudomonas* resinovorans str. c87 fj950593.1	9.2243	8.5118	-8%
OTU_3534	*Burkholderia sp.* z29_2	3.5618	3.2652	-8%

Microcosm Control F2. Stream sediment as seeding material, methanol as carbon substrate

OTU name	Taxonomy	OTU percentage		
		Before TCE dechlorination	After TCE dechlorination	Increased percentage
OTU_99	uncultured *Lutispora sp.*	0.0000	2.1300	#DIV/0!
OTU_207	*Clostridium* nitrophenolicum str. 1d am261414.1	0.0018	0.7224	40381%
OTU_58	uncultured *Acetivibrio sp.*	0.0178	4.1793	23320%
OTU_2209	*Methanosarcina* siciliae str. c2j u89773.1	0.0036	0.8123	22659%
OTU_106	*Lutispora* thermophila	0.0036	0.7472	20835%
OTU_3166	uncultured *Dehalobacter sp.*	0.0089	1.8509	20644%
OTU_6187	*Methanosarcina* mazei	0.0018	0.3596	20053%
OTU_3439	uncultured *Acetivibrio sp.*	0.0018	0.3534	19706%
OTU_797	*Methanosarcina* mazei	0.0018	0.3410	19011%
OTU_13	uncultured *Dehalobacter sp.*	0.0161	2.6942	16675%
OTU_4727	uncultured *Acetivibrio sp.*	0.0054	0.8309	15420%
OTU_132	*Clostridium sp.* 619	0.0143	1.9315	13430%
OTU_6437	*Sporomusa sp.* an4	0.0054	0.6821	12641%
OTU_5964	uncultured *Methanosarcina sp.*	0.0036	0.4341	12062%

OTU_6905	*Sporomusa sp.* gt1	0.0018	0.1860	10324%
OTU_6858	*Lutispora* thermophila	0.0018	0.1705	9456%
OTU_2325	*Methanosarcina sp.* str. fr af020341.1	0.0018	0.1643	9108%
OTU_1386	*Methanosarcina sp.* met5bhj	0.0018	0.1612	8934%
OTU_2472	uncultured *Acetivibrio sp.*	0.0036	0.3162	8761%
OTU_2354	uncultured *Methanosarcina sp.*	0.0071	0.6170	8543%
OTU_4736	*Lutispora* thermophila	0.0107	0.9053	8355%
OTU_3551	*Methanosarcina sp.* met5bhj	0.0036	0.3007	8326%
OTU_1750	*Methanosarcina* mazei	0.0018	0.1457	8066%
OTU_4041	uncultured *Dehalobacter sp.*	0.0089	0.7007	7753%
OTU_2862	*Methanosarcina* siciliae str. c2j u89773.1	0.0018	0.1271	7023%
OTU_8	uncultured *Methanosarcina sp.*	0.1071	7.4378	6847%
OTU_1651	uncultured *Methanosarcina sp.*	0.0018	0.1209	6676%
OTU_3435	uncultured *Dehalobacter sp.*	0.0018	0.1116	6155%
OTU_2124	*Sedimentibacter sp.* mo_sed	0.0018	0.1085	5981%
OTU_2840	uncultured *Dehalobacter sp.*	0.0018	0.1023	5633%
OTU_2587	uncultured *Methanosarcina sp.*	0.0071	0.4000	5503%
OTU_3706	*Sporobacterium* olearium	0.0054	0.2511	4591%
OTU_554	candidatus soleaferrea massiliensis ap7	0.0054	0.2511	4591%
OTU_886	uncultured *Sedimentibacter sp.*	0.0500	2.2447	4392%
OTU_4391	*Sedimentibacter sp.* mo_sed	0.0107	0.4806	4388%
OTU_6313	*Methanosarcina* siciliae str. c2j u89773.1	0.0018	0.0775	4243%
OTU_3953	*Methanosarcina* mazei	0.0036	0.1426	3896%
OTU_5974	uncultured *Methanosarcina sp.*	0.0018	0.0682	3722%
OTU_4481	uncultured *Methanosarcina sp.*	0.0018	0.0651	3548%
OTU_716	*Methanosarcina sp.* str. fr af020341.1	0.0036	0.1271	3462%
OTU_56	*Sedimentibacter sp.* mo_sed	0.0518	1.8354	3447%
OTU_2457	uncultured *Acetivibrio sp.*	0.0036	0.1240	3375%
OTU_6720	*Methanosarcina* mazei	0.0071	0.2418	3288%
OTU_5300	uncultured *Acetivibrio sp.*	0.0054	0.1674	3027%
OTU_30	*Sporobacterium* olearium	0.2302	7.1619	3011%
OTU_2515	*Methanosarcina* siciliae str. c2j u89773.1	0.0018	0.0527	2854%
OTU_4007	*Sporobacterium* olearium	0.0036	0.0961	2593%

OTU_1094	uncultured *Methanosarcina sp.*	0.0018	0.0465	2506%
OTU_2900	*Sporomusa sp.* an4	0.0054	0.1178	2101%
OTU_3918	*Sporobacterium* olearium	0.0178	0.3906	2089%
OTU_5774	uncultured *Acetivibrio sp.*	0.0018	0.0372	1985%
OTU_147	uncultured *Clostridium sp.*	0.0018	0.0372	1985%
OTU_629	uncultured *Clostridium sp.*	0.0036	0.0682	1811%
OTU_3547	*Methanosarcina* mazei	0.0018	0.0341	1811%
OTU_1748	uncultured *Clostridium sp.*	0.0089	0.1643	1742%
OTU_4496	uncultured *Acetivibrio sp.*	0.0071	0.1271	1681%
OTU_1067	uncultured *Ruminococcus sp.*	0.0018	0.0310	1637%
OTU_3912	*Clostridium sp.* enrichment culture clone cs5	0.0143	0.2325	1529%
OTU_2188	*Clostridium sp.* 619	0.0018	0.0248	1290%
OTU_168	uncultured *Clostridium sp.*	0.0071	0.0930	1203%
OTU_1172	uncultured *Turicibacter sp.*	0.0054	0.0651	1116%
OTU_2742	*Clostridium sp.* 619	0.0018	0.0217	1116%
OTU_1201	uncultured *Clostridium sp.*	0.0054	0.0620	1058%
OTU_1405	uncultured *Ruminococcus sp.*	0.0036	0.0403	1029%
OTU_560	uncultured *Turicibacter sp.*	0.0089	0.0868	873%
OTU_76	uncultured *Clostridium sp.*	0.0036	0.0341	856%
OTU_1668	uncultured *Clostridium sp.*	0.0018	0.0155	769%
OTU_6560	*Lutispora* thermophila	0.0036	0.0310	769%
OTU_1038	*Ornithinibacillus sp.* hme7715	0.0036	0.0279	682%
OTU_73	*Sedimentibacter* hongkongensis	0.1856	1.4479	680%
OTU_3026	uncultured *Methanosarcina sp.*	0.0018	0.0124	595%
OTU_2506	uncultured *Alkalibaculum sp.*	0.0018	0.0124	595%
OTU_4169	uncultured *Methanosarcina sp.*	0.0018	0.0124	595%
OTU_2786	human infant gastrointestinal tract microbiota stool mother babies 13 and 14 7 months after birth clone c245 ef434363.1	0.0018	0.0124	595%
OTU_644	uncultured *Clostridium sp.*	0.0018	0.0124	595%
OTU_3770	uncultured *Acetivibrio sp.*	0.0018	0.0124	595%
OTU_3736	*Sporobacterium* olearium	0.0054	0.0372	595%
OTU_837	uncultured *Clostridium sp.*	0.0071	0.0496	595%
OTU_6373	*Sedimentibacter sp.* mo_sed	0.0054	0.0341	537%
OTU_3048	uncultured *Anaerobranca sp.*	0.0054	0.0341	537%
OTU_684	uncultured *Clostridium sp.*	0.0054	0.0341	537%
OTU_33	uncultured *Anaerobranca sp.*	1.9844	2.4462	23%

OTU_79	*Desulfotomaculum* nigrificans str. cw_01 ay742958.1	1.9291	0.3441	-82%
OTU_5304	uncultured *Anaerovorax sp.*	3.5387	0.8185	-77%
OTU_1217	uncultured *Anaerovorax sp.*	1.8506	0.4217	-77%
OTU_18	*Gracilibacter* thermotolerans	6.3869	2.4958	-61%
OTU_3138	*Burkholderia* cenocepacia	5.3750	2.3532	-56%
OTU_5698	*Burkholderia* cenocepacia	24.3143	12.0636	-50%
OTU_6485	*Burkholderia* arboris	1.3045	0.6759	-48%
OTU_1165	*Clostridium sp.* k11	2.5947	1.3425	-48%
OTU_3	*Bacteroides* xylanolyticus	3.1140	1.6060	-48%

VITA

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Candidate for the Degree of

Doctor of Philosophy

Thesis: BIOSTIMULATION OF TRICHLOROETHYLENE DECHLORINATION BY ORGANOHALIDE RESPIRING BACTERIA WITH CHLOROPEROXIDASE PRODUCED ORGANOCHLORINES

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