STIMULATION OF ORGANOHALIDE RESPIRING BACTERIA WITH CHLOROPEROXIDASE PRODUCED ORGANOCHLORINES

By

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I was a chemical engineering student when I came to Oklahoma State University (OSU) as an undergraduate transfer student. By not knowing that OSU offered environmental engineering for bachelor's degree, I chose an engineering major which is closely related to the environment, chemical engineering. I decided to switch my major when I found out OSU offered environmental engineering as a bachelor's degree from an OSU student who worked as a cashier at ROSS. I want to thank her and my best friend Siew Juan Ee, who challenged and reminded me what my initial academic career goal is.

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Title of Study: STIMULATION OF ORGANOHALIDE RESPIRING BACTERIA WITH CHLOROPEROXIDASE PRODUCED ORGANOCHLORINES

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Abstract: Anaerobic microcosms were set up with CPO-produced organochlorines or control organic matter. Organohalide respirers from the phyla *Chloroflexi*, *Proteobacteria* and *Firmicutes* were measured with qPCR. Among all dechlorinators, *Dehalogenimonas* 16S rRNA had the most obvious and sustained growth from CPO-produced organochlorines. Other *Dehalococcoides*-like *Chloroflexi* were enriched as well and *Dehalobacter* had a temporary enrichment from CPO-produced organochlorines. The ratio of *Dehalogenimonas*/*Bacteria* in the microcosms amended with CPO increased from -1.40 ± 0.27 on Day 7 to -0.60 ± 0.40 (log_{10}) on Day 82 while in the CTRL microcosms the ratio increased slightly from -1.58 ± 0.19 (log_{10}) to -1.42 ± 0.10 (log_{10}). In the TRFLP analysis, putative dechlorinating *Chloroflexi* was examined and a single OTU with a restriction fragment length (RFL) of 276 base pair (bp) was a major component in all three CPO microcosms on Days 61 and 84. The relative peak area of the OTU was 25.8, 15.1 and 72.6% on Day 61 compared to minimal abundance in controls. Another OTU with fragment length of 278 bp likely represents a non-chlorinating species, and had a significance presence in all microcosms after Day 0. The results in this research has shown that the CPO-produced organochlorines are able to stimulate the growth of *Dehalococcoides*-like *Chloroflexi* group and other organohalide respirers that are also known to dechlorinate PCBs and PCE in contaminated soils and sediment.

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CHAPTER I

INTRODUCTION

Polychlorinated biphenyls (PCBs) were first synthesized in 1881 and commercial production was widespread in the United States (US) by 1929 (EPA, 2013[a]). PCBs are man-made organic chemicals that are made in mixtures containing up to a theoretical 209 unique congeners (ATSDR, 2014; EPA, 2013[a]). PCBs are able to bioaccumulate throughout the food chain as a result of quick absorption into the fatty tissue of animals (Johansen et al., 1993; Muir et al., 1992; Neff et al., 1984; Safe et al., 1993; Schecter et al., 1994 and Fagervold et al, 2011). Epidemiological studies in humans and animals have shown that PCBs cause cancer and affect immune systems, reproductive systems, neurological systems, endocrine receptors, blood pressure, serum triglyceride, and serum cholesterol (EPA, 2013[b]). In 1979, the US government banned all production of PCBs (EPA, 2013[a]).

Perchloroethylene (PCE) also known as tetrachloroethylene was first synthesized in 1821 and it was introduced as a dry-cleaning solvent in the 1930s (Partington et al., 1964; Martin et al., 1958; Ni et al., 2014; ITRC, 2005; Longstaff et al., 1992). PCE has become a concern because PCE, its dechlorination products, and other similarly structured chlorinated compounds are toxic, suspected carcinogens and widespread

groundwater contaminants (Aulenta et al., 2006; Ziv-El et al., 2014; Ni et al., 2014; Friis et al., 2007; Grindstaff et al., 1998; Henry et al., 2002). Inhalation exposure to PCE includes effects in the kidney, liver, immune system, and hematologic system, irritation of the upper respiratory tract and eyes, and neurological effects such as reversible mood and behavioral changes, impairment of coordination, dizziness, headache, sleepiness, and unconsciousness as well as cancer (EPA, 2012). PCE and PCBs are toxic pollutants that can be degraded by the same type of bacteria – the organohalide respirers.

Anaerobic reductive dechlorination of PCBs and PCE is a process that provides a means of detoxification and, especially when coupled with aerobic degradation, completely destroys the contaminant (Bedard et al., 2008; Bedard et al., 2006; Brown et al., 1987[a]; Brown et al., 1987[b]). Engineers have concluded that observed microbial dechlorination patterns at different contamination sites is likely due to the presence and activity of unique species and consortia of organohalide-respiring bacteria (Fagervold et al., 2011). Several anaerobic bacteria in the phylum *Chloroflexi* have been shown to be obligate organohalide respirers and these bacteria include several strains of *Dehalococcoides mccartyii*, "*Dehalobium chlorocoercia"* DF-1, bacterium o-17, phylotypes SF-1 and DH-10, and *Dehalogenimonas lykanthroporepellens* strains BL-DC-8 and BL-DC-9 (Kjellerup et al., 2012; Bedard et al., 2008; Fagervold et al., 2011; Payne et al., 2011, Löffler et al., 2013; Brown et al., 2013; May et al., 2008; Wu et al., 2002; Yan et al., 2009). Many of these microorganisms appear at contaminated and uncontaminated environments (Krzmarzick et al., 2012; Krzmarzick et al., 2013). Other

than the *Chloroflexi*, strains of the phyla *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).

In 1998, Bedard et al. primed PCB dechlorination with brominated biphenyls and the effectiveness of this process was twice as effective as using PCBs primed by other PCBs (Bedard et al., 1998). Ahn et al. discovered five more 'priming' compounds for dehalogenating compounds, tetrachlorobenzene, tetrachloroanisole, tetrachlorophenol, tetrachlorobenzoic acid and trichloroacetophenone (Ahn et al. 2007; Ahn et al. 2008). The feasibility of stimulating PCB and PCE dechlorination with other chlorinated compounds has been limited since these other compounds are also man-made contaminants. Other chlorinated compounds (organochlorines or organochlorides) though are naturally-occurring and play an important role in chlorine and carbon cycles as well (Gribble et al., 1994; Myneni et al., 2002; Öberg et al., 2002; Leri et al. and Myneni et al., 2010; Redon et al., 2011). Chloroperoxidase (CPO) enzyme plays a key role in the production of soil organochlorines and chlorinate aliphatic and aromatic structures during the breakdown of large molecular weight lignin molecules (Ortiz-Bermúdez et al., 2003; Leri et al. and Myneni et al., 2010; Bastviken et al., 2009). Research has already connected one narrow group of organohalide-dechlorinators to the dechlorination of

CPO-produced organochlorines (Krzmarzick et al., 2012). In my study, the dechlorinating microbial communities that grow during natural organochlorine amendments were further studied to better understand the potential for biopriming PCB and PCE dechlorination. Furthermore, the dechlorination of PCBs was investigated with natural soil communities with and without the presence of natural organochlorines to determine if natural organochlorines can stimulate, or conversely if they compete, with PCB dechlorination.

My hypothesis is that the CPO-produced organochlorines are able to stimulate the growth of *Dehalococcoides*-like *Chloroflexi* group and other organohalide respirers that are also known to dechorinate PCBs and PCE in contaminated soils and sediments. To test this hypothesis, anaerobic microcosms were set up and amended with CPO-produced organochlorines or control organic matter and the growth of known dechlorinating microorganisms were measured with quantitative real-time polymerase chain reaction (qPCR). Furthermore, terminal restriction fragment length polymorphism (TRFLP) analysis was used to further identify any other members of the dechlorinating *Chloroflexi* that were stimulated to grow from CPO-produced organochlorines. Lastly, microcosms were used to determine if the microbial dechlorination of PCBs could be stimulated by co-amendments with natural organochlorines.

CHAPTER II

REVIEW OF LITERATURE

2.1 Contamination of PCBs and PCEs

Polychlorinated biphenyls (PCBs) were first synthesized in 1881 and commercial production was widespread in the United States (US) by 1929 (EPA, 2013[a]). PCBs are man-made organic chemicals known as chlorinated hydrocarbons that are made from a mixture of many individual chlorinated chemicals compounds, up to 209 congeners (ATSDR, 2014; EPA, 2013[a]). The properties of PCBs including non-flammable, chemically stable, having a high boiling point and are useful as an electric insulator (ATSDR, 2014). Due to the properties of PCBs, they have been used in hundreds of industrial and commercial applications (ATSDR, 2014). Commercial PCB mixtures in the US were trademarked and produced by the Monsanto Chemical Company. These PCBs were trade-named as Aroclors, which contained a four digit numbers, which reflected the percent mass of the mixture that was chlorine (Aroclor 1260, for example, is 60% chlorine by mass). Commercial products that widely used PCBs include transformers and capacitors, oil used in motors and hydraulic systems, old electrical devices or appliances containing PCB capacitors, fluorescent light ballasts, cable insulation, thermal insulation material, adhesives and tapes, oil-based paint, caulking,

plastics, carbonless copy paper and floor finish (EPA, 2013[a]). PCBs have a range of toxicity, can travel a long distance in the air, remain dissolved, stick to organic particles and bottom sediments in water and bind strongly in soil (ATSDR, 2014). PCBs were first found to contaminate the Great Lakes in 1968, and soon thereafter were found to be pervasive in all environments including distant and isolated polar regions. Particularly, the ability to bioaccumulate in marine mammals far from any sources of direct pollution alarmed environmental scientists. In 1979, the US government banned all production of PCBs (EPA, 2013[a]). Even though PCBs' production was banned, today, they are still present and may be released into the environment from old capacitors and other equipment manufactured before 1979 (EPA, 2013[a]).

PCBs went from being viewed as a 'miracle chemicals' to being outright banned because they demonstrated many adverse health effects. Epidemiological studies in humans and animals have shown that PCBs cause cancer and affect immune systems, reproductive systems, neurological systems, endocrine receptors, blood pressure, serum triglyceride, and serum cholesterol (EPA, 2013[b]). The nature of PCBs, which are stable and hydrophobic, causes them to bioaccumulate and biomagnify in the food chain, which is still a major concern (Yan et al., 2006a; Bedard et al., 2008). PCBs bioaccumulate throughout the food chain as a result of quick absorption into the fatty tissue of animals, such as fish and marine mammals, and within humans, PCBs have been detected in human adipose tissue, milk, and serum (Johansen et al., 1993; Muir et al., 1992; Neff et al., 1984; Safe et al., 1993; Schecter et al., 1994 and Fagervold et al, 2011). Research has

been done in order to identify microorganisms to biologically remediate PCBs from polluted environments.

Perchloroethylene (PCE), also known as tetrachloroethylene, was first synthesized in 1821 and was introduced as a dry-cleaning solvent in the 1930s (Partington et al., 1964; Martin et al., 1958; Ni et al., 2014; ITRC, 2005; Longstaff et al., 1992). PCE is widely used for dry-cleaning fabrics and metal degreasing operations (EPA, 2012). PCE has become a concern because its daughter products, trichloroethene (TCE), *cis*-dichloroethene (*cis*-DCE), trans-dichloroethene (*trans*-DCE), and vinyl chloride (VC), which are commonly called volatile chlorinated ethenes (VOCls), and other similarly structured chlorinated compounds such as 1,1,2-trichloroethane (TCA) and chloroform (CF) are considered toxic, suspected carcinogens and widespread groundwater contaminants (Aulenta et al., 2006; Ziv-El et al., 2014; Ni et al., 2014; Friis et al., 2007; Grindstaff et al., 1998; Henry et al., 2002). Inhalation exposure to PCE include effects in the kidney, liver, immune system, and hematologic system, irritation of the upper respiratory tract and eyes, and neurological effects such as reversible mood and behavioral changes, impairment of coordination, dizziness, headache, sleepiness, and unconsciousness as well as cancer (EPA, 2012). Research has been done in order to identify methods and microorganisms to remediate PCE and related chemicals from polluted environments, particularly aquifers. PCE and PCBs are toxic pollutants that can be degraded by the same type of bacteria – the organohalide respirers.

2.2 Organohalide Respiring Bacteria

Some anaerobic microorganisms may catalyze the reductive dechlorination of chlorinated compounds in a process is known as "organohalide respiration" (Cutter et al., 2001). Organohalide respiration is defined as the activity of microorganisms that couple growth to the dechlorination of alkyl and aryl compounds. Several anaerobic bacteria in the phylum *Chloroflexi* have been shown to be obligate organohalide respirers and these bacteria include several strains of *Dehalococcoides mccartyii*, "*Dehalobium chlorocoercia"* DF-1, bacterium o-17, phylotypes SF-1, DH-10 and *Dehalogenimonas lykanthroporepellens* strains BL-DC-8 and BL-DC-9 (Kjellerup et al., 2012; Bedard et al., 2008; Fagervold et al., 2011; Payne et al., 2011, Löffler et al., 2013; Brown et al., 2013; May et al., 2008; Wu et al., 2002; Yan et al., 2009). Many of these microorganisms are capable of the microbial dechlorination of PCBs and/or PCE, thus reducing toxicity (Cutter et al., 2001). All of these bacteria form the class *Dehalococcoidia*, and among this group of currently isolated organohalide respiring bacteria, *Dehalococcoides mccartyi* (*Dhc*) is one of the most studied genus and species because it was the first to be isolated and has the ability of fully dechlorinate PCE to the nontoxic end product ethene (Löffler et al., 2013; Maymó-Gatell et al., 1997). *Dhc* also able to dechlorinate aromatic pollutants, including PCBs, and other aliphatic pollutants (Loffler et al., 2013; Yan et al., 2006; Bedard et al., 2006; Kube et al., 2005).

Other than these, 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). Among these other bacteria, only several *Dehalobacter* are similar to the *Dehalococcoidia* organisms in that they exclusively respire organohalides for energy; the other isolates have a wide range of other metabolic capabilities (Maphosa et al., 2010; Lee et al., 2012; Justicia-L et al., 2012; Nonaka et al., 2006). There are even more bacteria that can dechlorinate pollutants co-metabolically (Lohner et al., 2013, Nzila et al., 2013).

Reductive dehalogenase (rdh) genes code for the enzymes that catalyze organohalide respiration reactions (Hug et al., 2013). Although much research about rdhs has been done, only a few of the rdhs are biochemically characterized and these usually, though not always, have fairly wide substrate ranges (Hug et al., 2013; Waller et al., 2005; Krajmalnik et al., 2004; Magnuson et al., 2000; Fung et al., 2007; Ni et al., 1995; Neumann et al., 2002; van de Pas et al., 1999; Buttet et al., 2013). 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 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 organohalide respiring bacteria such as the *Dehalococcoidia* and *Dehalobacter* usually contain numerous rdh genes, as many as 39, but the organohalide respiring bacteria with broader physiologies 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 induced are often broadly transcribed (Wagner et al., 2013; Waller et al., 2005). With only a few enzymes characterized despite the large number of genes found, the divergence and diversity of rdhs are much still unknown (Hug et al., 2013).

2.3 PCB Dechlorination Processes

The first definitive study that found PCBs might be reductively dechlorinated was done in 2001 (Cutter et al., 2001). In order to identify 16S rRNA genes of that first PCBs dechlorinating enrichment culture, also known as the *ortho*-dechlorinating culture, Cutter et al. used denaturing gradient gel electrophoresis (DGGE) and PCR analysis (Cutter et al., 2001). *Ortho*- dechlorination can be understood as a sequential *ortho*-dechlorination process; for example, 2,3,5,6-tetrachlorobiphenyl (2,3,5,6-CB) dechlorinated to 2,3,5-trichlorobiphenyl and 3,5-dichlorobiphenyl (Cutter et al., 2001). In this experiment, cysteine-HCl was used to function as a reductant and a source of carbon, sulfur and energy (Cutter et al., 2001). 2,3,5,6-CB was supplied in the experiment as the only

chlorinated substrate and as a potential electron acceptor (Cutter et al., 2001). From DGGE, Cutter et al. identified an Operational Taxonomic Unit (OTU), named simply *o*-17, as the PCB dechlorinator based on three lines of evidence (Cutter et al., 2001). First, 16S rRNA genes of *o*-17 were always detected during PCB dechlorination and are only detected when PCB is included in the medium (Cutter et al., 2001). Second, dechlorination could not be recovered when *o*-17 was systematically eliminated from the culture (Cutter et al., 2001). Third, 16S rRNA genes of *o*-17 was most similar to that of *Dehalococcoides* spp., which was already known at the time to reductively dechlorinate organochlorines such as chlorinated ethenes (Cutter et al., 2001). The effect of acetate and hydrogen toward dechlorination of 2,3,5,6-chlorobiphenyl was also established in the *ortho*-dechlorinating culture (Cutter et al., 2001). Acetate could not be substituted for other tested carbon sources and competition for acetate by methanogens was found to reduce PCB dechlorination rates (Cutter et al., 2001). The relative amount of hydrogen was also found to be important in the dechlorination of PCBs. Hydrogen was found to be an electron donor, but at higher concentrations the PCB dechlorination pathways changed and the overall rate decreased; low levels of hydrogen were found to best support the reaction kinetics of *o-17* (Cutter et al., 2001).

A year before Cutter et al. published their study on *o*-17, Wu et al. used sediment from Charleston harbor as inoculum for the development of an anaerobic enrichment culture that specifically dechlorinates doubly flanked chlorines of PCBs (Wu et al., 2000). Additionally, this culture preferably dechlorinated at the *para* position, but also

could at the *meta* position; it did not remove *ortho* chlorines (Wu et al., 2000). The bacterium identified was named DF-1 (now isolated and called "*Dehalobium chlorocoercia"*) and is the first microorganism identified that strictly dechlorinated PCBs with doubly flanked chlorines (Wu et al., 2002). To implicate bacterium DF-1 as the PCB dechlorinator, Wu et al. used similar methods as Cutter et al. and proved that bacterium DF-1 was the dechlorinator and not the other co-culture organisms (Wu et al., 2002). "*Dehalobium chlorocoercia*" DF-1 was eventually isolated from a persistent co-culture with *Desulfovibrio* spp. (May et al, 2008; Wu et al., 2002). This *Desulfovibrio*, or extracts of *Desulfovibrio* cultures, was necessary for growth in a sediment-free medium and PCB dechlorination by the DF-1 organism was confirmed again with the use of hydrogen as electron donor (May et al., 2008). DF-1 was described as an ultramicrobacterium for being unusually tiny and hypothesized that this small size offers advantages when growth relies on hydrophobic compounds that are minimally soluble (May et al., 2008).

To further characterize PCB dechlorinators with the presence of PCBs, Yan et al. had set up experiment using three different sediments and amended elemental iron or a mixture of fatty acids (Yan et al., 2006[b]). The hypothesis was that PCB dechlorinators would be enriched only when PCBs were present, and the bacterial community structures of PCB-amended cultures would grow unique PCB dechlorinating strains compared to microcosms in which no PCBs were amended (Yan et al., 2006[b]). Sediment samples for the experiment were collected from Baltimore Harbor (BH), Palos Verdes Harbor (PV),

and Hudson River (HR) (Yan et al., 2006). PCB analysis, statistical analysis and combined techniques of 16S rRNA gene analysis, cloning and DGGE were used for this experiment to determine PCB dechlorination physiology and putative dechlorinators (Yan et al., 2006[b]). The amount of sulfate, phosphate, nitrate, total organic carbon, total inorganic carbon, iron, manganese, zinc, copper, lead, cadmium, and chromium and water pH were measured to ensure that these were not affecting the results of the experiment (Yan et al., 2006[b]). Dechlorination in PV and HR sediments began with the removal of doubly flanked chlorines, but PV culture dechlorinated to the greatest extent and HR cultures proceeded only to the di-chlorinated congener 2,5-chlorobiphenyl (Yan et al., 2006[b]). In BH sediment, removal of double flanked chlorines from 2,3,4,5-chlorobiphenyl was the most obvious dechlorination activity and the culture was modified with either Fe(0) or a mixture of fatty acids (Yan et al., 2006[b]). The differences between the Fe(0)-fed cultures and fatty acid-fed cultures in BH sediment is that Fe(0)-fed cultures had significantly shorter lag times before dechlorination began but both amended cultures stimulated predominately doubly flanked dechlorination processes (Yan et al., 2006[b]). Three OTUs were present in the PCB-amended cultures with the presence of fatty acids and absent in the controls, and only one unique OTU was present in the population in the PCB-amended culture with Fe(0) that was absent in the control cultures; this organism was most similar to *Dhc* (Yan et al., 2006[b]). The correlation between the population dynamics in the PCB-amended culture and the dynamics of dechlorination was based on the presence or absence of the OTUs at a given time and the

number of chlorines removed from 2,3,4,5-CB at the time (Yan et al., 2006[b]). Throughout the experiment, the presence of the *Dehalococcoides*-like OTU correlated in three different sediments to the removal of double flanked (DF) chlorines from the PCB congener 2,3,4,5-tetrachlorobiphenyl (Yan et al., 2006[b]). This paper suggests that these organisms were initially present in the sediments at low concentrations and enriched over time as a result of the niche provided by amended 2,3,4,5-CB (Yan et al., 2006[b]).

Yan et al. had also tested the effect of sodium bicarbonate toward 2,3,4,6-CB dechlorination in Hudson River (HR) sediment cultures (Yan et al., 2006[a]). The hypothesis of this experiment was the amendment of bicarbonate to the cultures could directly stimulate reductive dechlorination by providing additional inorganic carbon for the growth of PCB dechlorinators or the addition of bicarbonate could stimulate homoacetogenesis, which would generate acetate and thus carbon for dechlorinators (Yan et al., 2006[a]). The dechlorination of 2,3,4,5-CB began with the removal of double flanked *meta* and *para* chlorines by forming 2,3,5-CB and 2,4,5-CB (Yan et al., 2006[a]). The process followed by the removal of singly flanked (SF) meta or para chlorines and form 2,4-CB and 2,5-CB (Yan et al., 2006[a]). The purpose of this experiment was to identify the difference between adding less than 500 mg/L (low concentration) of bicarbonate and adding 500 or 1000 mg/L (high concentration) of bicarbonate into the sediment and the differences between these two were obvious in the following process (Yan et al., 2006[a]). In the treatment which added less than 500 mg/L of bicarbonate, the increases of 2,3,5-CB dechlorination products was not proportional to the decreases of 2,3,5-CB and this

had suggested that the simultaneous production and degradation of 2,3-CB, 2,5-CB and 2-CB (Yan et al., 2006[a]). In the microcosms to which 500 or 1000 mg/L bicarbonate was added, 2,3,5-CB dechlorination was either slow or did not occur (Yan et al., 2006[a]). When the amount of bicarbonate decreased to 100 mg/L, 2,3,5-CB dechlorination happened rapidly in the microcosms (Yan et al., 2006[a]). Thus, higher concentrations of sodium bicarbonate resulted in increased acetate concentrations and reduced rates and extent of dechlorination (Yan et al., 2006[a]). High bicarbonate either decreased the consumption of acetate, which thus had some negative effect on the enrichment for PCB dechlorinators, or stimulated homoacetogenesis, which altered the flow of nutrients and electron donor away from organohalide respiration thus creating an environment unfavorable to the enrichment of dechlorinating populations (Yan et al., 2006[a]). Intermediate amounts of sodium bicarbonate exhibited more extensive dechlorination of 2,3,4,5-CB and more rapid dechlorination of its daughter products, likely because acetate served as a carbon source (Yan et al., 2006[a]). This study had shown that PCB dechlorination is likely sensitive to other microbial processes, electron donor availability and carbon concentrations (Yan et al., 2006[a]).

Cultures of bacteria dechlorinating mixed PCB mixtures have been found to follow one of four unique processes: Process N, Process P, Process LP and Process H (Bedard et al., 2008; Adrain et al., 2009). Process N dechlorination removes all flanked *meta* chlorines except those on 2,3-CB rings (Bedard et al., 2008; Bedard and Quensen et al., 1995; Bedard et al, 1998; Quensen et al., 1990; Van et al., 1997). Key dechlorination

products for this process are 2,4-2',4'-CB, 2,4-2',6'-CB and 2,4,6-2',4'-CB because the meta chlorine (in the 3 and 5 positions) are readily removed (Bedard et al., 2008; Bedard and Quensen et al., 1995; Van et al., 1997). This process has been shown to be induced by alternative electron acceptors such as brominated benzoates, brominated benzonitriles, brominated nitrobenzenes and especially 2,6-dibromobiphenyl which is able to stimulate a 2000-fold growth in both the organisms that dehalogenate Aroclor 1260 (Bedard et al., 2008; DeWeerd et al., 1999). Process P, conversely removes flanked *para* chlorines (Bedard et al., 2008, Bedard and Quensen et al., 1995; Bedard et al., 1996). Key dechlorination products for this process are 2,5-2',5'-CB, 2,3,5-2',5'-CB and 2,3-2',5'-CB (Bedard et al., 2008; Bedard and Quensen et al., 1995; Bedard et al., 1996). Bacteria that dechlorinate according to process H, originally introduced by Brown and Wagner et al., was shown with 2,3,4,5,6-CB, 2,3,4,5-CB, 2,3,4,6-CB, 2,3,4-CB, 2,4,5-CB, and 3,4-CB (Adrian et al., 2009; Brown and Wagner et al., 1990). The dechlorination pathways for individual congeners was removal of *para* chlorines from 2,3,4,5-CB, 2,4,5-CB, and 3,4-CB rings and removal of doubly flanked *meta* chlorines from 2,3,4-CB and 2,3,4,6-CB rings (Adrian et al., 2009). Process H has been proven using the mixture Aroclor 1260 with a single pure strain of *Dhc* (sp. CBDB1) by the complex pattern of dechlorination (Adrian et al., 2009; Brown and Wagner et al., 1990; Erickson et al., 1997). There are similarities between Process H dechlorination and Process P dechlorination leading to the production for many of the same products (Adrian et al., 2009; Bedard and Quensen et al., 1995). However, Process P dechlorination exclusively

removes chlorines from the *para* position, which Process H does not (Adrain et al., 2009; Bedard and Quensen et al., 1995). The chlorobenzene rdh, *cbrA*, and a tetrachloroethene rdh, *pceA* which also appears to be also responsible for chlorophenol dehalogenation, were identified in CBDB1 and thus may assist in this process (Adrain et al., 2009; Adrain et al., 2007[a]; Adrain et al., 2007[b]; Fung et al., 2007; Morris et al., 2007). Process LP removes unflanked *para* chlorines from 2,4-CB and 2,4,6-CB (Bedard et al., 2008; Bedard et al., 2003; Wu[a] et al., 1997; Wu[b] et al., 1997). Process LP can also remove isolated *para* chlorine on 4-chlorophenyl group of some congeners and *meta* chlorine in position 3 from 2,3-CB, 2,3,4-CB and 2,3,5-CB groups (Bedard et al., 2008; Bedard et al., 1996; Bedard et al., 2005). This process does not efficiently dechlorinate Aroclor 1260 but it does further dechlorinate terminal products of Process N to *ortho*-substituted *di*- and *tri*-CBs (Bedard et al., 2008; Bedard et al., 1996). The resulting lesser chlorinated products are degradable by aerobic bacteria and are thus of high interest for remediation (Bedard et al., 2008; Bedard et al., 1986).

An important culture developed to study PCB dechlorination is the JN culture, which is sediment-free and has resulted in isolated organisms responsible for the dechlorination of PCBs (Bedard et al., 2008; Bedard et al., 2006). JN cultures have the ability of decreasing the proportion of PCBs with six or more chlorines when incubated with Aroclor 1260 at 5 to 500 µg per ml in the presence of acetate and hydrogen and carry out extensive Process N dechlorination (Bedard et al., 2008; Bedard et al., 2006). Dechlorination of 2,3,4,5-tetrachlorobiphenyl also has been shown to occur in a culture

named KFL, with the absence of *Dehalococcoides* sp. CBDB1 and strain DF-1 of *Chloroflexi* but with the presence of *Dehalobacter* spp. showing that PCB organohalide respiration extends beyond the *Chloroflexi* (Yoshida et al., 2009).

The presence of PCB dechlorinating microorganisms for *in situ* bioremediation has the potential for lower cost and reduced negative environmental impacts associated with dredging and capping (Fagervold et al., 2011). Bioremediation is also valuable for minimal disruption to benthic habitats in sensitive rivers and wetlands and for the ability to treat shallow locations or those with restricted accessibility (Fagervold et al., 2011). Bacteria cultures studied for bioaugmentation potential are bacterium *o-17, "Dehalobium chlorocoercia"* strain DF-1, phylotype DEH10 and phylotype SF1 (Fagervold et al., 2011). A culture containing a strain with a rare *ortho* dechlorination activity and a non-indigenous strain that attacks double-flanked chlorines, was inoculated into sediment microcosms amended with 2,2',3,5,5',6-hexachlorobiphenyl (PCB 151) and Aroclor 1260 to recognize dechlorination for *in situ* bioaugmentation (Fagervold et al., 2011). The dechlorination of PCB 151 was recognized initially through two pathways; first, *meta* dechlorination to PCB 95 (2,2', 3,5', 6-CB), which is further dechlorination in the *meta* position to PCB 53 (2,2', 5,6'-CB) (Fagervold et al., 2011). The second pathway is a dechlorination in the *ortho* position to PCB 92 (2,2', 3,5,5'-CB), which can be dechlorinated either in the *meta* position to PCB 52 (2,2' , 5,5'-CB) or in the *ortho* position to PCB 72 (2,3', 5,5'-CB) (Fagervold et al., 2011). The pattern of dechlorination was altered depending on the initial combination of microorganisms added (Fagervold et

al., 2011). Dechlorination of Arcolor 1260 was enhanced with bioaugmentation of PCB dechlorinating bacteria (Fagervold et al., 2011). The ability of bioaugmentation to redirect dechlorination reactions in the sediment microcosms indicate that the inoculated PCB organohalide respiring microorganisms effectively competed with the indigenous microbial populations (Fagervold et al., 2011; Payne et al., 2011). Discrepancies in the rates and extent of dechlorination could possibly occur due to number of factors including available nutrients, presence of inhibitory contaminants, the strain used and the growth state and numbers of cells used for bioaugmentation (Payne et al., 2011). Payne et al. stated that bioavailability does not prevent bioaugmentation from treating low levels of weathered PCBs in sediment microcosms and that granulated activated carbon actually enhanced the overall process (Payne et al., 2011). These observations indicate that bioaugmentation with PCB organohalide respiring microorganisms is a potentially tractable approach for *in situ* treatment of PCB impacted sites (Fagervold et al., 2011).

PCB concentrations in the Great Lakes decrease with increases of latitude and longitude over a period of time (Li et al., 2009). Li at el. stated that the concentrations and flux of PCBs in the surface sediment decrease in a log linear trend with increasing latitude (N) and longitude (W) of the sampling sites (Li et al., 2009). This latitude dependence reflects the combined effect of the south-to-north decrease in population density and industrialization in the region, and the general direction of the long range transport of PCBs in the northern hemisphere (Li et al., 2009). The dependence on longitude may be related to the fact that, in general, chemical industries are more densely

located in the east than in the west within the geographic region of the northeast United States (Li et al., 2009). Li et al. also found out PCB levels in sediments of all the Great Lake have either leveled off or declined (Li et al., 2009).

PCBs are often mentioned as needing both anaerobic and aerobic processes for complete destruction. A complete microbial degradation for PCBs may require anaerobic reductive dechlorination of extensively chlorinated congeners followed by subsequent aerobic cleavage of the biphenyl ring and mineralization of the less extensively chlorinated congeners (Kjellerup et al., 2012). Aerobic microorganisms are restricted to attacking lesser-chlorinated congeners (Cutter et al., 2001). Compared to anaerobic dechlorination, aerobic degradation can performed from many bacterial species including *Burkholderia xenovorans* strain LB400 and *Rhodococcus* sp. strain RHA1; these types of bacteria able to catabolize biphenyls are universally distributed in aerobic environments (Kjellerup et al., 2012). Complete anaerobic dechlorination may be possible (Quensen et al. 1988, Sower et al. 2013), but has yet to be definitely discovered in studies to date.

2.4 Natural Chlorinated Organic Matter

Despite their specialized niche of organohalide respiration, *Dhc*-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 organohalide-respiring *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 produced organochlorines were dechlorinated, thus strongly supporting the hypothesis that organohalide respiring bacteria occupy a niche in terrestrial soils using natural organochlorines as terminal electron acceptors (Krzmarzick et al., 2012, Adrain et al., 2007[a]; Bunge et al., 2008, Hiraishi et al., 2008; Kittelmann and Friedrich et al., 2008[a]; Kittelmann and Friedrich et al., 2008[b]). Further research has shown that a group, named the "Gopher group", were heavily enriched during the dechlorination of a chlorinated xanthones, which are broad class of natural organochlorines (Krzmarzick et al., 2014). The "Gopher group" contains 16S rRNA sequences exclusively collected from dechlorinating cultures, including PCB dechlorinating cultures and thus are putative organohalide respirers (Krzmarzick et al., 2014). Results obtained from these experiments gives encouragement for future research because if organisms that respire natural organochlorines can also dechlorinate compounds such as PCBs and PCE, a natural ability to stimulate pollutant degraders may exist. Additionally, these organohalide respirers could be quickly grown to a high density on natural organochlorines *ex situ* and then bioaugmented to contaminated sites (Krzmarzick et al., 2012). It also suggested that organohalide respiring *Chloroflexi* plays an integral role in the biogeochemical chlorine cycle (Krzmarzick et al., 2012).

Chlorinated compounds, usually called organochlorines or organochlorides, that are naturally-occurring in biogeochemical cycles are known by geologists to play an important role in chlorine and carbon cycles (Gribble et al., 1994; Myneni et al., 2002; Öberg et al., 2002; Leri et al. and Myneni et al., 2010; Redon et al., 2011). In terrestrial systems, chlorine undergoes transformations between inorganic and organic forms and many terrestrial organisms produce organochlorine and other organohalogen compounds as irritants, biocides, and other uses (Leri et al. and Myneni et al., 2010; Gribble et al., 1994). There are more than 4700 natural organohalogens that have been identified (Gribble et al., 1994; Myneni et al., 2002; Gribble et al., 2010; van Pée et al., 2012; Rohlenová et al., 2009; Bastviken et al., 2009; Aeppli et al., 2013). Studies on the degradation of naturally occurring organochlorines have demonstrated that in terrestrial systems the chloroperoxidases (CPO) enzymes, found in a variety of plants and fungi, chlorinate natural organic matter with both aliphatic and aromatic moieties (van Pée et al., 2012; Aeppli et al., 2013; Reina et al., 2004; Ortiz-Bermúdez et al., 2003). CPO plays a key role in the production of soil organochlorines (Leri et al. and Myneni et al., 2010; Bastviken et al., 2009). CPOs chlorinate aliphatic and aromatic structures during the breakdown of large molecular weight lignin molecules (Ortiz-Bermúdez et al., 2003). CPOs function by releasing hypochlorous acid (HOCl) or reactive "Cl^{+}" species that then target phenolic-rich portions of natural organic matters (Leri et al. and Myneni et al., 2010). 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).

2.5 Stimulation of dechlorination processes by stimulating with organohalides

As mentioned above, amending organochlorides to stimulate the dechlorination of pollutants, a process termed stimulation, has been shown to be effective. In 1998, Bedard et al. stimulated PCB dechlorination with brominated biphenyls and the effectiveness of this process was twice as effective as using PCBs stimulated by other PCBs (Bedard et al., 1998). The stimulated culture degraded PCBs via Process N and Process P as introduced in previous sections (Bedard et al., 1998). Congeners containing a *meta* bromine stimulated dechlorination Process N (flanked *meta* dechlorination), and congeners containing an unflanked *para* bromine stimulated dechlorination Process P (flanked *para* dechlorination) (Bedard et al., 1998). Two *ortho*-substituted congeners, 2-bromobiphenyl and 2,6-dibromobiphenyl also stimulated Process N dechlorination (Bedard et al., 1998). The most effective stimulators through the study were 2,6-dibromo-biphenyl, 2,4,5- dibromo-biphenyl, 2,5-3'-dibromo-biphenyl and 2,5-4'-dibromo-biphenyl (Bedard et al., 1998). Using a mixture of PCBs, Bedard et al. later showed that at least 64 congeners of PCBs may be induced to be dechlorinated (Bedard et al., 2006). In the enrichments, the presence of *Thauera*-like *Betaproteobacteria, Geobacter*-like *Deltaproteobacteria, Pseudomonas* species, various *Clostridiales,*

Bacteroidetes, Dehalococcoides of the *Chloroflexi* group, and unclassified *Eubacteria* had been identified (Bedard et al., 2006).

Ahn et al. discovered five more stimulation compounds for dehalogenation including tetrachlorobenzene, tetrachloroanisole, tetrachlorophenol, tetrachlorobenzoic acid and trichloroacetophenone (Ahn et al. 2007; Ahn et al. 2008). These stimulating compounds were added with 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (TeCDD) in a mixed culture containing "*Dehalococcoides ethenogenes"* strain 195 and these compounds successfully stimulated the dechlorination of TeCDD (Ahn et al. 2007; Ahn et al. 2008). Ahn et al. stated that halogenated additives were found to stimulate dechlorination of model dioxin (Ahn et al. 2007). Ahn et al., had shown that haloprimers with more analogous structure to dibenzofurans (CDD/Fs), such as 1,2,3,4-tetrachlorobenzene (1,2,3,4-TeCB) and 2,3,4,5-tetrachloroanisole (2,3,4,5-TeCA), were most effective in enhancing the dechlorination of 1,2,3,4-TeCDD and there was research that showed that haloprimers not only affected dechlorination rates but also affected the dechlorination pattern (Ahn et al., 2008; Ahn et al., 2005; Fu et al., 2005). Microbial populations were enriched with each halogenated co-amendment (Ahn et al., 2007; Ballerstedt et al., 2004).

How is degradation of TeCDD related to PCBs degradation? *Dhc* st. CBDB1 and *Dhc* st. 195, which dechlorinate PCBs, have been shown to dechlorinate CDD/Fs as well, even though energetically CDDs has only shown for *Dehalococcoides sp.* CBDB1 (Bunge et al., 2003; Fennell et al., 2004; Liu & Fennell, 2008; Ahn et al., 2008). *Dhc* st.

195 and other *Dhc* spp. contain a multitude of putative reductive dehalogenase genes and *Dhc* st. 195 is able to dechlorinate 1,2,3,4-TeCDD/F and 1,2,3,4,7,8-hexa-chlorodibenzofuran (1,2,3,4,7,8-HxCDF) (Hölscher et al., 2004; Seshadri et al., 2005; Fennell et al., 2004; Liu & Fennell, 2008). Based on research done for TeCDD and the relationship between CDDs/Fs and PCBs dechlorination, stimulating PCB dechlorination by adding haloprimers (alternate halogenated electron acceptors/ co-substrated) such as 2,3,4,5,6-PCB (PCB116), 2,6-dibromobiphenyl (2,6-DBB), halobenzoates, tetrachlorobenzene (TeCB), pentachloronitrobenzene (PCNB) and chlorobenzenes and chlorophenols may be an effective strategy (Van Dort et al., 1997; Bedard et al., 1998; Deceerd & Bedard, 1999; Cho et al., 2002; Krumins et al., 2009).

2.6 Summary

Anaerobic reductive dechlorination of PCBs and PCE is a process that provides a means of detoxification and, especially when coupled with aerobic degradation, completely destroys the contaminant (Bedard et al., 2008; Bedard et al., 2006; Brown et al., 1987[a]; Brown et al., 1987[b]). In conclusion, engineers have concluded that the variation of microbial dechlorination patterns observed in different sites is likely due to the presence and activity of specific species and consortia of organohalide respiring bacteria (Fagervold et al., 2011). It has become increasingly evident that the metabolism of these substrates in natural environments does not occur via the linear pathways that are familiar to us from pure culture studies (Abraham et al., 2002). Multiple community

members exchange metabolites and regulate carbon flow according to the availability of other substrates and nutrients, prevailing physical-chemical conditions, and community needs (Abraham et al., 2002). Method of using organohalide respiring bacteria and stimulated the process by injecting stimulation bacteria or haloprimers to dechlorinate PCBs and PCEs from the environment, is the easiest and the most efficient way. In addition, these bacteria are natural microorganisms that can easily found and not bringing any harm to the environment.

In my study, the dechlorinating microbial communities that grow in addition to response to natural organochlorine amendments were studied to better understand the potential for stimulation PCB and PCE dechlorination. Furthermore, the dechlorination of PCBs was investigated with natural soil communities with and without the presence of natural organochlorines to determine if natural organochlorines can stimulate, or conversely if they compete, with PCB dechlorination.

CHAPTER III

METHODOLOGY

3.1 Evaluation of Organohalide Respiring Bacteria using Natural Organochlorines

3.1.1 Soil and Sediment Collection

For microbial seed material, a 500-mL grab sediment sample was collected from a slow running stream at Ray Harrell Nature Park (Broken Arrow, OK) in March 2014. The sample was collected 1 foot from the stream edge in 6 inches of running water. The sediment was shoveled to about 4 inches of depth and funneled into a 500 mL bottle until it was filled completely. This park has no known history of direct anthropogenic contamination of chlorinated compounds. Approximately 2 kg of surface soil was collected from a forest with oak tree cover in Payne County, Oklahoma for organochlorine production. The material is rich with decaying detritus and collected only from the top 1 inch of the soil horizon.

3.1.2 Extraction of Organic Matter

To prepare organic matter for the synthesis of organochlorines, organic matter was first extracted from the soil into solvents. Approximately 10 g of soil material was added to a 15 mL Falcon centrifuge tube. Dissolved organic matters were separated from

bog or peat material by performing sequential extractions with methanol, acetone, dichloromethane and hexane. Extractions for each solvent was performed by filling the tube containing soil with solvent, mixing by vortex or hand for 30 seconds, sonication for 15 to 20 minutes, followed by another 45 seconds of vortexing. The solids were allowed to settle and the solvent was then transferred into a 500 mL with a silanized glass pipette. Each aliquot of soil was extracted with each solvent before finally being discarded, and a total of thirty 10 g aliquots of soil were subjected to organic matter extraction. After combining methanol, acetone, dichloromethane and hexane fractions in 500 mL Erlenmeyer flasks, the volume was split into two components: one for the production of organochlorines with CPO and the other as the organic matter control. The solvents were blown down to dryness using a stream of compressed air.

3.1.3. Synthesis of organochlorines

The synthesis of organochlorides was modified from Krzmarzick et al., 2012, Ortiz-Bermúdez et al., 2003, Niedan et al., 2000, Reina et al., 2004 and Aeppli et al., 2013. The CPO treated reactor were dosed with CPO enzyme (Sigma-Aldrich) and 0.1 % hydrogen peroxide (H_2O_2) solution while the controls were only dosed with 0.1% H_2O_2. The dried extracts from above were amended with 100 mL of a phosphate buffer (0.1 M K_2PO_4, 20 mM KCl). The pH value for each beaker was adjusted to 3.0 and maintained at 2.75- 3.25 during the chlorination reaction. To begin reaction, 10 µL of CPO was added to the 'CPO reactors' and 100 µL of 0.1% H_2O_2 was then amended to each reactor.

$$\text{Organic Extract} + H_2O_2 + K_2PO_4 + KCl \xrightarrow{\text{CPO enzyme}} H^+ + \text{Organochlorines} + K_2PO_4$$

The addition of H_2O_2 was added after 30 min and again after 30 additional min. The reaction mixture was left overnight and the addition of CPO and H_2O_2 sequence was repeated every day for four days total. Both the CPO and CTRL reactors where mixed gently after every addition of CPO and H_2O_2. The CPO-treated contents served as amendments in microcosms below while the control from this reaction served in control microcosms. After the reaction, the contents were extracted with dichloromethane by adding 100 mL of the solvent to the reactors, mixed vigorously for a few minutes, and then allowed to separate into two phases. The dichloromethane fraction was transferred to 160 mL microcosm bottles and then dried under a stream of compressed air to dryness.

3.1.4 Microcosms

Batch microcosms in this research were used to test for the degradation of PCBs. Table 1 shows summary of all microcosms used for this research. Each microcosms was operated in triplicate. For the determination of organohalide-respiring populations that are 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 extra. For DNA analysis, samples were collected at Days 0, 7, 14, 25, 39, 61, and 82.

Table 1. Summary of microcosms used in this experiment

Microcosm name	Amendments	Description
CPO microcosms	CPO Extract	Determine the organohalide respirers stimulated to grow from CPO-produced organochlorines
CTRL microcosms	Control Extract	
ClX and PCBs	Control Extract	Determine if 2,3,4,5-chlorobiphenyl (PCBs) could be dechlorinated more completely and faster with the priming of 7-chloro-1,3-dihydroxyxanthone (ClX) and with CPO-produced organochlorines. Controls were used to determine if the effects were from chlorinated compounds and not comparable organic matter or abiotic forces.
OHX and PCBs	1,3-OHX	
PCBs only	-	
CPO and PCBs	CPO Extract	
CTRL and PCBs	CPO Extract	
Autoclaved	Chloroxanthone, CPO extract and PCBs	

Microcosms were constructed in silanized 160 mL serum bottles capped with Teflon stoppers and aluminum crimps similar to previously published research (Krzmarzick et al., 2012; Krzmarzick et al., 2014). Each microcosms contained the respective organochlorine or control amendment, 5 g of sediment collect above, 130 mL of anaerobic mineral media reduced with sodium sulfide and cysteine, 10 mM acetate, and 1 mL of vitamin solution to provide cobalamin, an essential cofactor for rdhs (Shelton et al., 1984; Wolin et al., 1963; He et al., 2007; Krzmarzick et al., 2012). Microcosms were constructed in an anaerobic glovebag with a 3% H_2/ 97% N_2 headspace. Autoclaved controls were prepared and then autoclaved three times.

3.1.5 Sample collection

Samples were collected at Days 0, 7, 14, 25, 39, 61, and 82 for DNA analysis. Bottles were vigorously hand-shaken for 30 s before they were opened in the glovebag and approximately 1.6 mL of sediment slurry transferred to microcentrifuge tubes with sawed-off Pasteur pipettes (Yan et al., 2006, Krzmarzick et al., 2012). For DNA extraction, 1.6 mL of slurry was centrifuged at 4000 x g for 5 min, the supernatant was removed, the pellet was transferred to bead-beating tubes for DNA extraction with the PowerSoil DNA isolation kit (MoBio Laboratories) and frozen at -20^{o}C until further analysis. DNA was then extracted with the PowerSoil DNA kit according to the manufacturer's recommendations (MoBio Laboratories) and frozen until later analysis.

3.1.6 qPCR

Quantitative PCR (qPCR) was use to quantify several 16S rRNA genes from known dechlorinating bacteria and to quantify the overall *Bacteria* 16S rRNA. The phylogenetic targets, primers and references for qPCR approaches are listed in Table 2, for example primers Dhc 582F//Dhc 728R are specific for *Dhc* spp. and primers Dhc1154F//Dhc1286R are specific for *Dhc* spp. and also shown to amplify other *Chloroflexi* related to *Dehalococcoides*.

Table 2. Primers, methodologies and references used to study organohalide respiring organisms.

Phylogenetic Target	Primer Pair	Reference
Dhc-like spp.	1154 F//1286 R	Krzmarzick et al. 2012
Dhc-like spp.	1150F//1286F	Krzmarzick et al., 2013
Dhc	582F//728R	Duhamel et al., 2004
Dehalobium DF-1/*o*-17	866 F//1265 R	Fagervold et al., 2005; Watts et al., 2005
Dehalogenimonas spp.	634 F//799 R	Yan et al., 2009
Dehalobacter spp.	411 F// 645 R	Smits et al., 2004
Desulfitobacterium spp.	406 F// 619 R	Smits et al., 2004
"Gopher group"	163F//441R	Krzmarzick et al., 2014
Geobacter lovleyi	564 F// 840 R	Sung et al., 2006
Desulfomonile spp.	205F//628R	El Fantroussi et al., 1997
Desulfovibrio spp.	691F//826R	Fite et al., 2004
Sulfurospirillum spp.	114F//421R	Duhamel et al., 2006

For *Bacteria* 16S rRNA, primers 341F and 534R (Muyzer et al., 1993) were used as previously described (Krzmarzick et al., 2012). Each qPCR mixture totaled of 10 µl using 5 µl of iTaq SyberGreen Supermix with Rox master mix (BioRad), 300 nM of each primer, and 1.0 µl of undiluted DNA extract or standard. Analysis was on a CFX Connect Real Time System (Bio-Laboratories) with Bio-Rad CFX Manager software. Thermocycling protocol for each analysis was 95 ^{o}C for 3 min followed by 40 cycles of 95 ^{o}C for 15 s and 60 ^{o}C for 30 s. A melting curve analysis was performed after each complete run to ensure that primer-dimers were not amplified and that the amplification was specific. Standards for each qPCR were prepared from known concentrations of plasmid extracts containing the 16S rRNA gene of interest. Each sample was analyzed

with qPCR in duplicate, the duplicates were $\log_{10}$ transformed and averaged. Triplicate microcosms were then averaged and these means and standard deviations are shown in Appendix A. To normalize to *Bacteria* 16S rRNA, each sample was $\log_{10}$ transformed, averaged, untransformed, divided by the number of *Bacteria* 16S rRNA treated similarly, and then this ratio was $\log_{10}$ transformed, the averages and standard deviations of triplicate reactors were calculated, and these values are used for figures and Appendix A. If all triplicate microcosms were below the detection limit (BDL), the value was reported as BDL, but if one or two of the microcosms were not BDL, the detection limit was then used for samples that were BDL for the purposes of calculating and reported averages and standard deviations. These cases are specifically reported in the tables in Appendix A.

3.1.7 TRFLP Analysis

Terminal restriction fragment length polymorphism (TRFLP) was used in this study to further understand which *Chloroflexi* were enriched during CPO-amendment. The TRFLP method in this study was adapted from Krzmarzick et al (2013); the primary difference is that this study used the universal bacterial primer 8F (Reysenbach et al, 1994) instead of the *Dhc* specific primer 'Dhc553F' used in Krzmarzick et al (2013). Briefly, PCR was performed on DNA extracts from the samples using the universal bacterial primer 8F (Reysenback et al., 1994) and the *Chloroflexi* primer Chl1150R designed by Krzmarzick et al (2013) labeled with carboxyfluorescein. Primer Chl1150R

is specific to a broad swath of the *Chloroflexi* phylum, with specificity towards all isolated *Dehalococcoidia* as well as many *Anaerolineae* and *Caldilinea*; thus all putative dechlorinating *Chloroflexi* are expected to be amplified with this primer pair. PCR was performed in triplicate for each DNA extract. Triplicate products were then combined and an enzyme digestion was performed in duplicate using restriction enzymes Taq I, RsaI, and BamHI as described previously (Krzmarzick et al., 2013). This digestion gives unique sizes to all genera of the currently isolated strains of *Dehalococcoidia* and even distinguishes between some of the strains in *Dhc*. Each digestion product was analyzed by the DNA/Protein Core Facility at Oklahoma State University with an ABI 3730 DNA Analyzer using MapMarker1000 as a size standard (Bioventures). PeakScanner2 software (Life Technologies) was then used to analyze the data. Peaks and sizes were transferred to MS Excel, peak areas were normalized to total peak area for that run, duplicate enzyme digests were averaged, peaks less than 0.5% of the total peak area were deleted, remaining peaks were renormalized to total area, and the OTUs were binned according to size. After all samples were binned together, OTUs that were only present in 2 or fewer samples and less than 1% of their respective samples were removed from further analysis.

3.2 Dehalogenation for PCBs

3.2.1 Making Stock for PCBs (2,3,4,5-Tetrachlorobiphenyl)

First, a master stock was prepared 2,3,4,5-tetrachlorobiphenyl. A 15 mL silanized vial with a Teflon cap was weighed, approximately 0.05 g 2,3,4,5-tetrachlorobiphenyl

powder was added to the vial which was then reweighed to calculate the exact mass. Then, 5 mL of hexane was added to the vial which was then weighed again to determine final concentration. This master stock was used for addition to microcosms and was diluted from approximately 10 $\frac{mg}{mL}$ to 100 $\frac{\mu g}{mL}$.Concentration of the other PCB congeners, 2,3,5-chlorobiphenyl, 2,4,5-chlorobiphenyl, 2,3-chlorobiphenyl, 2,4-chlorobiphenyl, 2,5-chlorobiphenyl and 2-chlorobiphenyl were purchased as standards of 100 $\frac{\mu g}{mL}$ in isooctane (Accustandard).

3.2.2 Mix Solutions for Calibration

A mixed standard solution was prepared for calibration curves using a gas tight glass syringe for each stock of PCB congeners. Different concentrations of the mix standard solution were prepared by dilution with hexane. Standards and samples were analyzed with gas chromatography equipped with a micro-ECD detector. The method was optimized to separate major peaks and for a short run time. For 2-chlorobiphenyl, the peak appeared at approximately 8.419 min; 2,4 and 2,5-chlorobiphenyl appear as a coalescing peak at approximately 9.4 min; 2,3-chlorobiphenyl appear at approximately 9.6 min; 2,3,5 and 2,4,5-chlorobiphenyl appeared as a coalescing peak at approximately 10.4 min; 2,3,4,5-chlorobiphenyl appear at approximately 11.592 min.

3.2.3 Microcosms

Microcosms were used to study the dechlorination of PCBs when co-amended with natural organochlorines. Six treatments were tested, each in triplicate (see Table 1). The PCB amended was 2,3,4,5-tetrachlorobiphenyl, and microcosms contained either CPO-produced organochlorines, control organic extract, the chemical 7-chloro-1,3-dihydroxyxanthone (ClX), an analogue of natural organochlorines purchased from Princeton Biomolecular, or 1,3-dihydroxyxanthone (OHX) as a control. PCB stock was added to the microcosms as hexane solution prior to addition of media and it was left open for the hexane to evaporate. Xanthones were added as dry powder. PCBs and xanthones were added so that the beginning concentration was 100 µM. Microcosms were prepared as described above. On Day 88, 5 g of anaerobic digestor sludge and 0.003 g of 2,3,4,5-tetrachlorobiphenyl was amended to microcosms.

3.2.4 Sample collection

Bottles were vigorously hand-shaken for 30s before they were opened in the glovebag and approximately 1.5 mL of sediment slurry was withdrawn with a sawed-off Pasteur pipette and transfer to 20 mL serum vails for PCBs extraction (Yan et al., 2006, Krzmarzick et al., 2012). Samples were collected at 7, 14, 25, 39, 61, 82, 103 and 132 days.

3.2.5 Extraction

Next were prepared 20 mL serum vails contained approximately 1.5 mL of sediment slurry. PCBs were extracted according to the handshake method used in Yan et al. (2006). Samples were transferred to hexane. The amount of sample and amount of hexane after extraction were determined gravimetrically.

3.2.6 Weight of Soils in each Sample

After extraction, the amounts of solids extracted were determined to normalize the amount of PCBs to the solids. Disposable aluminum crinkle dishes were preweighed and extracted contents were transferred to the aluminum dish. Ethanol was used to assist in transferring all slurry particles, if needed. The dish was baked in a 105 ^{o}C oven overnight and weighed the next day for solids determination.

3.2.7 Gas Chromatography with Electron Capture Detector (GC-ECD) Analysis

Extracted hexane solution analyzed by using Agilent Technologies 7890B gas chromatograph (GC-ECD) system with an Agilent 19091J-413 column (30 m x 320 µm x 0.25 µm) that has a temperature range of -60 ^{o}C to 325 ^{o}C. The inlet of the GC was set for a splitless injection, the injector temperature was set at 250 ^{o}C, the pressure at 7.9566 psi and septum purge flow at 3 mL/min. The protocol for GC-ECD was 50 ^{o}C for 1 min, a 20 ^{o}C per minute ramp up to 270 ^{o}C and a final hold. The total run time was 14 min. The ECD detector was set at 100 ^{o}C with a makeup flow (argon methane gas) of 60

mL/min. The column flow rate (helium gas) was 1.6 mL/min. For analysis of standards and samples, manual injections of 2 µL were used. PCBs were analyzed using external calibration curves.

CHAPTER IV

FINDINGS

4.1 Growth of organohalide respirers from CPO-produced organochlorines

Several known groups of organohalide respiring bacteria were directly measured with qPCR to determine their concentrations over time in the microcosms with CPO-produced organochlorines versus the organic matter control. From the organohalide respiring *Chloroflexi*, *Dhc* were measured with three unique qPCR methods as was *Dehalogenimonas* and the PCB dechlorinator "*Dehalobium chlorocoercia*" DF1. Outside of the *Chloroflexi* group, the *Dehalobacter*, *Desulfitobacterium*, and "Gopher group" of the phylum *Firmicutes* were measured with qPCR and the dechlorinating *Sulfurospirillum*, *Geobacter*, *Desulfomonas*, and *Desulfovibrio* of the phylum *Proteobacteria* were measured. To be stimulated by the natural organchlorides, it is expected that the growth of the 16S rRNA genes in the triplicate microcosms amended with CPO produced organochlorines (CPO reactors) would be statistically significantly greater (Student T-test, $P < 0.05$) than the number of genes in the reactors amended with control organic extract (CTRL reactors). The means and standard deviations of dechlorinators in each set of microcosms are shown in Appendix A in both absolute measurement (per µL of DNA extract) and as a fraction of measured *Bacteria* 16S rRNA.

This latter value is favored for reasons of significance as it corrects for any variations in sampling and in DNA extraction efficiency.

The number of *Desulfitobacteria* 16S rRNA genes were found to be mostly below the detection limit of the qPCR analysis. No single time point showed an above BDL values for an entire set of triplicate microcosms. The "Gopher group" 16S rRNA genes additionally were BDL in all samples. The number of *Dehalobacter* 16S rRNA genes in triplicate microcosms amended with CPO in the controls started near the detection limit (Table A1). The ratio of *Dehalobacter* 16S rRNA per *Bacteria* 16S rRNA genes at day 7 for CPO microcosms is (-5.69 ± 0.13, $\log_{10}$ units) and CTRL microcosms is (-5.85 ± 0.07, $\log_{10}$ units) (Table Al). These two points (Figure 1) are close to each other and their error bars are small, which means the number of *Dehalobacter* in the triplicate microcosms amended were very similar. The ratio of *Dehalobacter/Bacteria* in CPO microcosms then increased significantly between Day 7 and Day 25 and then stabilized while in the CTRL microcosms, *Dehalobacter/Bacteria* increased slowly through the experiment (Figure 1). By Day 82, the number of *Dehalobacter/Bacteria* in CPO microcosms were very similar to the CTRL microcosms. The ratio of *Dehalobacter*/*Bacteria* were statistically significantly higher in the CPO microcosms versus the CTRL microcosms at Day 14 and Day 25. These results do suggest some stimulation of *Dehalobacter* by the CPO produced organochlorines during this part of the experiment, but since the two treatments become similar thereafter, it cannot be concluded that CPO produced organochlorines provided a sustained advantage in

stimulating *Dehalobacter* versus bulk organic matter extract. The overall slight growth of organohalide-respirers in the control is not unexpected, since the bulk organic matter likely contains some original natural organochlorines (Krzmarzick et al., 2012; Myneni et al., 2002).

None of the organohalide respiring strains of the phylum *Proteobacteria* showed significant enrichment due to the CPO or CTRL amendments. There are no statistically significant values of *Sulfurospirillum*/*Bacteria* in CPO microcosms compared to CTRL microcosms (Figure 2). The ratio of *Sulfurospirillum*/*Bacteria* in the CPO microcosms decreased from -2.36 ± 0.06 (log_{10}) on Day 7 to -2.79 ± 0.72 (log_{10}) on Day 82 and were never significantly higher than CTRL microcosms (Table A2) . *Sulfurospirillum* was below detection limits in the original sampling (Day 0), so the conditions of the reactor may have been favorable to some rapid, initial growth, theoretically due to non-dechlorinating metabolism such as sulfur reduction. Between Day 61 and Day 82, *Sulfurospirillum* 16S rRNA genes increased in CPO microcosms and continue decreasing in CTRL reactors though were still not significantly different (Figure 2).

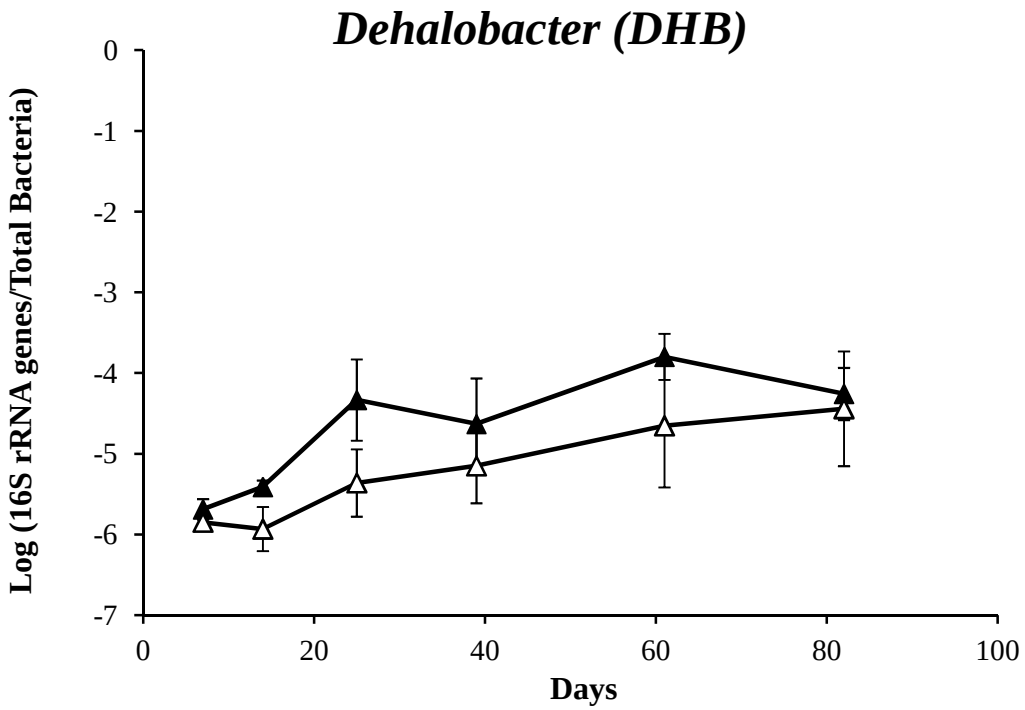

Figure 1. The numbers of *Dehalobacter* 16S rRNA genes/*Bacteria* 16S rRNA genes in microcosms amended with CPO produced organochlorines (▲) compared to microcosms amended the organic control (Δ). Error bars are the standard deviations of triplicate microcosms.

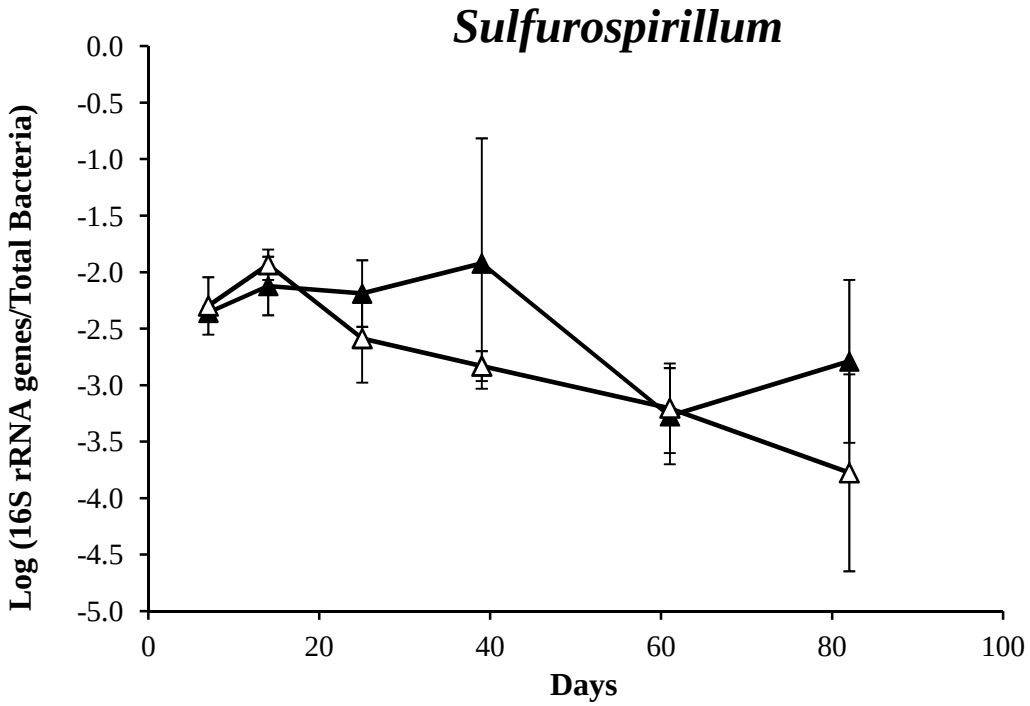

Figure 2. The numbers of *Sulfurospirillum* 16S rRNA genes/*Bacteria* 16S rRNA genes in microcosms amended with CPO produced organochlorines (▲) compared to microcosms amended the organic control (Δ). Error bars are the standard deviations of triplicate microcosms.

The number of *Geobacter*/*Bacteria* in the triplicate CPO microcosms and CTRL microcosms were relatively stable, but overall decreased from -2.30 ± 0.11 to -2.53 ± 0.35 (log_{10}) in the CPO reactors (Table A2; Figure 3). Extremely small or negligible error bars showed at Day 25 and Day 61 in the CPO reactors contributed to statistical significant differences between CPO and CTRL microcosms on those two days (Figure 3) but without growth in the CPO microcosms, there is no support to suggest that CPO-produced organochlorines stimulated the dechlorinating *Geobacter*. There was no statistically significant difference for *Desulfovibrio* 16S rRNA genes in the CPO versus the CTRL microcosms. *Desulfovibro* 16S rRNA genes decreases by an order of magnitude and the ratio of *Desulfovibrio*/*Bacteria* in CPO reactors decreased from -2.15 ± 0.59 (log_{10}) to -2.84 ± 0.28 (log_{10}) (Figure 4; Table A2) and the number of *Desulfovibrio* 16S rRNA genes in CTRL reactors decreased similarly (Table A2). *Desulfomonile* was never detected above the detection limit in any of the DNA extracts.

Some of the dechlorinating *Chloroflexi* appeared to show enrichment from the CPO-produced organochlorines. The bacterium "*Dehalobium chlorocoercia*" DF-1 was not detected in any sample. Conversely, the ratio of *Dehalogenimonas*/*Bacteria* in the triplicate amended with CPO increased from -1.40 ± 0.27 on Day 7 to -0.60 ± 0.40 (log_{10}) on Day 82 while in the CTRL microcosms increased slightly from -1.58 ± 0.19 (log_{10}) to -1.42 ± 0.10 (log_{10}) (Figure 5; Table A3).The ratio of *Dehalogenimonas*/*Bacteria* was statistically significantly higher in CPO microcosms versus CTRL microcosms from Day 39 to the end of the experiment. Among all dechlorinators in triplicate amended with

CPO and with CTRL, *Dehalogenimonas* 16S rRNA genes has the most obvious and stable growth (Figure 5). The number of *Dehalogenimonas* 16S rRNA was also higher than all other dechlorinating bacteria in the original sampling. Thus, *Dehalogenimonas* or a bacteria amplified with the same primers was enriched by CPO-produced organochlorines. Phylogenetic analysis of sequenced qPCR amplification products can elucidate how closely related these stimulated bacteria are to the *Dehalogenimonas*.

The number of *Dehalococcoides* 16S rRNA genes were studied with three primer sets. Primer set Dhc582F// Dhc782R and Dhc1154F//Dhc1286R are both specific for only *Dhc* spp. however the set Dhc1154F//Dhc1286R has been shown to amplify other *Chloroflexi* related to *Dehalococcoides* (Krzmarzick et al., 2012). The primer set Chl1150F//Dhc1286R has the *Dhc* specific primer Dhc1286R and a primer that covers all isolated *Dehalococcoidia* as well as some *Anaerolineae* and *Caldilineae* also in the *Chloroflexi* phylum (Krzmarzick et al., 2013). Because *Dhc* and related dechorinators in the *Chloroflexi* are relatively deeply branching and divergent, the specificity and range of these primers are less certain than those for the *Firmicutes* and *Proteobacteria*. Thus, any set of these primers should be viewed as measuring some subset of bacteria related to *Dehalococcoides* and thus called *Dehalococcoides*-like *Chloroflexi*.

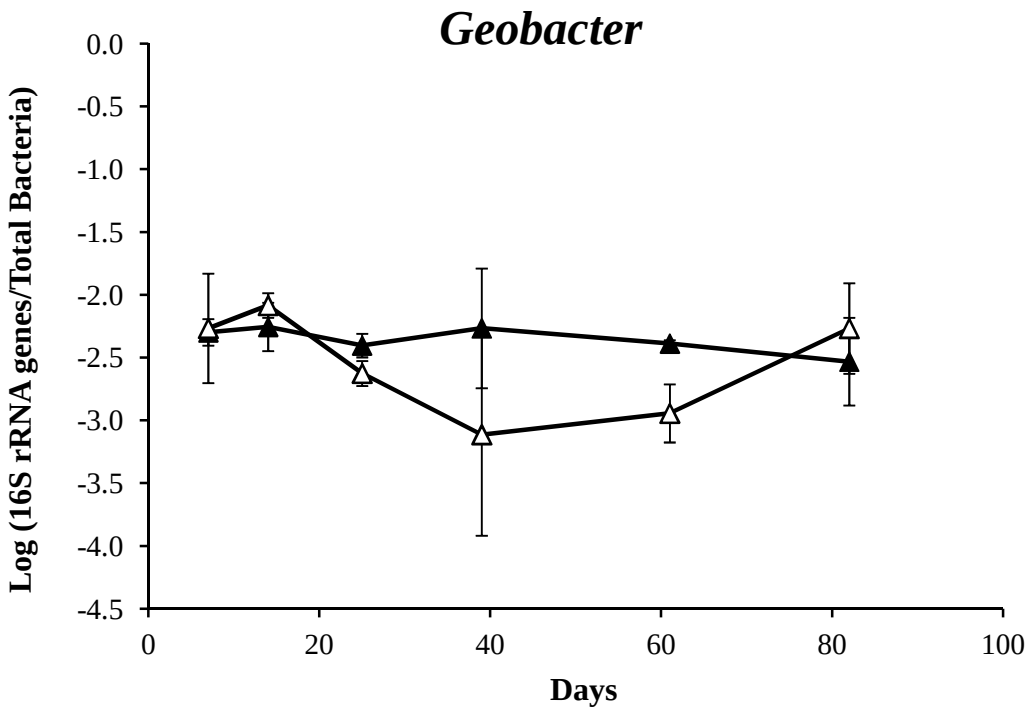

Figure 3. The numbers of *Geobacter* 16S rRNA genes/*Bacteria* 16S rRNA genes in microcosms amended CPO produced organochlorines (▲) compare to microcosms amended the organic extract control (Δ). Error bars are the standard deviations of triplicate microcosms.

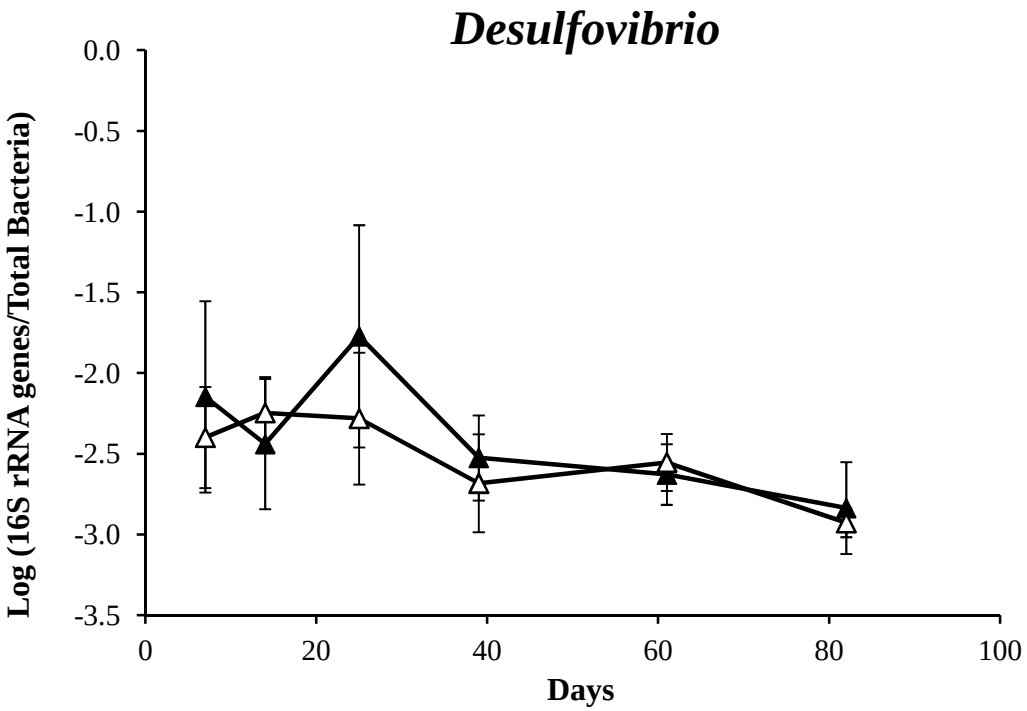

Figure 4. The numbers of *Desulfovibrio* 16S rRNA genes/*Bacteria* 16S rRNA genes in microcosms amended CPO produced organochlorines (▲) compare to microcosms amended the organic extract control (△). Error bars are the standard deviations of triplicate microcosms.

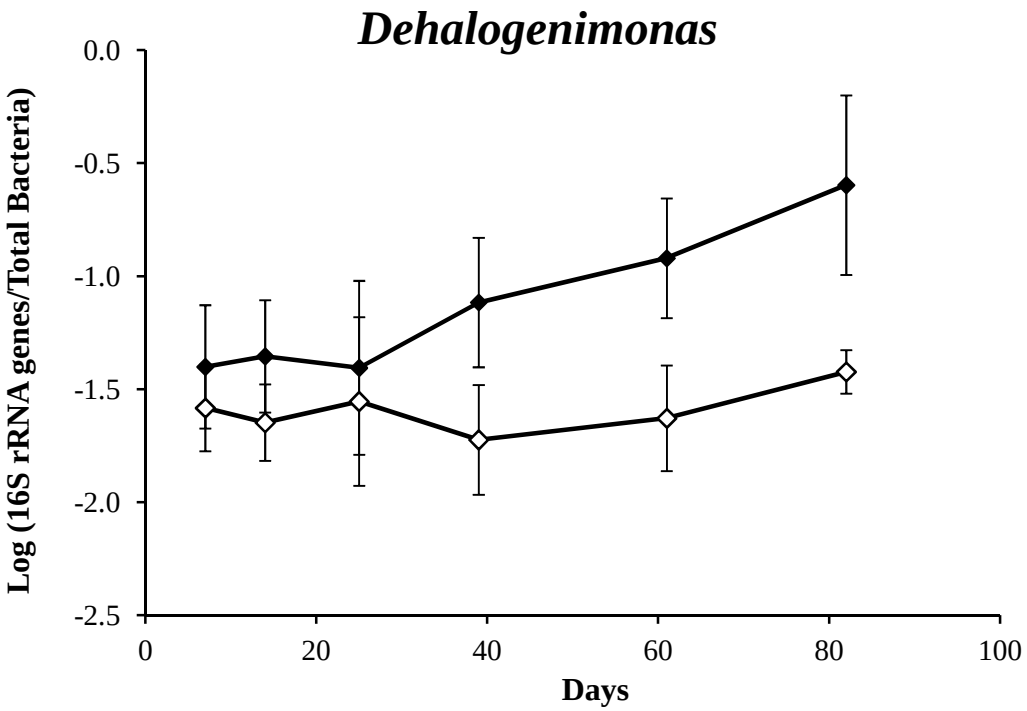

Figure 5. The numbers of *Dehalogenimonas* 16S rRNA genes/*Bacteria* 16S rRNA genes in microcosms amended CPO produced organochlorines (▲) compare to microcosms amended the organic extract control (△). Error bars are the standard deviations of triplicate microcosms.

Using the primer pair Dhc582F//Dhc782R, the *Dhc* 16S rRNA genes in CPO reactors increases nearly an order of magnitude while remaining relatively constant in the CTRL microcosms (Table A4). The ratio of *Dhc/Bacteria* in triplicate CPO microcosms increases from -3.55 ± 0.26 (log_{10}) on Day 7 to -2.61 ± 0.45 (log_{10}) on Day 61 before dropping to -3.13 ± 0.48 (log_{10}) on Day 82 (Figure 6). In the CTRL microcosms, the *Dhc* increased slightly (-3.87 ± 0.27 (log_{10}) on Day 7 to -3.56 ± 0.23 (log_{10}) on Day 82). There are statistically significant differences at Days 14, 39 and 61 showing that some *Dhc*-like organisms are stimulated by CPO-produced organochlorines.

Using the primer pair Chl1150F//Dhc1286R, the *Dhc* in the CPO microcosms increased by more than an order of magnitude between Day 7 and Day 82 while remaining similar in the CTRL microcosms (Table A4). The ratio of *Dhc*/*Bacteria* in CPO microcosms increased from (-1.32 ± 0.10 (log_{10}) to -0.42 ± 0.28 (log_{10}) and increased in the CTRL reactors from -1.30 ± 0.29 to -1.00 ± 0.10 (Figure 7; Table A4). Because of relatively large standard deviations in the triplicates, the *Dhc*/*Bacteria* was only significantly higher at Day 82, but the relative differences and magnitude of growth again support that some *Dhc*-like *Chloroflexi* were stimulated to grow.

Unexpectedly, there was no statistically significant differences of *Dhc* between treatments when using the primer pair Dhc1154F//Dhc1286R. The ratio of *Dhc*/*Bacteria* using these primers were typically higher for CPO microcosms compared to CTRL microcosms, but never statistically significantly so (Figure 8; Table A4). This result

contrasts the other *Dhc* measurements and insinuates that the entire breadth of *Dhc*-like *Chloroflexi* were not stimulated, but rather specific strains that were able to be measured with some primer pairs but not all; since all primer pairs should have affinity for *Dehalococcoides mccartyi*, these bacteria are likely not strictly members of that genera. These bacteria must also differ from the *Dehalococcoides*-like *Chloroflexi* from Krzmarzick et al., which found significance growth of *Dhc*-like *Chloroflexi* using the Dhc1154F//Dhc1286R primers (Krzmarzick et al., 2012). This difference may be due to the largely divergent methods of handling produced organochlorines prior to microcosm preparation. Krzmarzick et al., 2012 used solid phase extraction C-18 cartridges to extract the CPO-produced organochlorines from the production aqueous solution, which likely biased against larger molecular weight components (Krzmarzick et al., 2012). In this study, produced organochlorines were extracted with dichloromethane, transferred to a vial and then blown down extensively under a stream of gas to dryness, giving semivolatile organic compounds, including those which were chlorinated, time to volatilize while the larger weight chlorinated compounds, including those that clumped together, were transferred wholly intact. These differences may have also contributed to the longer time frames of enrichment in these cultures compared to those in Krzmarzick et al., (2012).

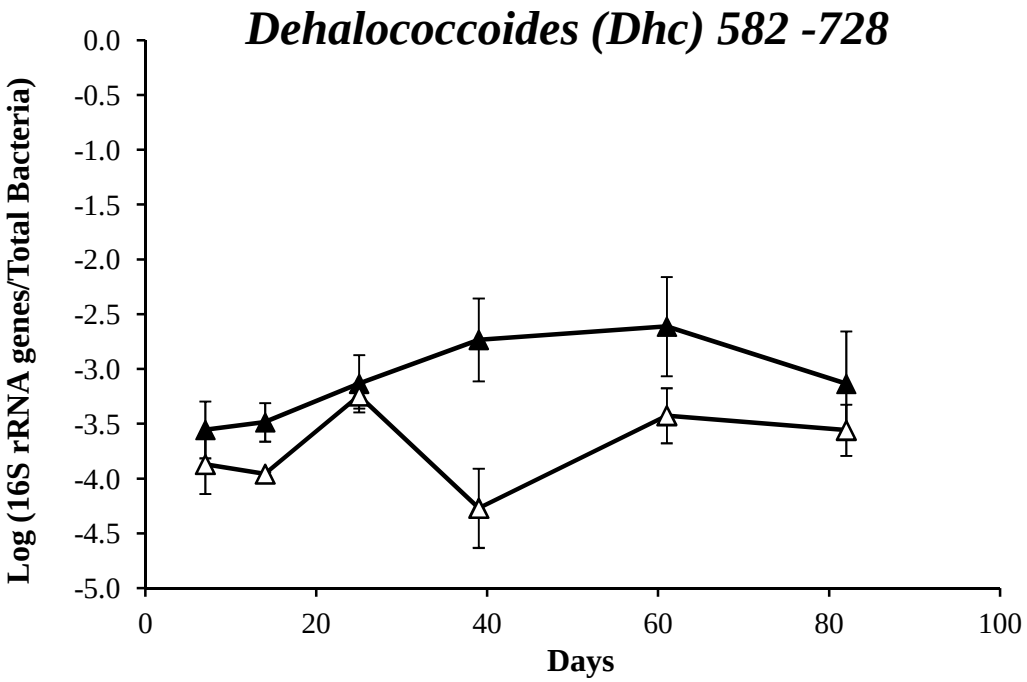

Figure 6. The numbers of *Dhc* 16S rRNA genes/*Bacteria* 16S rRNA genes as measured with primers Dhc582F and Dhc728R in microcosms amended CPO produced organochlorines (▲) compare to microcosms amended the organic extract control (△). Error bars are the standard deviations of triplicate microcosms.

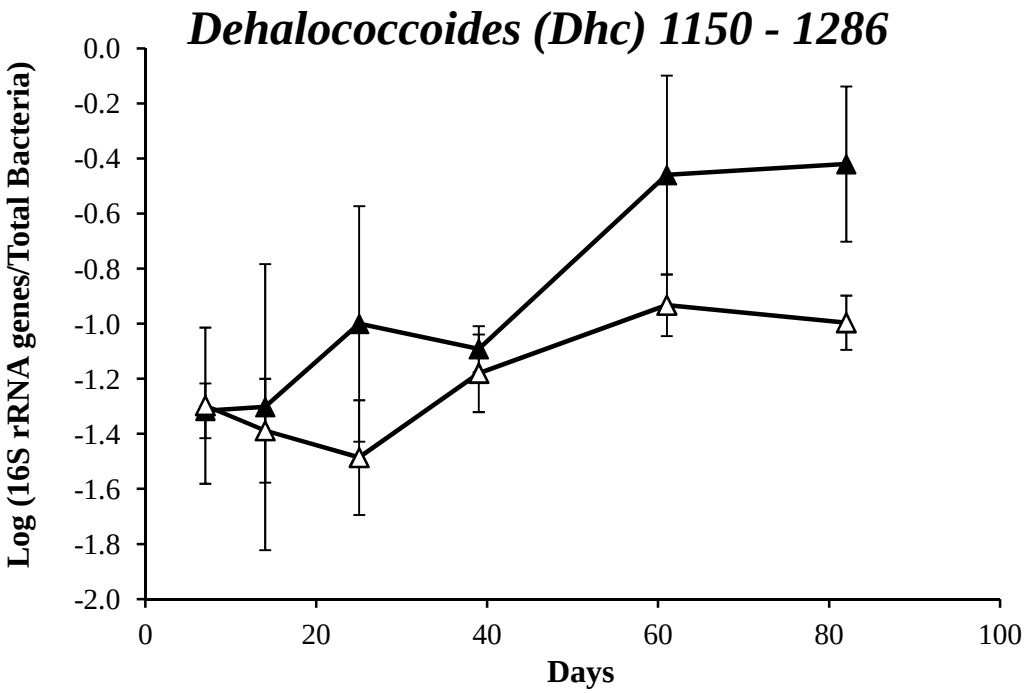

Figure 7. The numbers of *Dhc* 16S rRNA genes/*Bacteria* 16S rRNA genes as measured with primers Chl1150F and Dhc1286R in microcosms amended CPO produced organochlorines (▲) compare to microcosms amended the organic extract control (Δ). Error bars are the standard deviations of triplicate microcosms.

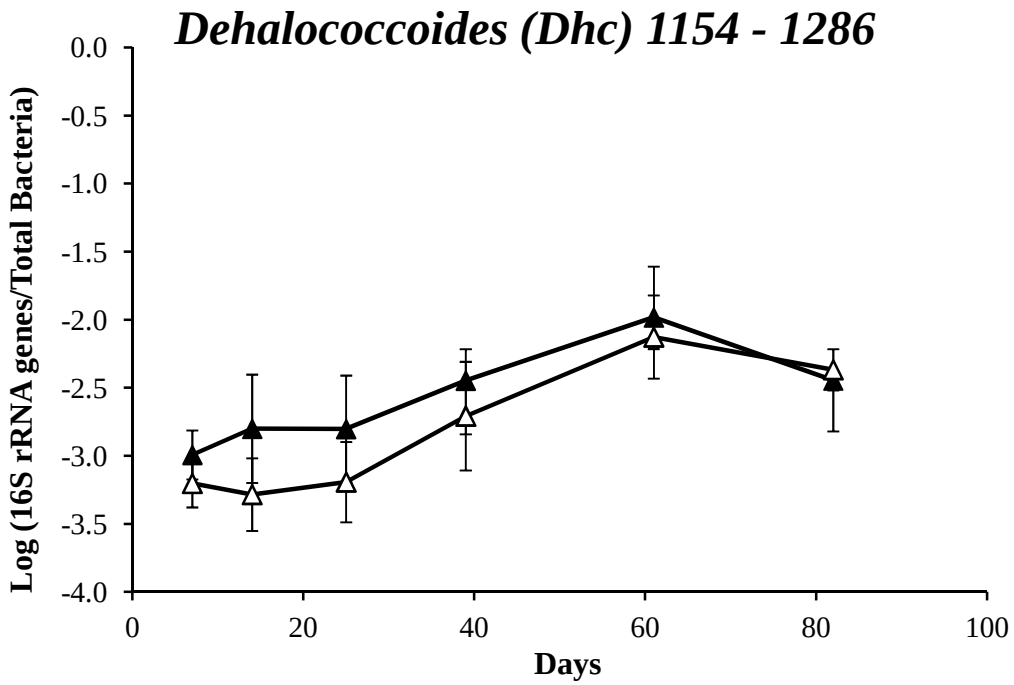

Figure 8. The numbers of *Dhc* 16S rRNA genes/*Bacteria* 16S rRNA genes as measured with primers Dhc1154F and Dhc1286R in microcosms amended CPO produced organochlorines (▲) compare to microcosms amended the organic extract control (Δ). Error bars are the standard deviations of triplicate microcosms.

4.2 Terminal Restriction Fragment Length Polymorphism (TRFLP) Analysis

TRFLP analysis was used to further examine the putative dechlorinating *Chloroflexi.* This method used PCR and primers to amplify all known *Chloroflexi* that are in the *Dehalococcoidia* class as well as many known in the related *Anaerolineae* and *Caldilineae* classes. Restriction enzymes then digested the product, giving unique fragment lengths for each known genus and species in this group (see methods). A total of 75 different fragment lengths, or operational taxonomic units (OTUs), were found in the analysis for the microcosms in this study. Upon visual inspection, nearly all of these OTUs were of relatively low abundance, not sustained through very many samples, and not unique to CPO or CTRL microcosms. Initial samples of the microcosms had 24-28 RFLs, but all later samples never had more than 19. Appendix C shows the data for all fragments that represented more than 5% of a peak area for at least one sample.

Using nonmetric multidemensional scaling of the data (nMDS) as described previously (McNamara and Krzmarzick, 2014), it appears that on days 7, 14 and perhaps 25, the diversity of *Chloroflexi* community in both CPO microcosms and CTRL microcosms, shifted significantly and similarly. On Days 25, 39, 61 and 82, the diversity of *Chlorofexi* in CTRL microcosms were very similar and it shows the diversity were kind of gathered together in one spot. While the diversity of *Chloroflexi* in CPO microcosms on days 25 to 82 shifted and separated from the controls which indicating a unique diversity of *Chloroflexi* arose from the CPO microcosms compared to the CTRL

microcosms (Figure 9). Upon close inspection of the data, these variances are perhaps dominated by a single OTU with a restriction fragment length (RFL) of 276 base pair (bp). This OTU first appeared in microcosms on Day 25 when it showed in two of the three CPO microcosms with just 1.6% and 5.0% of the total peak areas and in one CTRL microcosm with 3% total peak area. On Day 39, this OTU was again in two CPO microcosms, with 0.6% and 17.2% of the peak areas in respective microcosms while again appearing in 1 CTRL microcosm with a peak area of 0.7% total. On Day 61, the OTU was a major component in all three CPO microcosms with relative peak areas of 25.8, 15.1, and 72.6%, respectively while showing in two CTRL microcosms at 1.4% and 1.1%. On Day 82, the dominance of this OTU in the triplicate CPO microcosms was maintained with peak areas of 13.4, 49.4, and 33.9%, while only showing in one CTRL microcosm at 3.4% peak area. Occupying a sustained and major presence in the CPO microcosms while only showing at low concentrations inconsistently in the CTRL organisms indicate that this OTU was profoundly enhanced from the CPO-produced organochlorines. The timing of this difference between CPO and CTRL reactors is similar to that seen in *Dehalogenimonas* and *Dhc* in the qPCR results above, though 276 bp is not the fragment size predicted from current sequenced strains of these two bacteria, indicating this bacteria may be novel strain.

Other OTUs may represent stimulated dechlorinators, but none were as clear. An OTU with a length of 283 represented 9.2, 11.1 and 20.4% of the peak areas in respective triplicate CPO microcosms on Day 39 while showing in CTRL micocrosms as the lower

relative peak areas of 1.6, 4.2, and 0.05 % and again on Day 84 had relative peak areas of 27.9, 2.6, and 2.2 % in the triplicate CPO microcosms while only showing in 1 CTRL at 0.9%. There are many times, however, the CTRLs have upwards of 7% their peak area represented by this OTU, making any correlation of CPO produced organochlorines on this OTU unclear.

In both the CTRL and CPO microcosms, another OTU with a fragment length of 278 bp had a significance presence in all organisms after Day 0, often greater than 50% of the relative total peak area of the sample. This OTU likely represents a non-dechlorinating species enriched by the conditions of the microcosm and its dominance was reduced in the reactors in which the 276 bp fragment became a major fraction of the community.

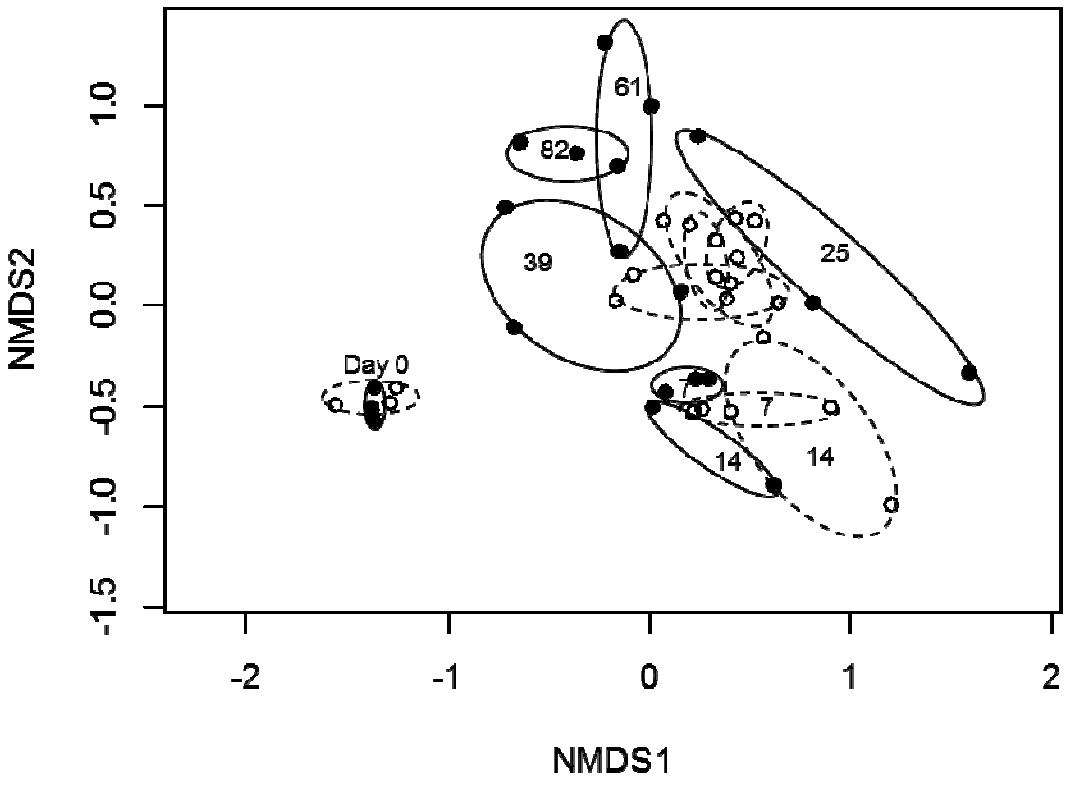

Figure 9. NMDS analysis of the dechlorinating *Chloroflexi* TRFLP data set for CPO-amended microcosms (solid line) and CTRL microcosms (dotted line) (all 74 RFLs used in analysis). Days 0, 7, and 14 are labeled for CPO-amended microcosms and CTRL microcosms, while days 25,39, 61 and 82, only CPO-amended microcosms being labeled since CTRL microcosms on these days had significant overlap in the middle of the figure.

4.3 PCB Analysis

Gas chromatography (GC-ECD) was used to analyze changes of PCBs concentration and recognized degradation activities of PCBs for each microcosm in batch experiments. PCB concentrations varied significantly making quantitative analysis difficult. Concentration of PCBs in microcosms did decrease from day 0 to day 82 and degradation activity became apparent due to the presence of dechlorination products on Days 103 and 132, likely due to amendment of digester sludge on Day 88 (Table D1 and Table D2). Because of the large amounts of error in measurement, and the presence of some degradation products in the autoclaved controls, it is not possible to elucidate whether CPO produced organochlorines or chlorinated xanthone truly affected the dechlorination rate of PCBs compared to controls. More precise PCB extraction methods needed and this experiment repeated to better determine the potential for priming PCB dechlorination with natural organochlorines.

CHAPTER V

CONCLUSION

This thesis is an progression of the work performed by Krzmarzick et al. in a paper published in 2012, which had showed that *Dhc*–like *Chloroflexi* grew quickly in a short amount of time while putatively dechlorinating an organochlorine mixture produced with CPO (Krzmarzick et al., 2012). This original work was limited in that it only investigated the *Dhc*-like *Chloroflexi* and did not measure the number of other groups of dechlorinators. In contrast to that work, the stimulation of *Dhc*-like *Chloroflexi* in this study was not found using the same set of primers in the Krzmarzick et al., study, but still found stimulation in the *Dhc*-like *Chloroflexi* using two other sets of primers suggesting different *Dhc*-like organisms were stimulated that had affinities for different primers. Additionally, this study found stimulation using primers targeting the *Dehalogenimonas* spp. and perhaps some limited stimulation of *Dehalobacter*, which were not studied in the previous experiment. The time frame of stimulation was also much longer in this study than the study by Krzmarzick et al., needing nearly 2 months of time.

The differences in the results in these two studies may be from the different sources of microorganisms. This study used a freshwater sediment from a forest with oak tree while Krzmarzick et al. used soils from pine dominated area, and two geographically

distinct inoculum sources has different relative abundances of initial communities, which could affect results. The most likely difference in results, though, is the difference in preparing organic matter for chlorination and handling the prepared chloroperoxidase produced organochlorines after production. Krzmarzick et al. extracted organic matter from soils using an accelerated solvent extractor with hexanes and acetone at high temperatures and pressures while this study extracted using a wide set of solvents and gentler handmixing and sonication techniques. In the handing after production, the organochlorines in Krzmarzick et al. were loaded onto a solid phase extraction C18 column and then extracted off of that column with acetone and hexane. The resulting CPO produced organochlorines thus would have had to been transported selectively through the column dissolved in hexane or acetone, thus many compounds were likely lost in the process that did not both effectively bind during initial loading and release during acetone and hexane extraction. Altogether, this process likely favored an enrichment of smaller organochlorines that could flow through the column. In this study, organochlorines were extracted with dichloromethane – a solvent that may be better suited for organochlorines than hexanes or acetone. Additionally, the dichloromethane was then simply transferred to the microcosm bottles and then blown under a stream of air to complete dryness, giving ample time for semivolatiles to evaporate also and allowing the larger and stickier components of the CPO production process to be transported wholly. This difference in results and methodologies is potentially significant, as it supports the likelihood that different ‘fractions’ of natural organochlorines stimulate

different organohalide-respirers. Thus, for effective biostimulation of a given contaminant, the fraction of amended natural organochlorines may be highly important for success. Further research elucidating the difference in niches between different fractions of organochlorines could be beneficial towards developing biostimulation strategies for bioremediation. As qPCR analysis approaches is required for prior sequence data for a specific target gene of interest and this has caused some limitation on the analysis, which this method can only used to target known genes and the possibility of recognizing unknown organisms through this analysis is almost none (Smith et al. & Osborn et al., 2008). For example, when primers Dhc582F and Dhc728R, which targeting for *Dehalococcoides* spp. only, were used for qPCR analysis, results that appeared will only for *Dhc* while other unknown organisms will not show in the result.

The TRFLP method in this study provided valuable insights. The effect of CPO was readily apparent on only a single OTU and possibly a second. These results suggest a rather limited diversity of bacteria were highly stimulated by the CPO-produced organochlorines, which is also concluded from the lack of stimulation of the *Proteobacteria* strains, "*Dehalobium chlorocoercia* DF-1", *Desulfitobacterium* and the Gopher group. This limited range of stimulation could be due to a number of factors, such as the starting microbial community, diversity of organochlorines produced, the physiochemical conditions of the batch microcosms, the limit of electron donor and carbon sources, vitamin concentrations, etc. The diversity of the *Chloroflexi* were

noticeably reduced immediately between Day 0 and Day 7, further showing how the batch microcosm limits the diversity compared to undisturbed sediments. The primary OTU stimulated by CPO produced organochlorines is of a fragment size not predicted from any known isolated strains of the *Chloroflexi*. Thus, this organism may represent a novel organohalide respiring member.

The concentration of PCBs in this research did show an obvious decrease from Day 0, and dechlorination products did appear after Day 103, suggesting that dechlorinators did dechlorinate some PCB. Without better quantitative data, however, determination of rates and extent of dechlorination between treatments cannot be elucidated. Further research is needed to determine whether natural organochlorines can serve as primers for PCB dechlorination. In conclusion, results obtained from qPCR and TRFLP analysis once again proven that organohalide respiring bacteria from *Proteobacteria, Firmicutes* and *Chloroflexi* groups able to be stimulated by stimulation bacteria and existed in uncontaminated environment. Although the stimulation is not significantly shown in all the organisms tested in this research, but it does showed that the *Chloroflexi* diversity is taking most of the stimulating activity. The unknown organisms found in TFRLP analysis is very encouraging for future research because it showed that there are more organohalide respiring bacteria in *Chloroflexi* group other than *Dehalococcoides*- like *Chloroflexi* that are taking part in PCBs dechlorination.

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APPENDICES

Appendix A: qPCR Analysis

Table A1. Logarithmic mean ± standard deviation of 16S rRNA genes in the triplicate microcosms amended with chloroperoxidase (CPO) and those amended with control organic extract (CTRL). Bolded are pairs that are statistically significantly different (P<0.05). Shown are *Firmicutes* groups and *Bacteria*.

		[A]*Bacteria*	[A]*Desulfitobacteria* (DSB)	[B]DSB/Bacteria	[A]*Dehalobacter* (DHB)	[B]DHB/ Bacteria	"Gopher Group"
Day 0	CPO	6.40 ± 0.24	[C]1.75 ± 0.44	[C]-4.81 ± 0.33	[C]1.54 ± 0.07	[A]-4.86 ± 0.18	[D]BDL
	CTRL	6.29 ± 0.06	[C]2.20 ± 0.74	[C]-4.14 ± 0.84	BDL	BDL	BDL
Day 7	CPO	7.23 ± 0.16	BDL	BDL	[C]1.54 ± 0.07	[C]-5.69 ± 0.13	BDL
	CTRL	7.39 ± 0.05	BDL	BDL	[C]1.54 ± 0.07	[C]-5.85 ± 0.07	BDL
Day 14	CPO	7.18 ± 0.05	[C]1.58 ± 0.12	**[C]-5.59 ± 0.08**	1.77 ± 0.12	**-5.41 ± 0.08**	BDL
	CTRL	7.61 ± 0.09	[C]1.61 ± 0.19	**[C]-6.01 ± 0.23**	[C]1.68 ± 0.24	**[C]-5.93± 0.27**	BDL
Day 25	CPO	7.08 ± 0.12	[C]1.54 ± 0.08	[C]-5.53 ± 0.10	2.74 ± 0.5	**-4.33 ± 0.50**	BDL
	CTRL	7.20 ± 0.10	BDL	BDL	[C]1.83 ± 0.32	**[C]-5.36 ± 0.42**	BDL
Day 39	CPO	6.50 ± 0.40	BDL	BDL	[C]1.87 ± 0.53	[C]-4.63 ± 0.56	BDL
	CTRL	7.32 ± 0.40	BDL	BDL	[C]2.17 ± 0.62	[C]-5.15 ± 0.46	BDL
Day 61	CPO	7.00 ± 0.11	BDL	BDL	3.20 ± 0.20	-3.80 ± 0.28	BDL
	CTRL	7.26 ± 0.16	BDL	BDL	2.61 ± 0.69	-4.65 ± 0.76	BDL
Day 82	CPO	7.36 ± 0.10	BDL	BDL	3.10 ± 0.40	-4.26 ± 0.32	BDL
	CTRL	7.28 ± 0.11	BDL	BDL	2.84 ± 0.80	-4.44 ± 0.71	BDL

[A]Values are the logarithmic mean ± standard deviation of 16S rRNA genes per µL of DNA extract
[B]Values are the logarithmic mean ± standard deviation of the ratios of 16S rRNA genes of dechlorinator to 16S rRNA genes of *Bacteria*
[C]At least one microcosm measured below the detection limit (BDL). BDL value used in averages of triplicates
[D]All microcosms measured below the detection limit (BDL)

Table A2. Logarithmic mean ± standard deviation of 16S rRNA genes in the triplicate microcosms amended with chloroperoxidase (CPO) and those amended with control organic extract (CTRL). Bolded are pairs that are statistically significantly different (P<0.05). Shown are *Proteobacteria* groups[A].

		[B]*Sulfurospirillum*	[C]*Sulfurosprillum/ Bacteria*	[B]*Geobacter*	[C]*Geobacter/ Bacteria*	[B]*Desulfovibrio*	[C]*Desulfovibrio/ Bacteria*
Day 0	CPO	[D]BDL	BDL	4.93 ± 0.38	-1.48 ± 0.15	3.46 ± 0.47	-2.95 ± 0.25
	CTRL	BDL	BDL	4.60 ± 0.04	-1.68 ± 0.04	3.31 ± 0.12	-2.98 ± 0.16
Day 7	CPO	4.87 ± 0.11	-2.36 ± 0.06	4.93 ± 0.06	-2.30 ± 0.11	5.08 ± 0.62	-2.15 ± 0.59
	CTRL	5.09 ± 0.26	-2.30 ± 0.25	5.12 ± 0.45	-2.27 ± 0.44	4.99 ± 0.31	-2.40 ± 0.31
Day 14	CPO	5.06 ± 0.22	-2.12 ± 0.26	4.92 ± 0.21	-2.26 ± 0.19	4.74 ± 0.44	-2.44 ± 0.40
	CTRL	5.64 ± 0.14	-1.93 ± 0.13	5.53 ± 0.17	-2.09 ± 0.10	5.37 ± 0.29	-2.25 ± 0.22
Day 25	CPO	4.89 ± 0.31	-2.19 ± 0.29	4.67 ± 0.07	**-2.40 ± 0.09**	5.31 ± 0.67	-1.77 ± 0.69
	CTRL	4.67 ± 0.40	-2.59 ± 0.39	4.57 ± 0.00	**-2.63 ± 0.10**	4.91 ± 0.44	-2.28 ± 0.41
Day 39	CPO	4.58 ± 0.90	-1.92 ± 1.11	4.23 ± 0.53	-2.27 ± 0.48	3.97 ± 0.62	-2.53 ± 0.26
	CTRL	4.49 ± 0.24	-2.83 ± 0.13	4.21 ± 0.49	-3.11 ± 0.81	4.64 ± 0.31	-2.68 ± 0.30
Day 61	CPO	3.73 ± 0.42	-3.27 ± 0.43	4.61 ± 0.14	**-2.39 ± 0.03**	4.37 ± 0.19	-2.63 ± 0.19
	CTRL	4.06 ± 0.54	-3.20 ± 0.40	4.32 ± 0.23	**-2.94 ± 0.23**	4.71 ± 0.03	-2.55 ± 0.18
Day 82	CPO	4.57 ± 0.79	-2.79 ± 0.72	4.83 ± 0.27	-2.53 ± 0.35	4.52 ± 0.25	-2.84 ± 0.28
	CTRL	3.51 ± 0.79	-3.77 ± 0.87	5.02 ± 0.46	-2.27 ± 0.36	4.36 ± 0.18	-2.92 ± 0.09

[A]*Desulfomonile* was also measured as below detection limit for all samples.
[B]Values are the logarithmic mean ± standard deviation of 16S rRNA genes per µL of DNA extract
[C]Values are the logarithmic mean ± standard deviations of the ratios of 16S rRNA genes of dechlorinator to 16S rRNA genes of *Bacteria*
[D]All microcosms measured below the detection limit (BDL)

Table A3. Logarithmic mean ± standard deviation of 16S rRNA genes in the triplicate microcosms amended with chloroperoxidase (CPO) and those amended with control organic extract (CTRL). Bolded are pairs that are statistically significantly different (P<0.05). Shown are selected *Chloroflexi*.

		[A]*Dehalogenimonas*	[B]*Dehalogenimonas/Bacteria*	"*Dehalobium DF-1*"
Day 0	CPO	5.60 ± 0.12	-0.58 ± 0.09	[C]BDL
	CTRL	5.25 ± 0.18	-0.78 ± 0.12	BDL
Day 7	CPO	5.25 ± 0.42	-1.40 ± 0.27	BDL
	CTRL	5.14 ± 0.23	-1.58 ± 0.19	BDL
Day 14	CPO	5.26 ± 0.33	-1.35 ± 0.25	BDL
	CTRL	5.21 ± 0.15	-1.65 ± 0.17	BDL
Day 25	CPO	5.13 ± 0.44	-1.41 ± 0.38	BDL
	CTRL	5.05 ± 0.47	-1.55 ± 0.37	BDL
Day 39	CPO	5.02 ± 0.24	**-1.12 ± 0.29**	BDL
	CTRL	4.93 ± 0.32	**-1.72 ± 0.24**	BDL
Day 61	CPO	5.67 ± 0.41	**-0.92 ± 0.26**	BDL
	CTRL	4.99 ± 0.41	**-1.63 ± 0.23**	BDL
Day 82	CPO	**6.43 ± 0.55**	**-0.60 ± 0.40**	BDL
	CTRL	**5.25 ± 0.11**	**-1.42 ± 0.10**	BDL

[A]Values are the logarithmic mean ± standard deviation of 16S rRNA genes per µL of DNA extract
[B]Values are the logarthmic mean ± standard deviations of the ratios of 16S rRNA genes of dechlorinator to 16S rRNA genes of *Bacteria*
[C]All microcosms measured below the detection limit (BDL)

Table A4. Logarithmic mean ± standard deviation of 16S rRNA genes in the triplicate microcosms amended with chloroperoxidase (CPO) and those amened with control organic extract (CTRL). Bolded are pairs that are statistically significantly different (P<0.05). Shown are *Dehalococcoides*.

		[A]*Dehalococcoides (DHC) 582-728*	[B]*DHC 582-728/Bacteria*	[A]*DHC 1150-1286*	[B]*DHC 1150-1286/Bacteria*	[A]*DHC 1154-1286*	[B]*DHC 1154-1286/Bac*
Day 0	CPO	3.47 ± 0.13	-2.94 ± 0.36	5.16 ± 0.36	-1.25 ± 0.16	4.09 ± 0.15	-2.32 ± 0.32
	CTRL	3.38 ± 0.08	-2.91 ± 0.06	5.08 ± 0.06	-1.20 ± 0.11	4.14 ± 0.14	-2.14 ± 0.09
Day 7	CPO	3.68 ± 0.11	-3.55 ± 0.26	5.91 ± 0.23	-1.32 ± 0.10	4.24 ± 0.31	-2.99 ± 0.18
	CTRL	3.52 ± 0.28	-3.87 ± 0.27	6.09 ± 0.29	-1.30 ± 0.29	4.19 ± 0.18	-3.20 ± 0.18
Day 14	CPO	3.69 ± 0.21	**-3.49 ± 0.18**	5.88 ± 0.54	-1.30 ± 0.52	4.38 ± 0.43	-2.80 ± 0.40
	CTRL	3.66 ± 0.06	**-3.96 ± 0.02**	6.23 ± 0.17	-1.39 ± 0.19	4.33 ± 0.22	-3.28 ± 0.27
Day 25	CPO	3.94 ± 0.24	-3.13 ± 0.26	6.08 ± 0.42	-1.00 ± 0.43	4.27 ± 0.37	-2.80 ± 0.39
	CTRL	3.95 ± 0.20	-3.24 ± 0.11	5.69 ± 0.34	-1.49 ± 0.21	4.00 ± 0.32	-3.19 ± 0.30
Day 39	CPO	**3.77 ± 0.19**	**-2.73 ± 0.38**	5.65 ± 0.10	-1.09 ± 0.08	4.05 ± 0.50	-2.45 ± 0.39
	CTRL	**3.05 ± 0.03**	**-4.27 ± 0.36**	6.14 ± 0.31	-1.18 ± 0.14	4.62 ± 0.18	-2.71 ± 0.40
Day 61	CPO	4.39 ± 0.44	**-2.61 ± 0.45**	6.54 ± 0.24	-0.46 ± 0.36	5.02 ± 0.16	-1.98 ± 0.23
	CTRL	3.84 ± 0.11	**-3.43 ± 0.25**	6.33 ± 0.04	-0.93 ± 0.11	5.13 ± 0.33	-2.13 ± 0.31
Day 82	CPO	4.23 ± 0.53	-3.13 ± 0.48	**6.94 ± 0.29**	**-0.42 ± 0.28**	4.91 ± 0.28	-2.45 ± 0.38
	CTRL	3.72 ± 0.14	-3.56 ± 0.23	**6.29 ± 0.09**	**-1.00 ± 0.10**	4.92 ± 0.06	-2.37 ± 0.15

[A]Values are the logarithmic mean ± standard deviation of 16S rRNA genes per µL of DNA extract
[B]Values are the logarthmic mean ± standard deviations of the ratios of 16S rRNA genes of dechlorinator to 16S rRNA genes of *Bacteria*

Appendix B: qPCR Standard Curve

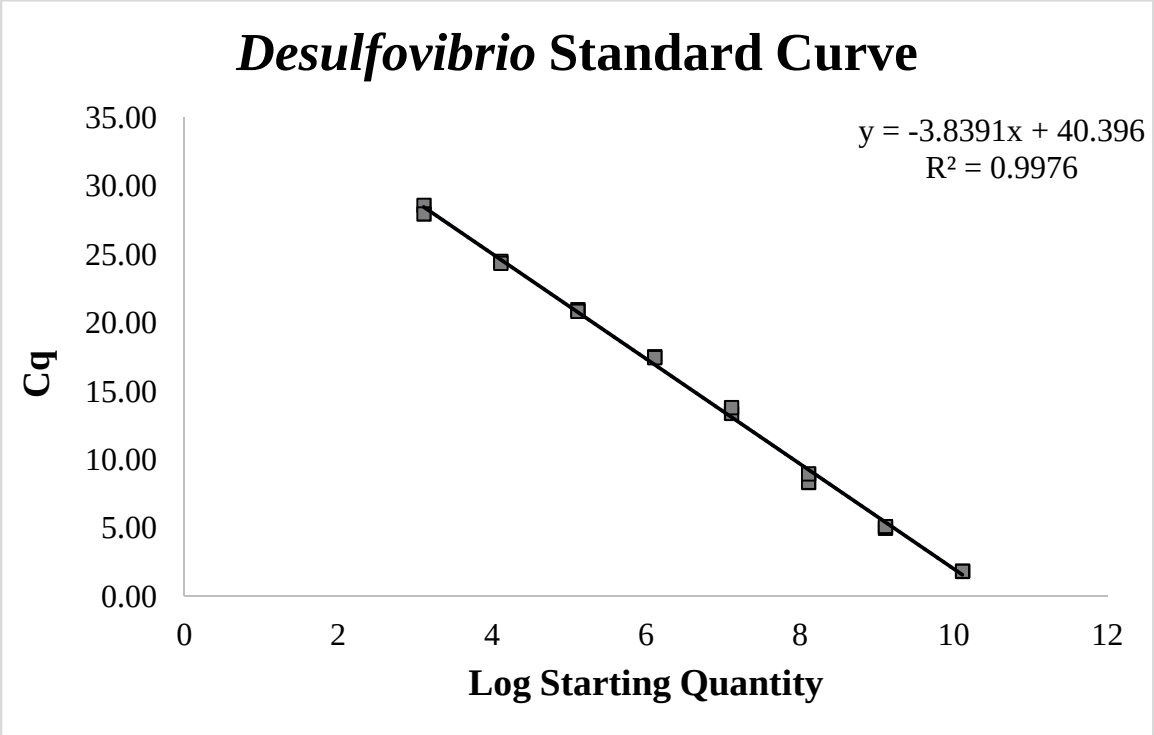

Figure B1. *Desulfovibrio* standard curve plotted with measured Cq values from qPCR against the log of the relative concentration. R^2 is 0.9976, which means log starting quantity values are closely related to Cq values.

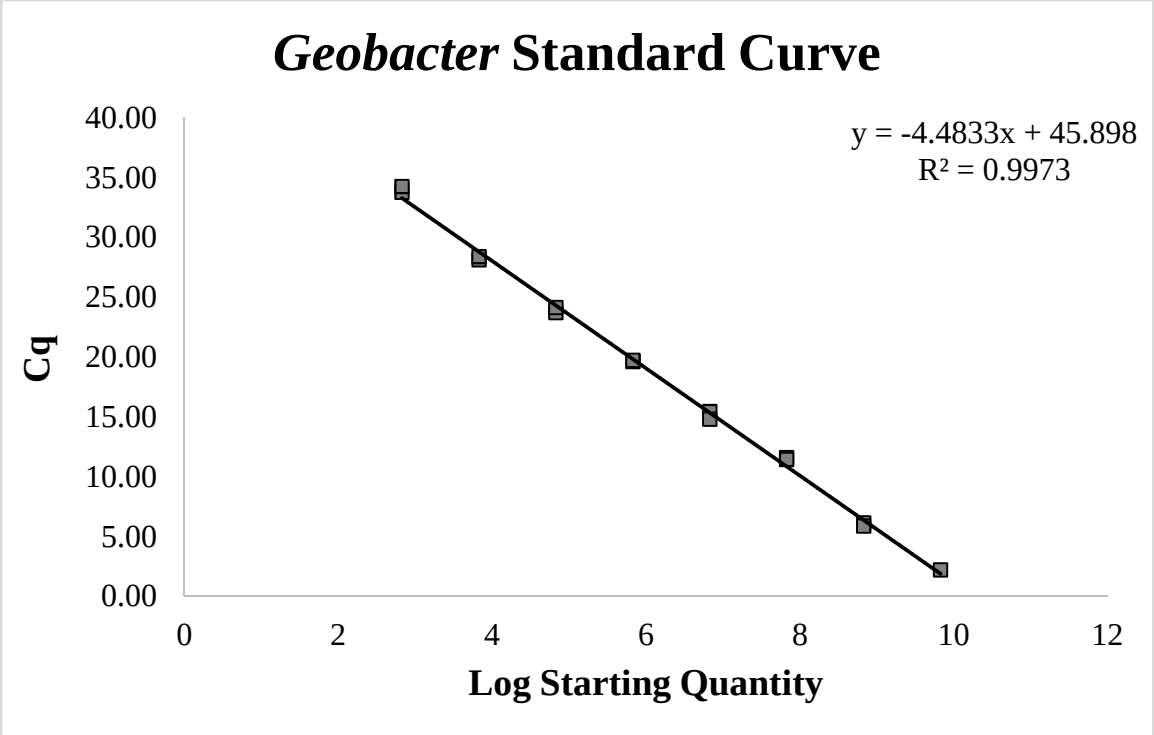

Figure B2. *Geobacter* standard curve plotted with measured Cq values from qPCR against the log of the relative concentration. R^2 is 0.9973, which means log starting quantity values are closely related to Cq values.

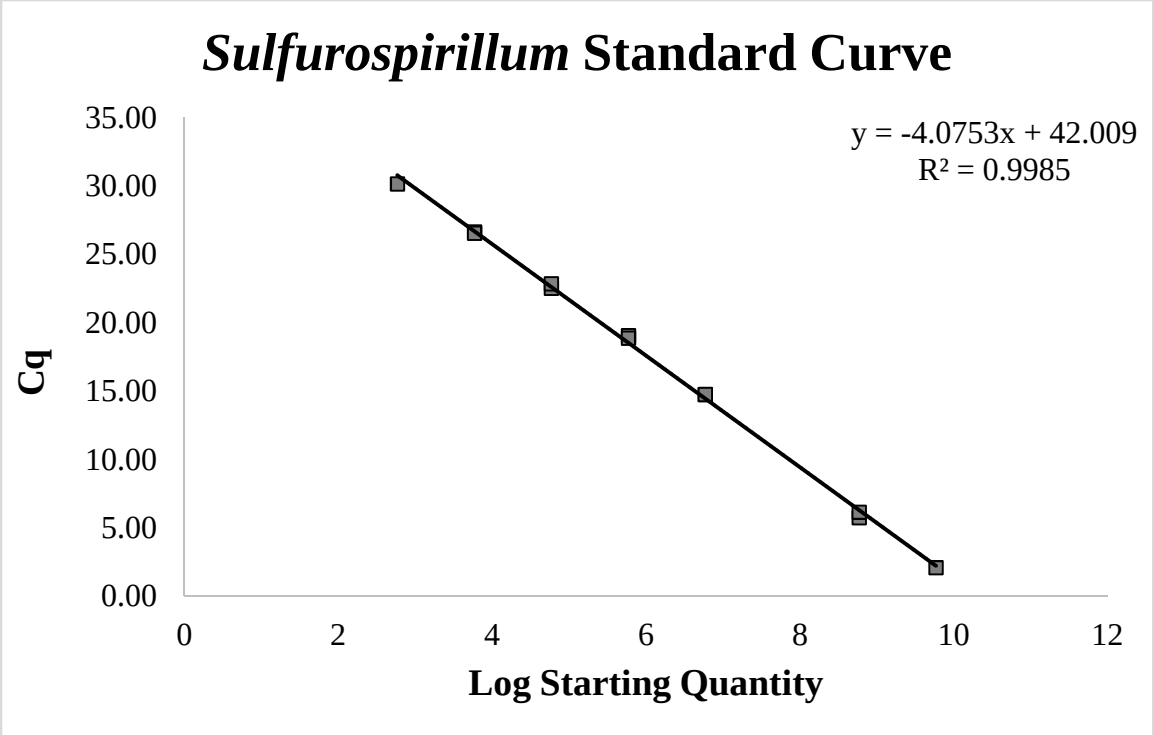

Figure B3. *Sulfurospirillum* standard curve plotted with measured Cq values from qPCR against the log of the relative concentration. R^2 is 0.9985, which means log starting quantity values are closely related to Cq values.

Appendix C: TRFLP Analysis

TABLE C1. Fractions of peak area for samples from the *Chloroflexi* TRFLP analysis for Days 0 and 7. Shown are all bp that represent greater than 5% of the peak area for at least one sample.

	Day 0						Day 7					
Size	CPO Microcosms			CTRL Microcosms			CPO Microcosms			CTRL Microcosms		
53.6	0.012		0.009		0.039	0.029			0.008	0.067		
86.1										0.171		
96.3	0.011	0.010		0.011	0.012	0.011						
153.8						0.015						
175.5												
194.2	0.050	0.060	0.069	0.057	0.058	0.068						
196.3	0.040	0.058	0.061	0.038	0.036	0.050						
214.5	0.045	0.041	0.045	0.048	0.052	0.060						
236.6	0.079	0.061	0.060	0.069	0.046	0.069						
247.9												
261.1												
263.4	0.147	0.112	0.118	0.136	0.115	0.153		0.005	0.025			
264.4	0.126	0.120	0.178	0.114	0.154	0.139			0.006			
267.8	0.051	0.032	0.015	0.033	0.033	0.031	0.005					0.005
273.0												
273.6	0.006						0.011	0.023	0.047	0.139	0.042	0.069
275.7												
276.5	0.088	0.069	0.088	0.078	0.068	0.069	0.195	0.307	0.197	0.165	0.238	0.347
278.0	0.101	0.122	0.114	0.148	0.161	0.063	0.675	0.583	0.581	0.408	0.597	0.452
279.6	0.054	0.051	0.037	0.041	0.035	0.057						
280.8							0.015	0.015	0.018	0.051	0.010	0.015
282.0												
283.3	0.006							0.007				0.006
348.7	0.014	0.013		0.014	0.009			0.011	0.008		0.013	0.013

TABLE C2. Fractions of peak area for samples from the *Chloroflexi* TRFLP analysis for Days 14 and 25. Shown are all bp that represent greater than 5% of the peak area for at least one sample.

	Day 14					Day 25					
Size	CPO Microcosms[A]		CTRL Microcosms			CPO Microcosms			CTRL Microcosms		
53.6							0.048				0.007
86.1											
96.3			0.076				0.055		0.021		
153.8								0.096			
175.5					0.088						
194.2											
196.3						0.016					
214.5	0.009										
236.6											
247.9											
261.1		0.106									
263.4	0.025	0.064	0.013			0.005					
264.4				0.018							
267.8											
273.0						0.013					
273.6	0.090	0.028	0.034	0.034		0.007	0.029	0.116	0.031	0.023	0.025
275.7						0.016	0.050			0.031	
276.5	0.111	0.143	0.097	0.154	0.119		0.039		0.042	0.035	0.019
278.0	0.504	0.569	0.667	0.663	0.793	0.737	0.670	0.788	0.691	0.753	0.821
279.6	0.107										
280.8	0.038		0.011	0.010					0.032		0.013
282.0											
283.3						0.091	0.012		0.058	0.071	0.050
348.7				0.007							

[A] Duplicate microcosms are shown. The third microcosm resulted in outlier data.

TABLE C3. Fractions of peak area for samples from the *Chloroflexi* TRFLP analysis for Days 39 and 61. Shown are all bp that represent greater than 5% of the peak area for at least one sample.

	Day 39						Day 61					
Size	CPO Microcosms			CTRL Microcosms			CPO Microcosms			CTRL Microcosms		
53.6	0.021			0.005			0.037	0.005		0.012		
86.1												
96.3				0.022				0.013		0.035		
153.8	0.012											
175.5												
194.2												
196.3	0.011	0.007	0.026									
214.5	0.014											
236.6	0.008											
247.9	0.009	0.023			0.013	0.016	0.025	0.101	0.007		0.011	0.088
261.1												
263.4	0.196		0.025	0.007	0.010		0.039	0.027	0.037		0.035	
264.4												
267.8												
273.0			0.050									
273.6	0.016	0.005		0.030	0.028	0.010	0.012	0.025		0.029	0.031	0.018
275.7		0.006	0.172			0.007	0.258	0.151	0.726		0.014	0.011
276.5	0.065	0.028		0.041	0.015	0.034		0.021		0.024	0.020	0.023
278.0	0.351	0.744	0.242	0.758	0.752	0.823	0.185	0.528	0.108	0.771	0.677	0.791
279.6			0.168									
280.8		0.010		0.040	0.013	0.011				0.013		0.006
282.0							0.321					
283.3	0.092	0.112	0.204	0.051	0.043	0.025	0.030	0.017	0.013	0.016	0.042	0.005
348.7	0.010		0.010		0.055							

TABLE C4. Fractions of peak area for samples from the *Chloroflexi* TRFLP analysis for Day 82. Shown are all bp that represent greater than 5% of the peak area for at least one sample.

	Day 82					
Size	CPO Microcosms			CTRL Microcosms		
53.6		0.005			0.007	0.022
86.1						
96.3		0.006		0.015		
153.8						
175.5						
194.2						
196.3						0.007
214.5						
236.6			0.009			
247.9	0.021	0.119	0.023		0.172	
261.1						
263.4	0.011	0.029	0.013	0.006	0.033	0.013
264.4						
267.8		0.011				
273.0						
273.6		0.009		0.063	0.022	0.021
275.7	0.134	0.494	0.340			0.034
276.5			0.005		0.116	0.081
278.0	0.419	0.171	0.435	0.827	0.584	0.669
279.6						
280.8			0.036	0.015		
282.0	0.036	0.029				
283.3	0.279	0.026	0.022			0.009
348.7	0.009				0.011	0.016

Appendix D: PCBs Analysis

Table D1. Mean ± standard deviation for each PCB congeners concentration in the triplicate microcosms amended with the chlorinated xanthone 7-dichloro-1,3-dihydroxyxanthone (ClX) and 2,3,4,5-chlorobiphenyl (PCBs), amended with 1,3-dihydroxyxanthone (OHX) and PCB, and one amended with PCB only. Values are mean ± standard deviation for concentration of each PCB congeners as µg per gram

		2,3,4,5-CB	2,3,5-CB & 2,4,5-CB	2,3-CB	2,4-CB & 2,5-CB	2-CB
Day 0	ClX + PCBs	17.26 ± 8.82	BDL	BDL	BDL	BDL
	OHX + PCBs	7.22 ± 0.80	BDL	BDL	BDL	BDL
	PCB only	16.81 ± 9.47	BDL	BDL	BDL	BDL
Day 7	ClX + PCBs	8.72 ± 3.00	BDL	BDL	BDL	BDL
	OHX + PCBs	8.77 ± 0.67	BDL	BDL	BDL	BDL
	PCB only	13.23 ± 6.83	BDL	BDL	BDL	BDL
Day 14	ClX + PCBs	5.62 ± 1.20	BDL	BDL	BDL	BDL
	OHX + PCBs	4.91 ± 3.05	BDL	BDL	BDL	BDL
	PCB only	13.10 ± 6.99	BDL	BDL	BDL	BDL
Day 25	ClX + PCBs	19.61 ± 9.77	BDL	BDL	BDL	BDL
	OHX + PCBs	17.70 ± 7.45	BDL	BDL	BDL	BDL
	PCB only	3.08 ± 1.10	BDL	BDL	BDL	BDL
Day 39	ClX + PCBs	19.99 ± 3.55	BDL	BDL	BDL	BDL
	OHX + PCBs	11.02 ± 8.83	BDL	BDL	BDL	BDL
	PCB only	20.67 ± 10.21	BDL	BDL	BDL	BDL
Day 61	ClX + PCBs	27.62 ± 4.93	BDL	BDL	BDL	BDL
	OHX + PCBs	21.21 ± 2.67	BDL	BDL	BDL	BDL
	PCB only	14.38 ± 8.72	BDL	BDL	BDL	BDL
Day 82	ClX + PCBs	3.14 ± 2.16	BDL	BDL	BDL	BDL
	OHX + PCBs	2.66 ± 0.70	BDL	BDL	BDL	BDL
	PCB only	4.64 ± 0.75	BDL	BDL	BDL	BDL
			Add PCBs			
Day 103	ClX + PCBs	3.18 ± 0.52	BDL	BDL	3.17 ± 0.19	BDL
	OHX + PCBs	10.89 ± 12.29	BDL	BDL	3.36 ± 0.51	BDL
	PCB only	8.86 ± 8.62	1.50 ± 0.02	BDL	1.83 ± 0.23	BDL
Day 132	ClX + PCBs	15.23 ± 9.42	1.79 ± 0.80	2.31 ± 0.14	20.74 ± 15.69	BDL
	OHX + PCBs	15.24 ± 10.35	43.33 ± 45.04	BDL	6.10 ± 4.64	BDL
	PCB only	24.99 ± 4.56	BDL	2.14 ± 0.03	9.02 ± 5.88	BDL

Table D2. Mean ± standard deviation for each PCB congener concentration in the triplicate microcosms amended with chloroperoxidase produced organochlorines (CPO), amended with control organic extract (CTRL) and the autoclaved controls. Values are mean ± standard deviation for concentration of each PCB congeners µg per gram.

		2,3,4,5-CB	2,3,5-CB & 2,4,5-CB	2,3-CB	2,4-CB & 2,5-CB	2-CB
Day 0	CPO + PCBs	27.98 ± 1.72	BDL	BDL	BDL	BDL
	CTRL + PCBs	15.18 ± 6.52	BDL	BDL	BDL	BDL
	Autoclave CPO + PCBs	8.29 ± 3.15	BDL	BDL	BDL	BDL
Day 7	CPO + PCBs	12.49 ± 2.83	BDL	BDL	BDL	BDL
	CTRL + PCBs	6.26 ± 2.30	BDL	BDL	BDL	BDL
	Autoclave CPO + PCBs	9.24 ± 2.58	BDL	BDL	BDL	BDL
Day 14	CPO + PCBs	14.89 ± 7.60	BDL	BDL	BDL	BDL
	CTRL + PCBs	12.36 ± 9.54	BDL	BDL	BDL	BDL
	Autoclave CPO + PCBs	12.12 ± 3.08	BDL	BDL	BDL	BDL
Day 25	CPO + PCBs	18.60 ± 3.95	BDL	BDL	BDL	BDL
	CTRL + PCBs	13.15 ± 6.37	BDL	BDL	BDL	BDL
	Autoclave CPO + PCBs	2.58 ± 0.63	BDL	BDL	BDL	BDL
Day 39	CPO + PCBs	18.77 ± 6.76	BDL	BDL	BDL	BDL
	CTRL + PCBs	14.82 ± 4.40	BDL	BDL	BDL	BDL
	Autoclave CPO + PCBs	17.46 ± 9.38	BDL	BDL	BDL	BDL
Day 61	CPO + PCBs	17.69 ± 5.61	BDL	BDL	BDL	BDL
	CTRL + PCBs	9.03 ± 8.73	BDL	BDL	BDL	BDL
	Autoclave CPO + PCBs	16.08 ± 4.45	BDL	BDL	BDL	BDL
Day 82	CPO + PCBs	5.85 ± 23.35	BDL	BDL	BDL	BDL
	CTRL + PCBs	9.00 ± 2.46	BDL	BDL	BDL	BDL
	Autoclave CPO + PCBs	3.64 ± 3.48	BDL	BDL	BDL	BDL
		Add PCBs				
Day 103	CPO + PCBs	14.20 ± 9.35	2.62 ± 1.30	BDL	2.25 ± 0.71	BDL
	CTRL + PCBs	3.14 ± 0.46	2.06 ± 0.58	BDL	2.71 ± 0.15	BDL
	Autoclave CPO + PCBs	11.91 ± 8.41	3.54 ± 2.07	BDL	1.84 ± 0.37	BDL
Day 132	CPO + PCBs	28.11 ± 19.35	4.13 ± 2.97	11.53 ± 11.48	12.69 ± 8.46	BDL
	CTRL + PCBs	11.19 ± 9.71	2.18 ± 0.40	2.55 ± 0.59	6.00 ± 4.10	BDL
	Autoclave CPO + PCBs	28.76 ± 8.97	BDL	BDL	12.80 ± 7.81	BDL

Appendix E: PCBs Standard Curve

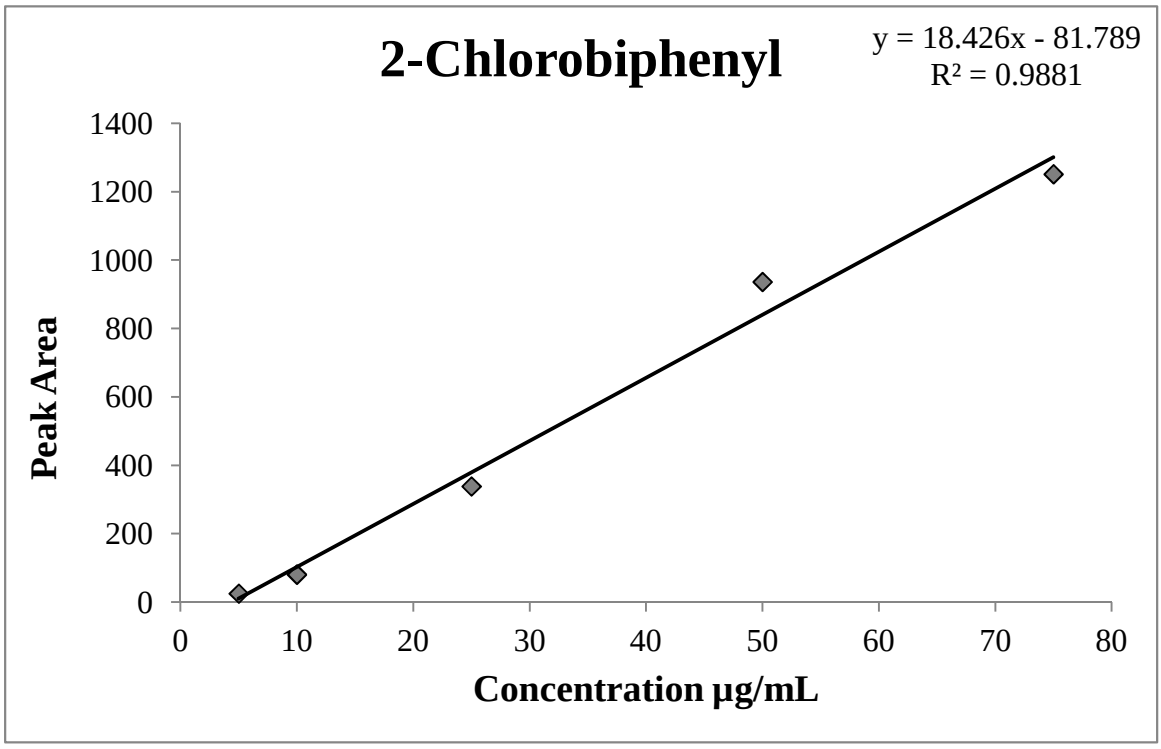

Figure E1. Calibration curve of 2-chlorobiphenyl plotted with peak area appeared at approximately 8.416 min against different concentration in µg/mL. R^2 value is close to one which means concentration of 2-chlorobiphenyl closely related to peak area.

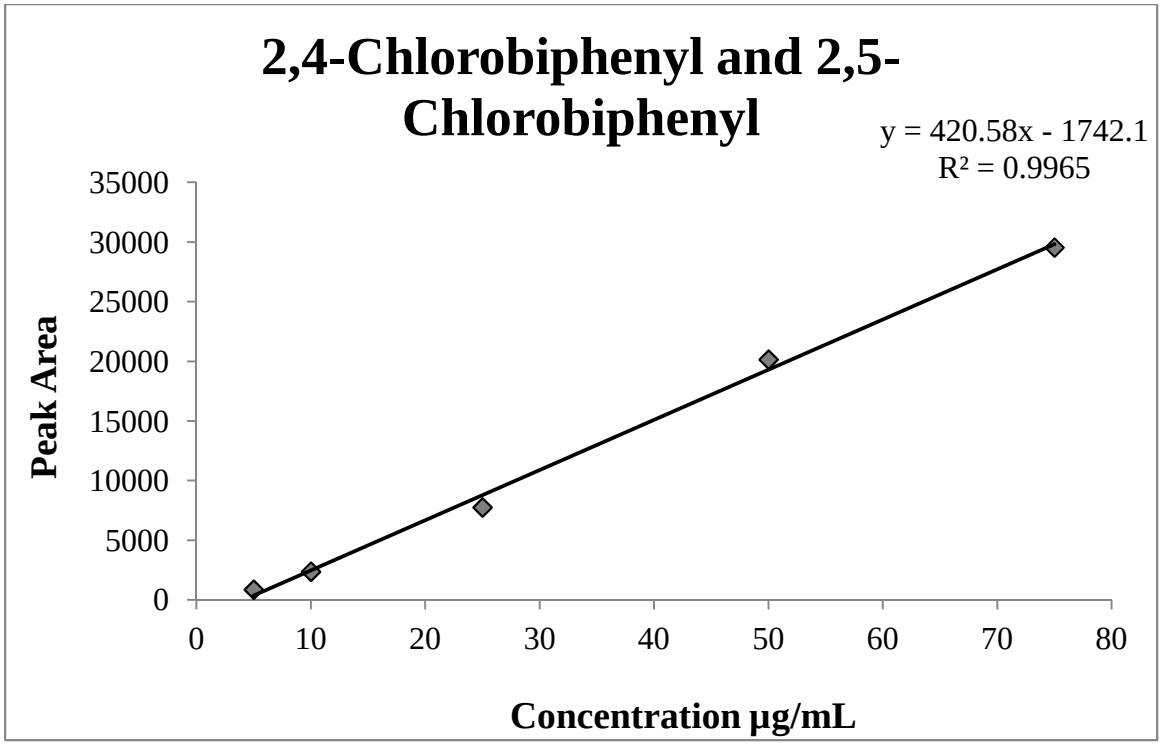

Figure E2. Calibration curve of 2,4–chlorobiphenyl and 2,5-chlorobiphenyl plotted with peak area appeared at approximately 9.4 min against different concentration in µg/mL. R^2 value is close to one which means concentration of 2,4–chlorobiphenyl and 2,5-chlorobiphenyl closely related to peak area.

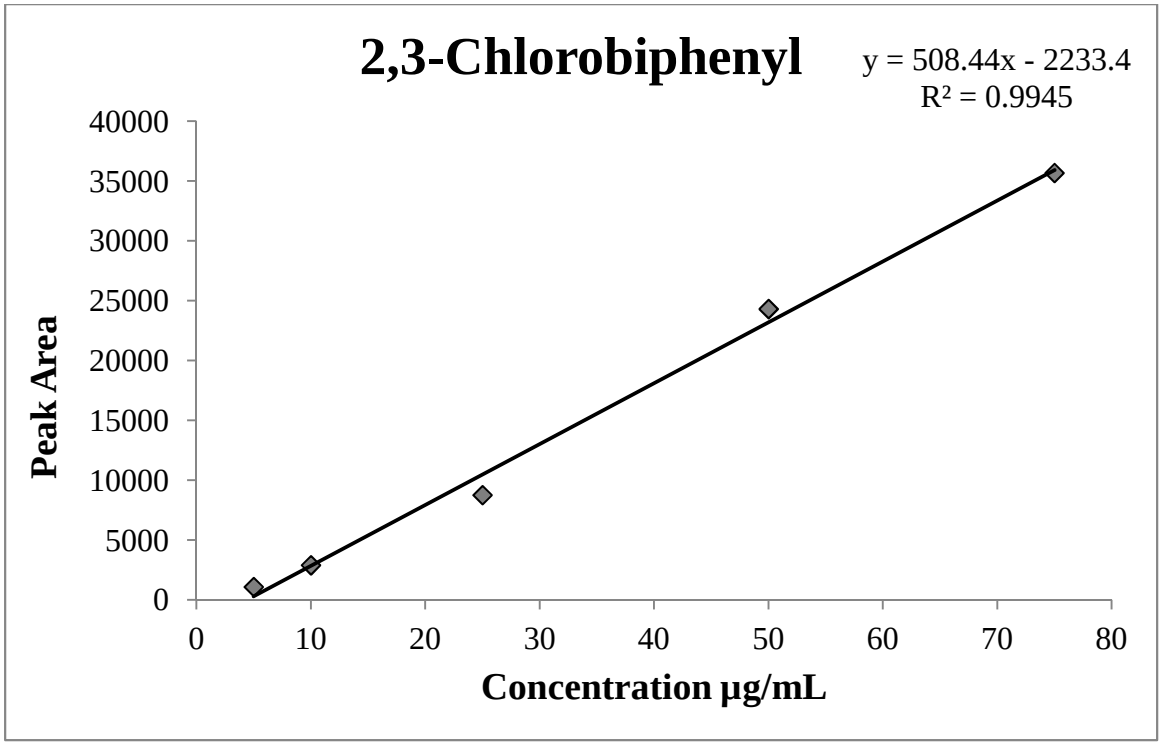

Figure E3. Calibration curve of 2,3–chlorobiphenyl plotted with peak area appeared at approximately 9.6 min against different concentration in µg/mL. R^2 value is close to one which means concentration of 2,3-chlorobiphenyl closely related to peak area.

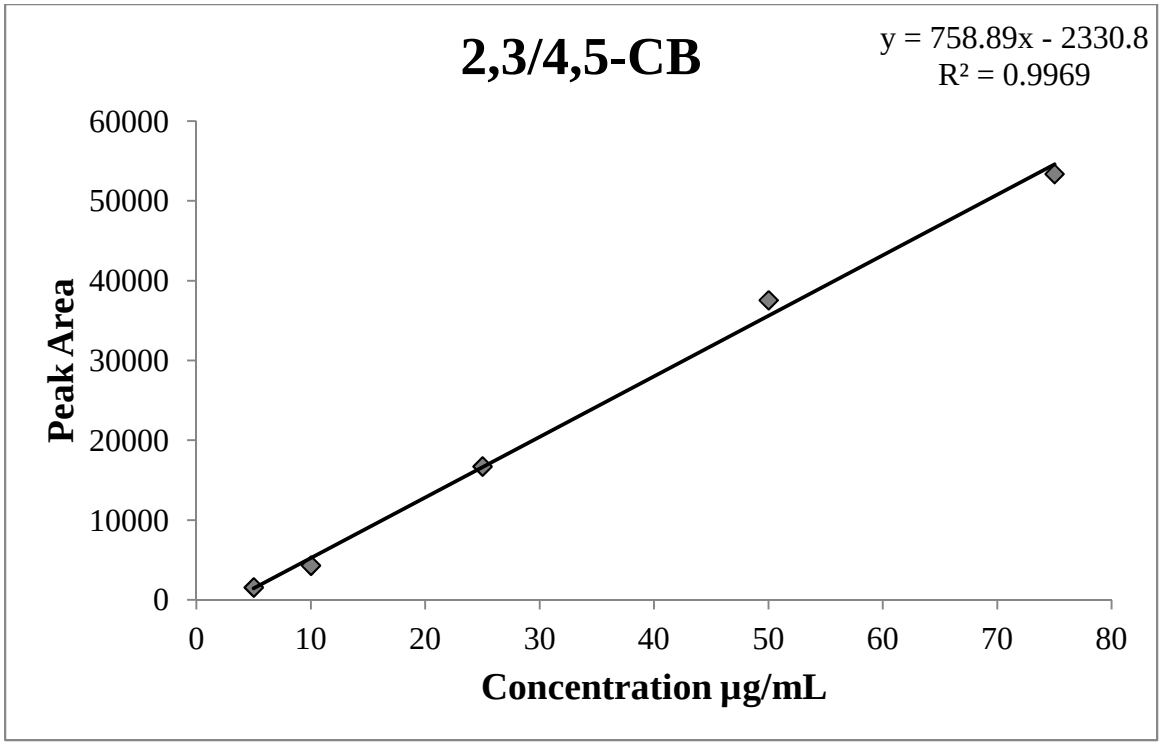

Figure E4. Calibration curve of 2,3,5–chlorobiphenyl and 2,4,5-chlorobiphenyl plotted with peak area appeared at approximately 10.4 min against different concentration in µg/mL. R^2 value is close to one which means concentration of 2,3,5–chlorobiphenyl and 2,4,5-chlorobiphenyl closely related to peak area.

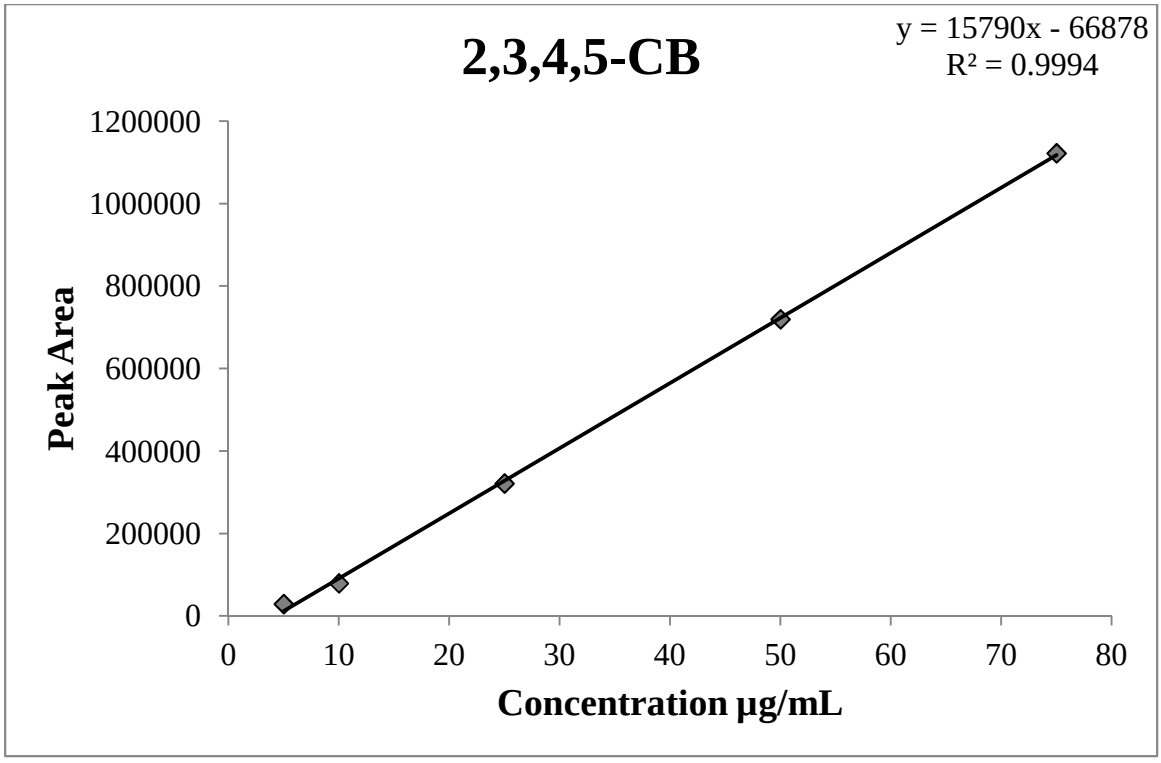

Figure E5. Calibration curve of 2,3,4,5–chlorobiphenyl plotted with peak area appeared at approximately 11.592 min against different concentration in µg/mL. R^2 value is close to one which means concentration of 2,3,4,5–chlorobiphenyl closely related to peak area.

VITA

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