Stimulating Bacteria to Degrade Industrial Chlorinated Solvents

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DEDICATION

This thesis is dedicated to any and every woman of color who is interested in environmental engineering.

ABSTRACT

The widespread use and improper storage of chlorinated solvents in industries like agriculture, dry cleaning, and manufacturing has resulted in these toxic chemicals being released into the environment. In Minnesota, perchloroethylene (PCE) has been identified in 50 to 100 remediation sites. Once in the environment, remediation of these solvents is difficult due to the stability of its carbon-halogen bond and its volatility. Biological dechlorination of chlorinated solvents such as PCE is a well-known method of remediation. Although these methods can be successful, additional work is needed to limit the formation of toxic intermediates that are present due to incomplete dechlorination. Current remediation methods also rely on the addition of an external carbon source, such as methanol or lactate, that acts as an electron donor, and presents an additional cost for these remediation techniques.

Biological dechlorination is performed by a variety of microorganisms. For this study, these microorganisms are referred to as obligate organohalide respiring bacteria (OHRB), facultative OHRB, and hydrolytic dechlorinators. Obligate OHRB perform reductive dechlorination and use chlorinated solvents as their sole terminal electron acceptors and an external carbon source as their electron donor. These bacteria contain reductive dehalogenase (rdh) genes that help facilitate dehalogenation and generate cellular energy. Facultative OHRB can use a variety of electron acceptors and contain rdh genes as well as dehalogenase (dh) genes to facilitate the dechlorination process. These organisms also use chlorinated solvents as an electron acceptor, but can use other electron acceptors, such as Fe(III). Hydrolytic dechlorinators have been found in both anaerobic and aerobic environments and can use a variety of electron donors and acceptors to perform a substitutive dehalogenation catalyzed by hydrolytic dhs.

The work presented in this thesis describes the effect of a substance known as chlorinated natural organic matter (Cl-NOM) on these groups of bacterial dechlorinators. Cl-NOM is derived

from natural organic matter that reacts in the environment with free chloride and reactive oxygen species. Batch reactors with known obligate OHRB were operated with varying levels of Cl-NOM and PCE to determine if Cl-NOM amendment would affect PCE dechlorination. Experiments showed that this amendment did in fact accelerate the dechlorination of PCE; it was unclear whether obligate OHRB grew on Cl-NOM itself or grew on PCE in the presence of Cl-NOM. A continuous flow reactor was also operated to better understand if Cl-NOM addition could enrich facultative OHRB or hydrolytic dechlorinators present in uncontaminated soil. Results showed that two facultative OHRB were slightly enriched during reactor operation. Rdh genes were found at higher gene copies than dh genes, suggesting that both facultative OHRB and hydrolytic dechlorinators were enriched. No known obligate OHRB were detected in the reactor.

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1.1 Chlorinated solvent contamination

The presence and concentration of man-made halogenated solvents at sites within the United States have been well documented (Gribble, 1998), (Major et al., 2002), (McCarty et al., 2020). Historical U.S. production volumes of tetrachloroethylene (PCE) ranged from 547 million pounds in 1982 to 271 million pounds in 1993 to with an estimated total U.S. production volume of less than 1 billion pounds (ATSDR, 2018). The widespread use and improper storage of these chemicals in industries like agriculture, dry cleaning, and manufacturing has resulted in environmental releases that require site remediation. PCE has been identified in at least 949 of the 1,854 hazardous waste sites that were proposed for the EPA National Priorities List (NPL) (ASTDR, 2017). In Minnesota, between 50 and 100 Resource Conservation and Recovery Act (RCRA) sites have been or are contaminated with chlorinated solvents like PCE and trichloroethylene (TCE) (U.S. EPA, 2004). PCE has been detected in groundwater and soil due to improper disposal and release from industrial facilities, with releases of PCE to the soil accounting for approximately 13% of total environmental releases according to the Toxic Release Inventory (TRI) (ASTDR, 2017), and PCE releases to surface water from metal degreasing and dry-cleaning industries accounting for less than 1% of its total environmental releases.

The stability of the carbon-halogen bond makes the fate and transport of solvents such as PCE and TCE a persistent concern. The average solubility of PCE in groundwater is 200 mg/L. Once PCE is present in groundwater, it can partition into the surrounding soil (Log K_{OW} of 3.30) and air (Log K_{OA} of 3.48). Indeed, PCE is expected to volatilize rapidly at the water-air interface and from moist soil surfaces because of its Henry's Law Constant (0.02 atm-m^3/mole). Perhaps as a result, ambient air levels in the United States range from 0.035 to 1.3 ppb (ASTDR, 2017). In Minneapolis, median indoor residential concentrations of PCE were present at 0.4 µg/m^3 (Nicole Nijhuis, 2010). Also, once present in soil and groundwater, vapor-phase PCE can migrate into the

air of homes and buildings via vapor intrusion. If released directly to the air, PCE is expected to exist solely as a vapor in the ambient atmosphere because of its high vapor pressure (18.47 mm Hg, 25°C).

More problematic than widespread and persistent contamination by chlorinated solvents is their negative effect on human health. The most important routes of exposure to PCE are inhalation in the indoor and outdoor air and ingestion of contaminated drinking water. Exposure to these solvents has several adverse effects on the human body, including impaired cognitive performance, autoimmune disease, and multiple types of cancer. The maximum contaminant limit (MCL) for PCE is 5 μg/L (ppb). Long-term exposures in drinking water above the MCL can cause adverse effects to the liver, kidneys, and central nervous system. At levels above $2x10^8$ $\mu g/m^3$ in air, PCE may cause eye irritation and light-headedness and at $1.5x10^9$ $\mu g/m^3$ (1.59 ppm) can cause extreme irritation to the eyes and respiratory tract as well as unconsciousness within 30 minutes.

PCE and TCE, along with other chlorinated solvents, can undergo a variety of reactions (biological and chemical) once released to the environment. In a biologically active environment, such as in the groundwater or soil, diverse bacteria are involved in the biodegradation of chlorinated solvents. The processes that these bacteria perform can be classified into four different groups: anaerobic reductive dechlorination, anaerobic oxidation, aerobic cometabolism, and aerobic assimilation (Mattes et al., 2010), with the process that occurs being controlled by the redox potential and the organisms present. In addition, PCE can adsorb onto organic matter and this adsorption has been shown to follow a linear or Freundlich isotherm. Studies have also shown that PCE is more strongly adsorbed than TCE (Ruffino & Zanetti, 2009). Previous studies have shown that PCE can undergo chemical reactions with oxidants, such as potassium permanganate ($KMnO_4$). The reaction between $KMnO_4$ and PCE can result in the release of chloride and hydrogen ions (Nelson MD, 2001). The chemical and biological degradation of PCE via reductive processes occurs preferentially under methanogenic or sulfate-reducing conditions,

which are commonly found in saturated zones of soil or aquifers (Schaerlaekens, Mallants, Simunek, Genuchten, & Feyen, 1999). Finally, in the atmosphere, PCE can be transformed via photochemically-produced hydroxyl radicals, forming phosgene and chloroacetyl chlorides (Organization, World Health, 1996). Although a variety of transformation pathways exist for these chlorinated solvents, remediation via biological processes is often pursued.

1.2 Cleanup of chlorinated solvents

1.2.1. Current remediation methods

Systems for the removal of chlorinated solvents from the environment consist of *ex situ* treatment (e.g., pump and treat systems, off-site disposal, and incineration) and *in situ* treatment, such as chemical remediation or biological remediation. Pump and treat methods are the most common for cleanup and are advantageous because they can prevent the further spread of contaminated plumes and can remove a wide range of contaminants. Incineration is commonly used because it destroys the contaminant of concern and has the potential to generate heat and power. The most significant disadvantage of these *ex situ* methods is the cost due to either system installation, hazardous waste disposal expenses, or significant energy input for incineration. As previously stated, chemical remediation takes advantage of redox conditions and often involves the addition of chemicals as electron donors. These conditions are not always conducive to efficient remediation. Biological remediation uses microorganisms and additional chemicals to remediate these pollutants. Studies have shown that the addition of electron donor such as acetate has a positive effect on dechlorinating communities (Fennell et al., 2001); however the concentrations needed are relatively high and contribute to a high operational cost. The disadvantages of these methods include cost and the formation of toxic products such as vinyl chloride (VC) because of incomplete dechlorination. Prior research has revealed that microbial communities where sulfate-reducing bacteria and/or iron-reducing bacteria are present were more likely to reduce chlorinated solvents like cis-dichloroethene (cDCE) or VC (Murray et al., 2020).

However, the presence of these communities does not always coincide with PCE plumes. PCE can be difficult to remediate, and remediation efforts do not always result in complete site restoration.

The use of bioremediation to clean up contaminated sites is a well-known remediation solution; nevertheless, the overall cost is high and the ability to achieve complete dechlorination to ethene is rare. The estimated cost for the cleanup of PCE at RCRA sites was approximately $45 billion for 3,800 sites (U.S. EPA. Office of Solid Waste and Emergency Response, 2004). At sites that have undergone bioremediation, there can still be detections of the bacteria that degrade these halogenated solvents when dechlorination has stopped (Gretchen L. W. Heavner, 2019). It is hypothesized that at lower levels of chlorinated solvents, concentrations are no longer high enough to sustain the degrading community. Unfortunately, this can result in contaminant concentrations that are still too high to be protective of human health. Previous studies have shown that dechlorinating communities are maintained when consistently fed with chlorinated solvents (Duhamel et al., 2002), (Major et al., 2002). However, the addition of toxic chemicals would indeed defeat the purpose of remediation. Therefore, a non-toxic electron acceptor that acts as an alternative to a chlorinated solvent is needed to maintain these communities.

1.2.2. Reductive Dechlorination

Current methods for bioremediation are useful; nevertheless, a more cost-effective and reliable approach to biological dechlorination is needed. The most common biological dechlorination processes are reductive dehalogenation and hydrolytic dehalogenation. The bacteria that perform reductive and hydrolytic dechlorination are shown in Figure 1.

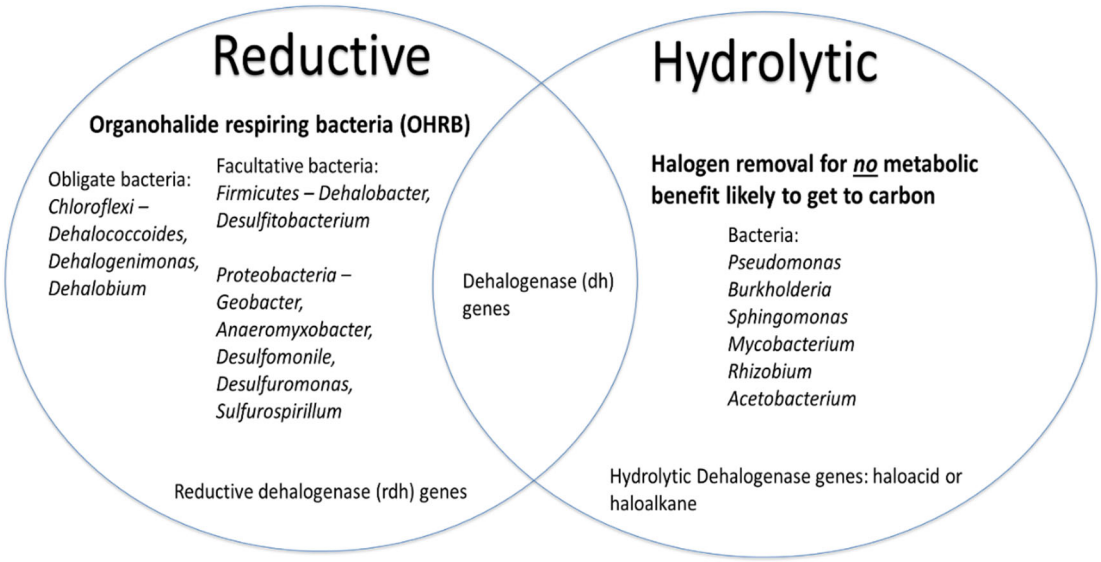

Figure 1 Comparison between reductive organohalide-respiring bacteria (OHRB) and hydrolytic bacteria that perform biological dechlorination.

The reductive dechlorination process occurs via the removal of one or more chlorine atoms on a chlorinated solvent with a transfer of electrons. This process requires an electron donor (hydrogen or acetate) and a terminal electron acceptor to generate energy and waste products. Specifically, organohalide respiration is an anerobic bacterial *respiratory* process that uses halogenated solvents as terminal electron acceptors during electron transport-based energy conversion (Hug et al., 2013). It occurs in anaerobic environments and is performed by organohalide respiring bacteria (OHRB). Many OHRB are slow-growing anaerobes that are typically sensitive to light and pH and are known to thrive in mutualistic anaerobic microbial communities (Maphosa et al., 2012). Obligate OHRB include the following: *Dehalococcoides, Dehalobacter,* and *Dehalogenimonas.* There are also facultative OHRB within the *Firmicutes* and *Proteobacteria* phyla and within this group, *Desulfitobacterium* is the most extensively studied organism that performs dechlorination, while *Geobacter* and *Desulfuromonas* are less studied, but still important for bacterial dechlorination.

OHRB contain reductive dehalogenases (rdhs), which are membrane-bound enzymes that help facilitate dehalogenation of organohalides to generate cellular energy. There are a number of rdhs that take part in dehalogenation that are still undefined (Temme et al., 2019). The limited number of rdhs that have been shown as active during reductive dechlorination are as follows: PceA in the dechlorination of PCE (Alfán-Guzmán et al., 2017) (Rupakula et al., 2013), TceA in the dechlorination of TCE (Molenda et al., 2015), VcrA in the dechlorination of VC (Waller et al., 2005), and TdrA in the dechlorination of trans-dichloroethene (tDCE) (Molenda et al., 2015). Anaerobic reductive dehalogenation is a critical step in bioremediation of chlorinated solvents; however, complete detoxification can require aerobic oxidation of daughter products such as DCE and VC (Coleman et al., 2002).

OHRB have been isolated from environmental samples as well as enriched in laboratory settings to create dechlorinating cultures and consortia. For example, KB1 is a microbial consortium dominated by *Dehalococcoides* and is capable of dechlorinating TCE to ethene (Duhamel M, 2002). ACT-3 is an anaerobic *Dehalobacter*-dominated culture that uses 1,1,1-trichloroethane (TCA) as an electron acceptor and ethanol and lactate as electron donors to produce 1,1-dichloroethane (DCA) and dichloromethane (DCM) (Tang & Edwards, 2013). WBC-2 is dominated by *Dehalogenimonas,* contains the reductive dehalogenase TdrA, and has been shown to be responsible for the hydrogenolysis of tDCE to VC (Molenda et al., 2015). Many other dechlorinating cultures exist, but these cultures are particularly well-known because they have been used in field bioremediation (Jugder et al., 2016).

1.2.3. Hydrolytic dechlorination

Although anaerobic reductive dechlorination performed by OHRB is important, non-respiratory hydrolytic dechlorination processes also contribute to the overall detoxification of chlorinated solvents. Hydrolytic dechlorination is a substitutive dehalogenation process catalyzed by hydrolytic dehalogenases and consists of a hydrolysis step where an active-site carboxylate group attacks the substrate C atom bound to the halogen atom to form an ester intermediate and a

halide ion. The chloride substituent is then replaced by a hydroxyl group derived from water. The dehalogenation process can be executed in the absence of oxygen but not in the absence of water. During the process, dehalogenases catalyze the cleavage of the carbon-halogen bond of organohalogens (Fetzner, 1998) after which the dechlorinated product can hypothetically be used as an electron donor. This process results in the removal of chlorine atoms from an organic backbone with *no energetic benefit* to the organism, other than the liberation of organic carbon that can be used catabolically (Fetzner, 1998). This process usually occurs in aerobic environments, but organisms that perform this process have also been found in anaerobic environments (Temme et al., 2019), (Munro, 2017). Organisms that perform this process can also use electron acceptors such as iron or oxygen or can dechlorinate during fermentation.

There are multiple types of hydrolytic dehalogenase enzymes, including 2-haloacid dehalogenase-like enzymes and haloalkane dehalogenase enzymes. Haloacid dehalogenase activity is said to exist in other enzymes, such as dehydrogenases or hydrolases, which can also be involved in the degradation of polychlorinated biphenyls (Ang et al., 2018). The 2-haloacid dehalogenases are classified into three types based on their substrate specificities: L-2-haloacid dehalogenases, D-2-haloacid dehalogenases, and D,L-2-haloacid dehalogenases. Haloalkane dehalogenases convert haloalkanes to their corresponding alcohols, halides, and protons (Ang et al., 2018) and can break the carbon-halogen bond in halohydrocarbons through a hydrolytic dechlorination mechanism. The first haloalkane dehalogenase identified, DhlA, was from *Xanthobacter autotrophicus* strain GJ10 and utilizes 1,2-dichloroethane as a carbon source (Fetzner, 1998). Both hydrolytic dehalogenase enzymes are found in a variety of organisms, including *Pseudomonas* sp. Strain ADP (Seffernick, 2002), *Rhizobium* sp. Strain PATR, and *Rhodococcus corallinus* (Fetzner, 1998).

1.3. Parameters that affect dechlorination

1.3.1. Electron donor

Enhancement of reductive dechlorination is accomplished through the addition of organic compounds and nutrients to produce favorable reducing conditions and a pool of external electron donors such as acetate, formate, or hydrogen to stimulate OHRB (Fennell et al., 2001). Previous studies have explored the addition of various electron donors (lactate, butyrate, benzoate, and propionate) to determine whether the dechlorination process could be stimulated to proceed past the DCE isomers. When electron donors were depleted, reactors were re-amended with donor to continue the stimulation of OHRB, which was a successful approach. During these experiments, all donors were readily fermented to hydrogen, and ethene was formed from PCE dechlorination (Fennell et al., 2001). Nevertheless, the continuous addition of external electron donor is costly and electron acceptors that compete with chlorinated solvents, such as sulfate or Fe(III), increase the quantity of donor needed for dechlorination. Therefore, the fate of electron donors cannot always be predicted due to these possible competing processes.

It is hypothesized that hydrolytic dechlorinators perform dechlorination to use freed carbon as an electron donor. Therefore, carbon addition via acetate or formate may not improve dechlorination performed by hydrolytic dechlorinators in the same way as it benefits OHRB. No studies have been executed to specifically study the effect of electron donor addition on hydrolytic dechlorination, however.

1.3.2. Electron acceptor

In reductive dechlorination, the chlorinated solvent serves as a terminal electron acceptor, whereas in hydrolytic dechlorination, iron or oxygen are common electron acceptors. Terminal electron acceptors can be dechlorinated slowly and sometimes incompletely. It has been hypothesized that the anaerobic bacteria that use chlorinated solvents as their electron acceptor slow, both with respect to dechlorination and growth, when the chlorinated solvent concentrations

are no longer high enough to sustain them (Temme & Novak, 2020). Adrian et al. determined that the presence of different halogenated aromatic compounds can affect the reductive dechlorination of PCBs, a process described as priming, and that *Dehalococcoides* strains can grow by respiratory dehalogenation of chlorophenols after being primed on halogenated aromatic compounds (Adrian et al., 2007). Ahn et al. observed something similar, dechlorination of a contaminant without the co-amendment of another halogenated compound lagged compared to reactors with co-amendment (Ahn et al., 2005). Priming is a specific type of biostimulation that involves the addition of a chemical, usually chlorinated or brominated, that can be co-degraded with a chlorinated contaminant, with both serving the same metabolic need as electron acceptors. Previous research has used toxic compounds, such as PCBs, as primers to stimulate dechlorination of a different toxic compound of concern. Bedard et al. observed that brominated biphenyls prime microbial reductive dehalogenation of a specific PCB congener (Bedard et al., 1998). The use of chemical primers could help stimulate dechlorinating communities and sustain them enough to achieve complete dechlorination without the formation of toxic intermediate products. Nevertheless, there is a lack of research on the use of less toxic primers and whether they could improve remediation efforts by stimulating the growth and activity of known dechlorinators.

1.4. Chlorinated natural organic matter and its production in the environment

Natural organic matter (NOM) is created over decades from decayed plants and organisms and their metabolism in the environment. It consists of over 5,000 chemical structures and approximately 58% of the mass exists as carbon (Edwards, 2022). Once stable, it can react with natural enzymes and products in the environment. Natural enzymes such as haloperoxidase are found in a variety of plants, bacteria, and fungi. Chloroperoxidase, a type of haloperoxidase, can react with NOM in the presence of low levels of chloride and a reactive oxygen species to form what is known as chlorinated natural organic matter (Cl-NOM) (Reina et al., 2004).

Previous research has shown that certain OHRB can be enriched with Cl-NOM (Lim et al., 2018), Krzmarzick et al., 2013, Temme & Novak, 2020). Specifically, *Dehalogenimonas* and *Dehaloccoides mccartyi* were enriched on Cl-NOM amended microcosms. However, facultative OHRB such as *Geobacter* were not reproducibly enriched on Cl-NOM (Lim et al., 2018). Specific organisms within the *Firmicutes, Chloroflexi*, and *Proteobacteria* phylum have been shown to dechlorinate Cl-NOM, with the dechlorination of Cl-NOM priming the dechlorination of chlorinated solvents (Temme et al., 2019). Additional research showed for the first time that hydrolytic dechlorination processes were involved in the dechlorination of Cl-NOM (Temme & Novak, 2020). It is hypothesized that microorganisms can dechlorinate Cl-NOM to use its carbon as an electron donor if no other donors are present. In this case, a lack of donor in the presence of ample Cl-NOM could stimulate hydrolytic dechlorination. We hypothesize that diverse organisms that respire toxic chlorinated compounds can also respire natural organochlorines and that hydrolytic dechlorination is also likely involved in the dechlorination of Cl-NOM, though it is unclear how the relative concentrations of Cl-NOM may influence this process.

1.5. Summary and research objectives

In this research, we tested the hypothesis that Cl-NOM could aid in bacterial dechlorination, acting as a chemical primer for a microbial community that contained OHRB. We also tested the hypothesis that Cl-NOM could act as an electron donor and be dechlorinated by facultative reductive dechlorinators and hypothesized hydrolytic dechlorinators, resulting in changes in their relative abundance during periods of low carbon exposure (NOM) and high Cl-NOM exposure. The objectives of this research were to determine how hydrolytic and reductive dechlorinators react to the addition of high and low levels of Cl-NOM and whether Cl-NOM acts as a primer for these communities to achieve more effective bacterial dechlorination.

Two sets of experiments were conducted, one with known OHRB in batch reactors and a second with unknown organisms in a low carbon flow-through reactor. The goal of these

experiments was to determine which organisms were stimulated when enriched with Cl-NOM and/or PCE. Over the course of each experiment, the concentrations of chloride, PCE, and its daughter products were monitored. The number of specific dechlorinating bacteria and the number of several functional genes were monitored as well.

Chapter 2 Materials and Methods

2.1 Materials

Seed cultures, batch reactors

Batch reactors were seeded with mixed cultures of known dechlorinators, provided by Dr. Elizabeth Edwards, University of Toronto. To maintain microbial activity, the mixed culture was maintained by feeding two solutions. Solution one contained a ratio of 1:1.67:1.67 electron equivalents of TCE:methanol:ethanol, where TCE was the electron acceptor and methanol and ethanol were the electron donors. Solution two contained filter-sterilized sodium lactate (3.5M) as an additional electron donor. The culture was fed with 100 mg/L of TCE every 30 days. Every 3 months, one third of the anaerobic medium was discarded and re-filled with fresh medium. Anaerobic medium contained a mixture of phosphate buffer, salt solution, trace mineral solution, magnesium sulfate solution, redox indicator, saturated bicarbonate solution, vitamin stock solution, and ferrous sulfide solution, as described in detail elsewhere (Edwards, 1994). Three defined consortia obtained from Dr. Edwards were mixed and added to batch reactors as described below: KB-1, dominated by *Dehalococcoides*, ACT-3, dominated by *Dehalobacter*, and WBC-2, containing a mixture of *Dehalococcoides*, *Dehalogenimonas* and *Dehalobacter* (Grostern & Edwards, 2006).

Seed cultures, continuous flow reactor

Pelican Lake, MN sediment was collected by H. Temme, as described previously, and stored anaerobically (Temme et al., 2019). Samples were stored in an anaerobic chamber (Coy) and the sediment pore water concentrations of chloride was determined by ion chromatography. Results are shown in Appendix A. The sediment was used to seed the continuous flow reactor (CFR) as described below.

Low chloride reduced anaerobic mineral medium (RAMM)

Salt and mineral solution ingredients were purchased from Fisher Scientific. The low chloride reduced anaerobic mineral medium (RAMM) contained the following, mixed into 1 L of ultrapure water (MilliQ, Millipore): 0.27 g $KH2PO_4$, 0.35 g $K2HPO_4$, 0.97 g NH_4Br, 51 mg

$CaBr_2$, 90.5 mg MgBr, 36.3 mg $FeBr_3$, and 10 ml of a filter sterilized (0.2 µm) reduced chloride trace element solution. The reduced chloride trace element solution contained 72.4 mg $MnBr_2$•4 H20, 5 mg H_3BO_3, 3.2 mg CuBr, 1 mg $NaMoO_4$•$2H_2O$, 80 mg $CoBr_2$•H2O, 4.5 mg $NiBr_2$, 5 mg $ZnBr_2$ and 5 mg Na_2SeO_3 in 1 L of ultrapure water (MilliQ, Millipore). Resazurin (2.5 mL of a 1,000 mg/L solution) was added as an indicator of redox potential. The media was pH adjusted to approximately 7.0 with H_3PO_4 (0.5 M). The chloride concentration of the prepared media was approximately 1.5 mg/L (0.04 µM).

Chlorinated natural organic matter (Cl-NOM)

Natural organic matter was extracted from Pelican Lake sediment and chlorinated according to previously established protocols (Temme & Novak, 2020). Chloroperoxidase (CPO) enzyme was purchased as a buffered aqueous suspension from Sigma Aldrich (CAS-No: 9055-20-3). Pelican Lake sediment samples were dried for 8 hours at 150°C and approximately 500 g was packed into 35-mL accelerated solvent extraction (ASE) vials (Thermo Scientific). Extractions were performed with a 50:50 hexane:acetone mixture using a static time of 20 minutes at 100°C and a 5-minute heating time with three extractions per vial, resulting in approximately 600 mL of solvent extract. Solvent extract was evaporated via Rotovap to a reduced volume of approximately 30 mL. The organic extract was then separated into equal volumes (15 mL) and placed into separate 250-mL flasks and suspended in 165 mL of phosphate/salt buffer in each flask (0.1 M K_2HPO_4 and 20 mM KCl, pH~3.0). Hydrogen peroxide (0.1M) was added (150 µL) daily to one flask (CPO-); no chloroperoxidase enzyme was added to that flask. Hydrogen peroxide (0.1M, 150 µL) and aqueous chloroperoxidase enzyme (600 units, 60 µL) were added to the second flask (CPO+). Four total additions of hydrogen peroxide (in both the CPO- and CPO+ flasks) and CPO (in the CPO+ flask) occurred every 20 minutes for 1 hour per day for 4-6 days (Lim et al., 2018). Samples (1 mL) were taken from each flask on Day 1 and Day 5 for chloride measurement via ion chromatography. Chloride incorporation was calculated by subtracting the final chloride concentration in each flask from the initial chloride concentration in each flask. The

difference in the two values was the Cl^- concentration assumed to have been incorporated into the natural organic matter.

After chloride incorporation was confirmed (approximately 0.5 mM), contents from each flask were added to separate C18 columns for cleanup of the organochlorines via solid phase extraction (SPE). C18 columns were added to a vacuum manifold with collection vials placed underneath each column. Contents from the CPO- and CPO+ flasks were added to separate C18 columns labeled CPO- and CPO+; columns were washed with approximately 100 mL each of methanol, acetone, 50:50 acetone/hexane and hexane. Solvents were allowed to sit in the C18 column for approximately 30 minutes, then eluted through each column via vacuum into a single vial containing the mixture of solvents. After column cleanup, solutions were placed in a Rotovap and blown to dryness with forced air. Syringe-filtered Milli-Q water was added to bring the total volume of each solution to 60 mL and solutions were pH adjusted to 7 with sodium hydroxide. The resulting solution contained partially dissolved Cl-NOM or NOM in water, as the substance did not completely dissolve in water.

2.2 Experimental setup

Batch reactor operation

Batch experiments were seeded with a 1:1:1 mixed culture of KB-1, ACT-3 and WBC-2 provided by Dr. Edwards (Molenda et al., n.d.),(Grostern & Edwards, 2006). The mixed culture provided by Dr. Edwards was stored in an anaerobic glovebag (Coy) until use. Additional experiments that did not use this mixed culture are described in Appendix B. Four sets of reactors (A, B, C, and D) were operated in 140-mL serum bottles and amended with varying amounts of carbon (NOM) and Cl-NOM, 100 µM of PCE-saturated Milli-Q water, sterilized RAMM media, and mixed culture, as shown in Table 1. Previously sterilized 140 mL serum bottles were placed in the anaerobic glovebag for batch reactors. The mixed culture was aliquoted (20 mL) into each reactor in the presence of low-chloride anaerobic media. Each reactor was spiked with PCE-saturated media

(10 mL) for an approximate concentration of 100 µM. Batch reactors were placed on a shaker table at room temperature immediately after spiking. Reactor headspace consisted of 40 mL of 96% N_2/4% H_2 from the anaerobic glovebag (Coy). Headspace measurements were taken within two hours of spiking to allow contents within the reactor to equilibrate. The PCE concentration and the concentration of the PCE daughter products TCE and DCE were monitored in all reactors via headspace and quantified via gas chromatography. Liquid from the reactors was sampled periodically (1.5 mL) and centrifuged for 10 min at 8,000 g. The supernatant (0.5 mL) was discarded, and the pellet and remaining solution were stored with 0.5 mL of CLS-TC buffer solution (MP Biomedicals) at -20°C until DNA extraction. The positive control, Group D, consisted of six reactors. After sufficient dechlorination of PCE, contents of the six reactors were homogenized in the anaerobic glovebag and aliquoted for the next phase of experiments into Groups A and B.

Table 1 Reactor amendments

Set	Set Name	NOM vol (µL)	Cl-NOM vol (µL)	RAMM media (mL)	Mixed culture (mL)
A	Low Cl-NOM[b]	0.25	0.005	69.745	20
B	High Cl-NOM[a]	0.005	0.75	69.245	20
C	Abiotic control	--	--	90	--
D	Positive control	--	--	70	20

[a] based on the quantity of chloride consumed during Cl-NOM generation, this is estimated to be an equivalent amendment of 375 µmol of chloride added to reactors

[b] based on the quantity of chloride consumed during Cl-NOM generation, this is estimated to be an equivalent amendment of 2.5E-6 µmol of chloride added to reactors

Continuous flow reactor operation

A CFR was operated May 2020 to January 2022. The CFR was maintained in an anaerobic chamber containing 96% N_2 and 4% H_2 (Coy Laboratory Products), fed with sterilized, pH-adjusted low chloride RAMM media at an influent flowrate of 0.04 mL/min and consistently amended with 0.03% v/v Cl-NOM. Additional discrete Cl-NOM amendments above this quantity occurred over the course of reactor operation, as shown in Table 2. Pelican Lake sediment was used to seed the reactor, and additional sediment was added periodically over the course of

operation, as shown in Table 2. The reactor was operated with a retention time of approximately 5 days. Chloride samples were collected weekly over the course of reactor operation to determine whether certain bacteria were actively dechlorinating Cl-NOM. Aqueous (12 mL) samples were collected weekly from the influent, effluent, and the reactor tank itself. Reactor tank samples were centrifuged for at 8,000 g for 10 minutes. The sample supernatant (approximately 7 mL), along with samples taken from the influent and effluent tanks were used to measure chloride via ion chromatography. The remaining pellet (approximately 5 mL) from the reactor tank was stored at -20°C for later DNA extraction.

Table 2 Cl-NOM added to Continuous Flow Reactor

Day	**Cl-NOM (% v/v)**	**Mass (g) Pelican Lake sediment**
0	0.03	400
191	--	300
289	0.3	400
409	0.3	--
478	0.3	--
495	0.2 NOM 0.3 Cl-NOM	--
587	0.032	--

2.3 Sample analysis

DNA analysis

Samples from the batch reactors, which were primarily aqueous in nature, were extracted with the FastDNA Extraction Kit (MP Biomedicals), according to the manufacturer's protocol, also producing 100 µL of DNA extract. The DNA extract from the batch reactors was analyzed by quantitative polymerase chain reaction (qPCR) with primers developed from previous studies to quantify the total number of *Dehalobacter, Dehalococcoides, Dehalogenimonas,* and 16S rRNA genes (Table 3).

Frozen biomass samples from the CFR were extracted with a FastDNA Soil Extraction Kit (MP Biomedicals) according to the manufacturer's protocol, generating 100 µL of DNA extract. The DNA extract from the CFR was analyzed by both quantitative polymerase chain reaction (qPCR) and Illumina sequencing of the 16S rRNA gene, as described below. Primers previously developed (as shown in Table 3) were used to quantify the number of hydrolytic and reductive

dehalogenase genes, the total number of Bacterial 16S rRNA genes, and the total number of *Geobacter, Desulfitobacterium and Desulfuromonas* in CFR samples.

The total number of 16S rRNA genes was quantified in all samples with general bacterial primers targeting the V3 region of the 16S rRNA gene (Muyzer et al., 1993). The qPCR mixture (20 µL) contained 1X EvaGreen Mastermix (Bio-Rad Laboratories), 100 nM of each primer, 1 mg/L BSA, and 1 µL of undiluted DNA extract. The number of gene copies in each sample was determined with a standard curve of tenfold dilution standards ranging from 10^{10} to 10^{0} for total *Bacteria* 16S rRNA genes and 10^{8} to 10^{2} for all other target organisms. Standards were purchased as G-blocks from Integrated DNA Technologies using sequences found in Table 3. A melting curve analysis was completed at the end of each run for quality control. Amplification efficiency and detection limit are shown in

Table 4.

Table 3 Primer and standard sequences of dechlorinators and dechlorinating genes for qPCR

Target gene Sample origin	**Genbank Accession Number or Standard Sequence**	**Primer Sequence**
Bacteria 16S CFR, Batch reactors	KR190116	338F – CCTACGGGAGGCAGCAG 518R - ATTACCGCGGCTGCTGG
Dehalococcoides ethenogenes Batch reactors	AY882434	Dhc385F – GGGTTGTAAACCTCTTTTCAC Dhc692R – TCAGTGACAACCTAGAAAAC
Dehalobacter Batch reactors	DQ250129	Dhb477F – GATTGACGGTACCTAACGAGG Dhb647 R – TACAGTTTCCAATGCTTTACG
Dehalogenimonas Batch reactors	JQ994266	Dhg273F – TAGCTCCCGGTCGCCCG Dhg537R – CCTCACCAGGGTTTGACATGTTAGAAG
Geobacter lovleyi CFR	AY914177	Geo196F – AATATGCTCCTGATTC Geo535R – TAAATCCGAACAACGCTT
Desulfitobacterium CFR	X95972	Dsb406F – GTACGACGAAGGCCTTCGGGT Dsb619R – CCCAGGGTTGAGCCCTAGGT
Desulfuromonas CFR	AF357915	DsfF – AACCTTCGGGTCCTACTGTC DsfR – GCCGAACTGACCCCTATGTT
PelicanRdh CFR	GGGGCCGATCGGGTGGGCATCACGCGCCTGAACCC GCTCTGGATCTACACGCACTGGGGGATGCAGAACGT CCACTACTCCGGGCGCGGCCCAGGCGGGCGACCCGAT CGACATccccccGAGTACCAGACGGTGATCGTCATGA	F –GCTCGCCACCTTCATTACT R – GCCGTTCCGTCCCATTT

	TCCACCGCATGGATTACGACGTGATCCTGAGGTCGC CGGCGGTCGAGCATGAAACCGACATCGGGTACTCCA AGGCCGCCTGGAGCGCGGCATCGCTCGCCACCTTCA TTACTGAACTTGGGTACAAGGCCATCCCCGCGTGCA ACGAGCTCGGCATCAGCATCGCCATGGCGGTCGATG CCGGCCTTGGCGAAATGGGACGGAACGGCCAGCTG	
Pelican2Haloacid-dhg CFR	ATGATCTTGGGCAGGTCGGCAAGCGCGGAGATTCGA AAGTCGGGTTCCATCCCGAACTCGTCCATTTGCATG CGCAGCGCCCTGAACATCGACAGCGGGCGCACGAG ATCGGCTTTCGCAAGCTCGGCAGCCATTGCTTGCGG CGTCACGCGTTCGATCCATGCCACATTCAGGCCAAA GGCTTTCGCGCCGCAGGCGTCGAACGGATTGGACGA CACGAACAGCACCTCATGCGGCTTTACGCCGAGATT CGATTCAATCAGTTCGTAGGTTCGCGGCGACGGCTT GAAGGTCTTCGTTGAGTCGATGCTGATGGTCGCGTC	F – CGATCCATGCCACATTCA R – GCGAACCTACGAACTGATT

Table 4 Annealing temperature, detection limits and efficiency of qPCR for each organism

Target rRNA gene	**Annealing Temperature (°C)**	**Detection Limit (copies/mL)**	**Efficiency (%)**	**Source**
Bacteria 16S	55	$1x10^1$	101.2	(Muyzer et al., 1993)
Geobacter lovleyi	50	$1x10^2$	79.99*	(Amos et al., 2007)
Desulfitobacterium	58	$1x10^2$	93.62	(Smits et al., 2004)
Desulfuromonas	58	$5x10^2$	87.74	(Löffler et al., 2000)
Dehalococcoides	55	$5x10^1$	77.98**	(Grostern & Edwards, 2006)
Dehalobacter	62.5	$5x10^1$	87.15	(Grostern & Edwards, 2006)
Dehalogenimonas	59	$5x10^0$	90.08	(Manchester et al., 2012)
PelicanRdh	59	$1x10^1$	89.45	(Temme et al., 2019)
Pelican2Haloacid-dhg	56	$1x10^1$	93.61	(Temme et al., 2019)

*Amplification efficiency of *Geobacter lovleyi* is in line with previous studies (Amos et al., 2007).
**Primers used for these analyses have been shown to have a low amplification efficiency but also a low detection limit (Major et al., 2002), which was beneficial for these experiments.

16S Illumina sequencing

For bacterial community analysis, Illumina sequencing (300 bp) was completed on the V4-V6 region of the 16S rRNA gene. Amplification and sequencing were completed at the University of Minnesota Genomics Center (UMGC). Analysis of the single end sequencing reads was completed with Quantitative Insights into Microbial Ecology (QIIME) through the Minnesota Supercomputing Institute (MSI). Forward reads were trimmed to 200 base pairs and reverse reads were trimmed to 160 base pairs. Forward and reverse reads were analyzed separately for each sample. Sequences with a Q score below 30 were removed. De novo OTU classification was performed using QIIME and taxonomy was assigned based on the Silva-138-99 database. The

V4-V6 was amplified with primers 515F-GTGCCAGCMGCCGCGGTAA and 806R-GGACTACHVGGGTWTCTAAT.

2.4 Analytical methods

Chloride

Samples taken from the continuous flow reactor were filtered through a 0.45 µm hydrophilic filter, diluted 1:1 with ultra-pure (MilliQ, Millipore) water, then injected into a Metrohm 930 Compact IC Flex with an eluent of carbonate buffer (3.2 mM Na_2CO_3 and 1.0 mM $NaHCO_3$). The flow rate of the eluent was 0.7 ml/min. The detection limit for chloride was 0.01 mg/L. Chloride in batch reactors was estimated based on PCE, TCE, and cis-DCE concentrations (Appendix C).

Chlorinated compounds

Perchloroethene (PCE), trichloroethene (TCE) and cis-dichloroethene (cDCE) were purchased neat from Sigma Aldrich. PCE, TCE, and cDCE were measured via headspace injection (5 µl) onto an Agilent HP-6890 gas chromatograph equipped with a micro-electron capture detector (GC-µECD). Standards were prepared in the same way as the cultures, with different known quantities of PCE, TCE, and cis-DCE added to 90 ml pure water (milli-Q, Millipore) in sealed 140-ml serum bottles. The GC method consisted of a constant oven temperature at 50°C, a split ratio of 90:1 and a split flow of 72 mL/minute with an injector temperature of 210 °C. All possible isomers of DCE were measured by this method (1,1-DCE, *trans*-DCE, and *cis*-DCE) but only *cis*-DCE was detected. The method detection limits for PCE, TCE, and cis-DCE were 1 µM, 0.1 µM, and 1 µM, respectively.

2.5 Statistical analysis

Paired and unequal variance T-tests were completed to determine whether dechlorination rates between Groups A, B, and D were statistically significant. Paired and unequal variance tests were also completed on CFR data to determine whether total counts of bacteria and rdh and dh genes were statistically different from each other during different periods of CFR operation – start up, poor dechlorination, and good dechlorination. Results were determined to be statistically

significant if the calculated P-value was less than 0.05. The Shannon index, a quantitative measure of community richness, was calculated using QIIME for sequencing data from the CFR. In addition to the Shannon index, the Bray-Curtis distance was also quantified via QIIME to visualize community dissimilarity (Appendix D).

Chapter 3 Results

3.1 Reductive dechlorination in Batch Reactors

Reductive dechlorination of PCE

Results of the initial dechlorination experiment in the absence of Cl-NOM and NOM are shown in Figure 2. PCE concentrations decreased to approximately 50 µM from 100 µM within 1 day of its addition and TCE formation was seen within 1 day. PCE concentrations decreased to as low as 12.48 $\pm$ 7.84 µM over the course of the experiment.

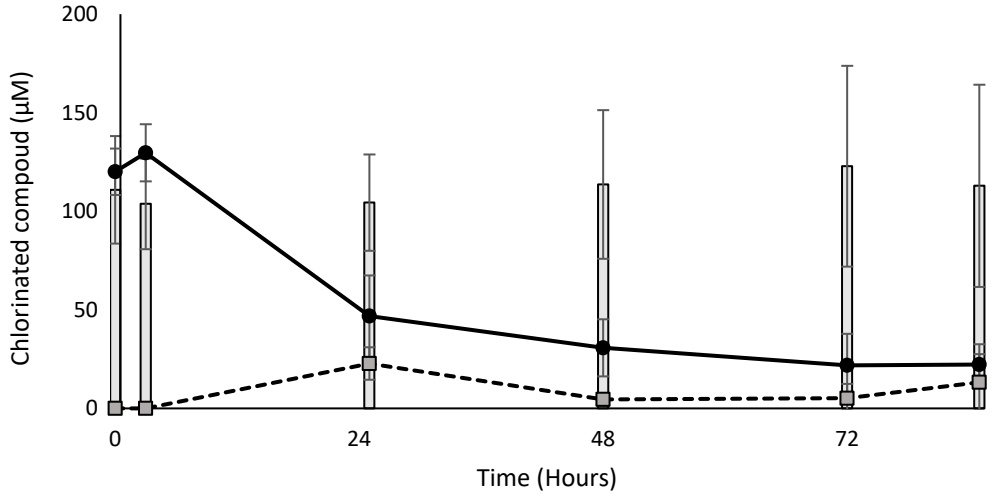

Figure 2 Dechlorination of PCE in group D (●, solid lines) *is achieved after approximately 80 hours in all 6 reactors. The abiotic control shown via bar graph maintained PCE levels around 100 µM throughout the experiment, indicating no abiotic dechlorination. TCE (*■, dashed lines*), a daughter product of PCE, was produced up to 30 µM. Dichloroethene isomers were not detected; VC and ethene could not be detected using our GC method.*

PCE was dechlorinated with the concomitant growth of Bacteria and the OHRB - *Dehalococcoides, Dehalogenimonas,* and *Dehalobacter* (Figure 3). In fact, the differences in initial and final concentrations of all organisms were statistically significant ($P<0.05$). This suggests that these OHRB were able to use PCE as an electron acceptor for growth.

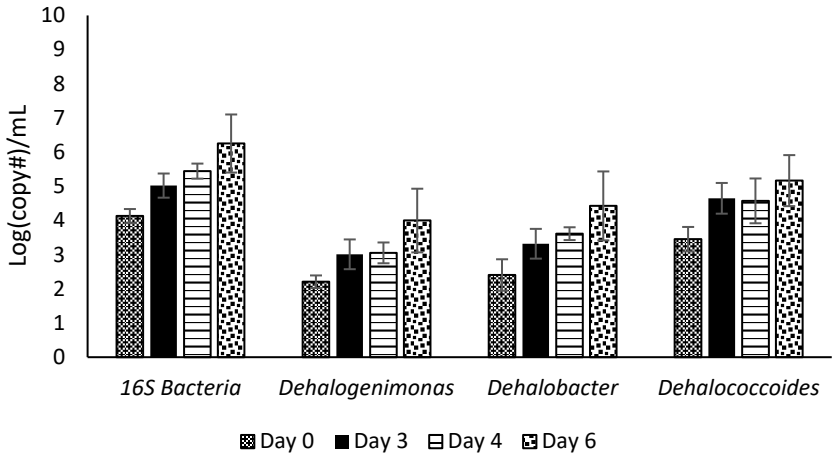

Figure 3 Growth of 16S Bacteria and OHRB over the course of reductive dechlorination of PCE without Cl-NOM or NOM.

Reductive dechlorination of PCE with Cl-NOM

Figure 4 shows dechlorination of PCE after amendment with NOM and Cl-NOM. Panel A shows results of reactors amended with low Cl-NOM, where Panel B shows results for high Cl-NOM. TCE was detected within 1 day and cis-DCE within 3 days, however, cis-DCE formation lagged for reactors amended with low Cl-NOM. Concentrations of PCE were seen as low as 3.59 ± 1.42 µM and 1.88 ± 0.21 µM for low Cl-NOM and high Cl-NOM amendment, respectively, approximately 1 order of magnitude lower than experiments without Cl-NOM added.

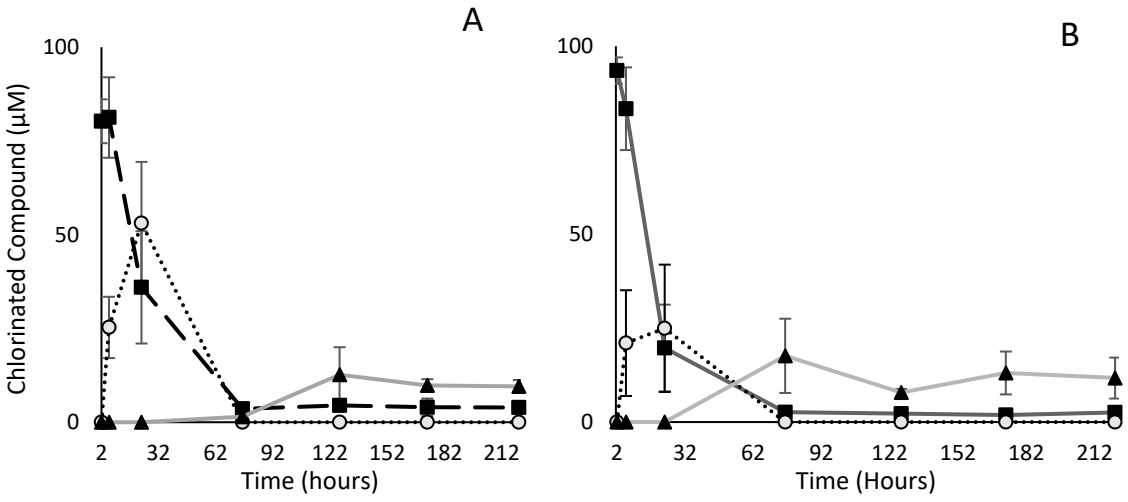

Figure 4 Mixed culture amended with low Cl-NOM in Panel A and high Cl-NOM in Panel B and PCE (■), *TCE* (○), *and cis-DCE* (▲) *concentrations over time.*

Table 5 shows the dechlorination rates for experiments without NOM and Cl-NOM compared to experiments with high and low Cl-NOM added. From these results, we can see that the addition of Cl-NOM accelerates the rate of dechlorination of PCE. In fact, when comparing dechlorination in reactors to which no Cl-NOM was added to those to which low Cl-NOM was added, dechlorination rates for PCE were not statistically different (P = 0.132). Nevertheless, when comparing dechlorination with no Cl-NOM amendments to that with high Cl-NOM added, results were significantly different (P = 0.016).

Table 5 Comparison between first order dechlorination rates and apparent chloride released per change in log copy number of OHRB for experiments with and without Cl-NOM and NOM during the reductive dechlorination of PCE.

Amendment	**First-order dechlorination rate (hr^{-1})**	**Apparent [Cl^-] released/Change in log(copy# of total bacteria) (mM/△log(copy#)**	**Apparent [Cl^-] released/Change in log(copy# of OHRB) (mM/$\Sigma_{\triangle log(copy\#)}$)**
D: no Cl-NOM or NOM	0.027 ± 0.01	0.212 ± 0.028	0.085 ± 0.011
A: low Cl-NOM, high NOM	0.038 ± 0.01	0.247 ± 0.027	0.125 ± 0.011
B: high Cl-NOM, low NOM	0.054 ± 0.009	0.188 ± 0.021	0.079 ± 0.008

Figure 5 shows an increase in total 16S rRNA genes for Bacteria and OHRB between the initial and final measurements of all reactors. When comparing this increase in reactors with low Cl-NOM added to that in reactors with high Cl-NOM added, the difference was not statistically different (P>0.05). However, when comparing the gene copy increase in reactors with low Cl-NOM addition to reactors with no Cl-NOM addition, results were statistically different for all bacteria (P<0.05), except *Dehalococcoides* (P = 0.09). So, we can say with 90% confidence that Cl-NOM addition seems to benefit dechlorination via obligate OHRB, but it is unclear what amount is needed.

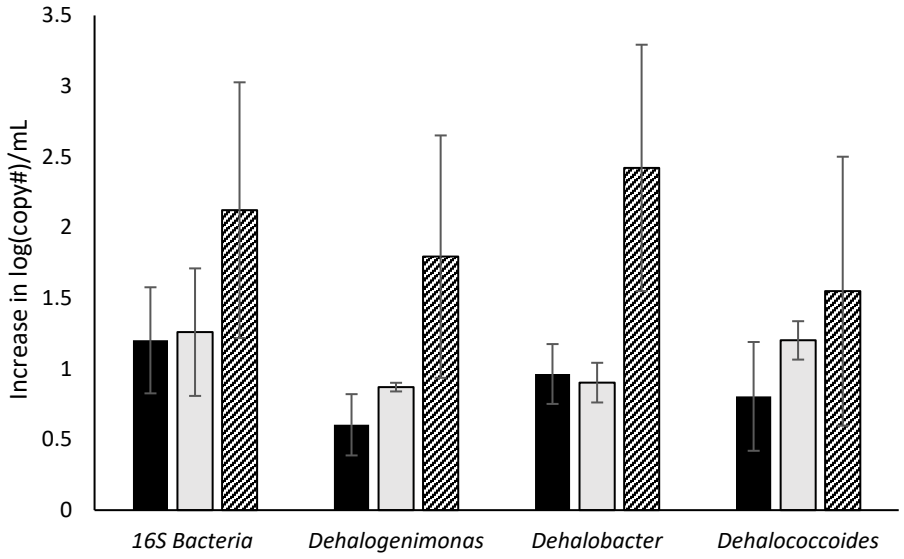

Figure 5 Increase in the log(copy#) of total 16S rRNA genes for Bacteria and OHRB for initial and final samples from Group A (■)- amended with low Cl-NOM, Group B (□) - amended with high Cl-NOM, and Group D (▨)– amended with no Cl-NOM.

3.3 Dechlorination in a Continuous Flow Reactor

Results from qPCR

Figure 6 shows the total number of 16S Bacteria rRNA gene copies as well as the free chloride concentration from the dechlorination of Cl-NOM over time. Days 0-79 are referred to as the Startup phase. We identified a period of poor dechlorination over days 80-225 and good dechlorination over days 225-614. As shown in Table 2, fresh sediment was amended on Day 191.

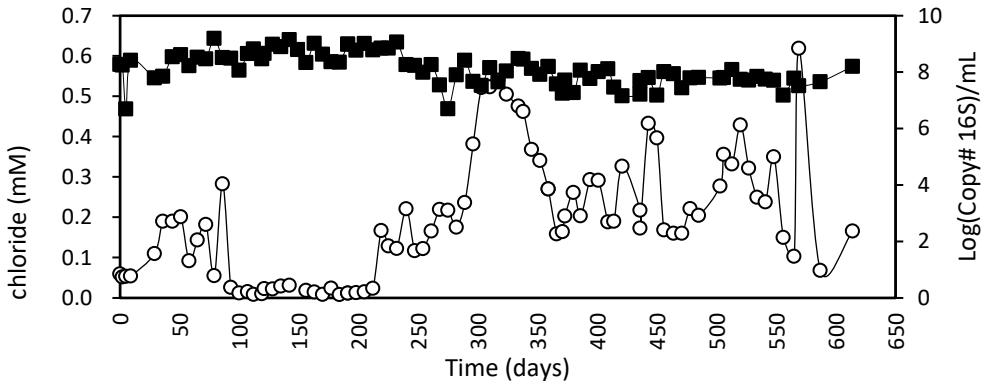

Figure 6 The concentration of chloride (○) is shown over time as result of dechlorination of chlorinated natural organic matter (Cl-NOM) for the same time series as the total number of Bacterial 16S rRNA genes (■) during the operation of the continuous flow reactor (CFR).

Interestingly, the total number of 16S rRNA genes for Bacteria decreased significantly during the good dechlorination period (Figure 7) ($P<0.05$). This could be because Cl-NOM is toxic or inhibitory to some bacteria, though more work is needed to better understand this decrease.

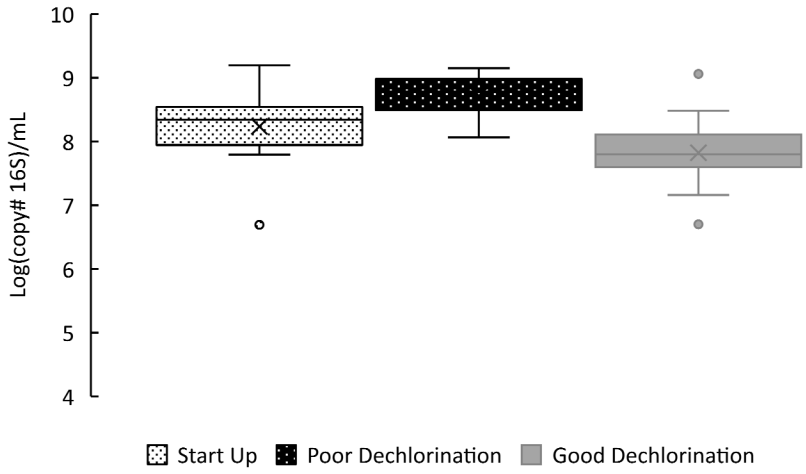

Figure 7 Total 16S rRNA gene copy numbers for Bacteria during three phases of CFR operation: startup, poor dechlorination and good dechlorination

The qPCR primers for *Geobacter, Desulfuromonas and Desulfitobacterium* were developed previously as shown in Table 3. Total gene copies/mL reactor liquid for these bacteria were grouped by corresponding median released chloride concentration in Figure 8. We hypothesized that *Geobacter* would dechlorinate amended Cl-NOM since it has been shown to have substrate versatility and consume electron donors at low threshold concentrations (Sung et al., 2006). This species was able to maintain high copy numbers throughout reactor operation at all levels of released chloride. A Pearson correlation between effluent chloride concentration and *Geobacter* was conducted, and results showed that there was not a strong correlation between concentrations of chloride and gene copy numbers of this organism.

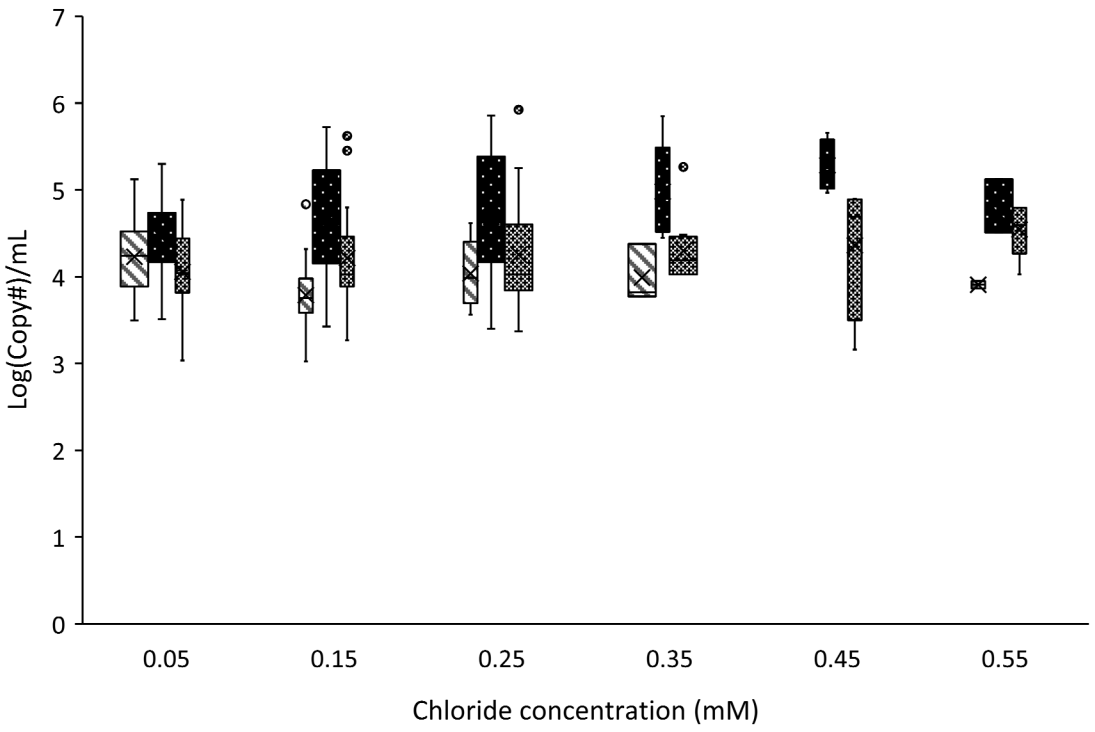

Figure 8 The total gene copy number (16S rRNA gene) of Desulfuromonas (▨), Geobacter (■),and Desulfitobacterium (▩) are shown with respect to increasing chloride concentrations because of dechlorination of Cl-NOM within the CFR. At higher chloride concentrations, Geobacter seems to dominate.

Figure 9 shows the difference in copy numbers of the facultative OHRB *Geobacter, Desulfuromonas and Desulfitobacterium,* during periods of poor vs. good dechlorination. From this, we can see that both *Geobacter* (P = 0.002) and *Desulfitobacterium* (P = 0.002) increase in a statistically significant matter when dechlorination is better. This suggests that during periods of good dechlorination, both *Geobacter* and *Desulfitobacterium* were enriched, while *Desulfuromonas* was not enriched.

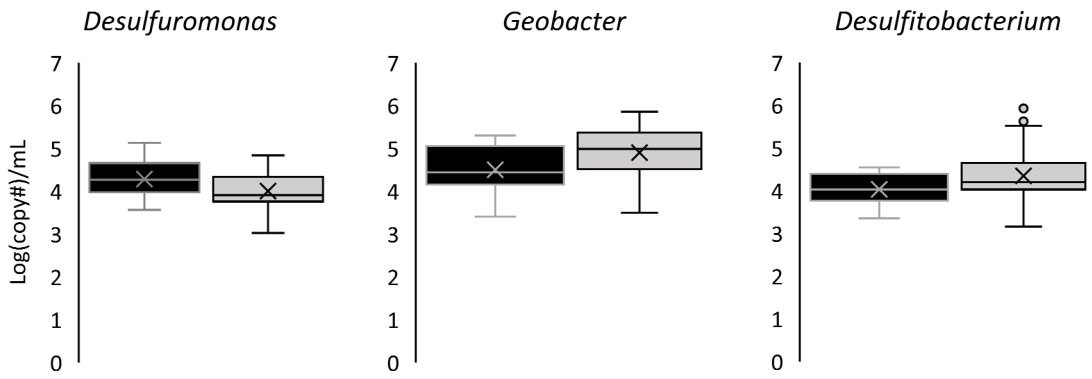

Figure 9 Gene copy numbers (16S rRNA genes) for facultative OHRB and hydrolytic dechlorinators for the poor and good dechlorination phases of CFR operation. Results for sediment samples taken during poor dechlorination are shown in black and those during good dechlorination are shown in gray.

Figure 10 shows copy numbers of the PelicanRdh reductive dehalogenase gene (rdh) and the Pelican 2-haloacidDhg dehalogenase gene (dh) (Temme et al., 2019) during startup, poor dechlorination, and good dechlorination periods. Rdhs and dhs were present in all DNA samples taken from the CFR. A paired t-test was performed to understand the significance between rdh and dh genes over the course of reactor operation. Results showed that rdhs were significantly higher than dhs ($P<0.05$). In addition, rdh genes were lower ($P = 0.0004$) during the good dechlorination phase while dh genes were higher during this phase ($P = 0.0262$). This could indicate that dechlorinators with dh genes are enriched during good dechlorination of Cl-NOM or that Cl-NOM selects for bacteria with dh genes over rdh genes.

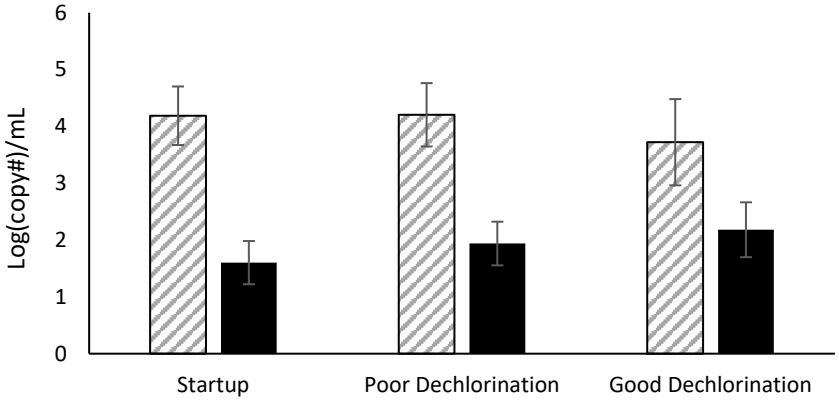

Figure 10 The qPCR results of Pelican rdh (▨) and Pelican 2-haloacidDhg (■) genes are shown over the three phases of operation of the CFR as result of dechlorination of chlorinated natural organic matter (Cl-NOM).

Results from 16S Illumina Sequencing

DNA samples taken from the CFR were submitted to UMGC for 16S Illumina Sequencing, and the microbial community was monitored over time to determine the relative abundance of key organisms, community richness and community diversity. Results from 16S Illumina Sequencing and analysis via the QIIME pipeline revealed the top three organisms present and their relative abundance during different reactor phases. Bacteria within the *Proteobacteria* phylum were present throughout operation of the CFR. During the startup and poor dechlorination phase, the maximum relative abundance of *Proteobacteria* was 11%, while during the good dechlorination phase, the maximum relative abundance was 31%. A steady increase or decrease was not seen for organisms within this phylum. Bacteria within the *Firmicutes* phylum were not seen at a relative abundance above 3%.Estimated absolute abundance was calculated at the phylum level using total 16S rRNA gene copy numbers and the relative abundance calculated via the QIIME pipeline. The estimated absolute abundance of *Proteobacteria* was significantly higher than *Firmicutes* ($P<0.05$). The estimated absolute abundance of Proteobacteria during the good dechlorination phase was significantly higher than in the startup phase ($P=0.035$) but not higher than in the poor dechlorination phase ($P=0.36$). No obvious obligate OHRB were observed from the sequencing data (e.g. *Chloroflexi)*, which suggests that under these conditions, facultative OHRB and hydrolytic dechlorinators were likely active, rather than obligate OHRB.

The sequencing data was analyzed via QIIME using the denovo clustering pipeline and core metrics phylogeny pipeline to calculate the Shannon index, a quantitative measure of community richness, and the Bray-Curtis distance, a quantitative measure of community dissimilarity. The average Shannon index during the startup, poor dechlorination, and good dechlorination phases were 2.7 ± 0.67, 3.06 ± 0.89, and 2.96 ± 0.78, respectively, indicating that the community within the CFR was less rich when dechlorination seemed to be occurring.

Chapter 4 Discussion

Results from batch experiments show that when amended with Cl-NOM, obligate OHRB dechlorinate PCE at an accelerated rate and to a greater extent (Table 5). Interestingly, the difference in dechlorination rate was not statistically different when cultures were amended with low versus high quantities of Cl-NOM (Figure 4). In fact, the first order dechlorination rate coefficient was only statistically different in the reactors amended with high Cl-NOM ($P = 0.016$). It is therefore unclear what amount of Cl-NOM is needed to accelerate this process. It was clear that obligate OHRB grew on PCE, but not clear whether they grew on Cl-NOM (Figure 5, Table 5). This is demonstrated by the fact that when low Cl-NOM was present, the growth of *Dehalogenimonas* ($P = 0.013$) and *Dehalobacter* ($P = 0.006$) was statistically lower than the growth of these organisms when no Cl-NOM was amended. Although the growth of both *Dehalogenimonas* ($P = 0.123$) and *Dehalococcoides* ($P = 0.188$) was higher in reactors amended with high Cl-NOM compared to reactors amended with low Cl-NOM, they were not statistically different. Therefore, we can say that Cl-NOM addition accelerates the dechlorination of chlorinated solvents, but that it does not necessarily contribute to the significant growth of these obligate OHRB.

Previous research has shown that *Dehalogenimonas* was enriched in microcosms amended with Cl-NOM while *Dehalobacter* was slightly enriched by Cl-NOM when amended for over 50 days (Lim et al., 2018). The growth of *Dehalococcoides* was not observed and it was hypothesized that the hydrogen peroxide additions during Cl-NOM generation could have inhibited its growth (Lim et al., 2018). Additional studies have shown that OHRB and non-respiratory dechlorinators can dechlorinate different fractions of Cl-NOM and that enrichment on different fractions can prime contaminant dechlorination (Temme & Novak, 2020). Further studies have explored the effect of co-amendment with halogenated compounds to stimulate dechlorination. Ahn et al. hypothesized that co-amendment of halogenated compounds with

greater structural similarity to chlorinated dioxins would more effectively stimulate dechlorination of those chlorinated dioxins and found that the addition of electron donors, such as lactate and propionate alone, did not result in effective stimulation. In fact, this study determined that other halogenated compounds other than chlorinated dioxins could be used to enrich and isolate organisms that have activity on these compounds. The study demonstrated that the addition of toxic halogenated co-amendments may be one tool to enhance dechlorination of chlorinated dioxins (Ahn et al., 2005). My research showed that Cl-NOM can accelerate the dechlorination of PCE, acting as a primer for reductive dechlorination of PCE; nevertheless, the mechanism of this priming activity cannot be determined from this data.

There are multiple reductive dechlorinating cultures that are currently used for bioremediation *in situ*. The cultures used in these batch experiments (KB-1, WBC-2, and ACT-3) are dominated by *Dehalococcoides, Dehalobacter,* and *Dehalogenimonas,* respectively, which are commonly used for bioaugmentation in the field. Because these cultures are in fact used for this purpose, the findings from the experiments described herein are particularly important in that they suggest that Cl-NOM can be added when these cultures are used for bioremediation to increase the rate (and possibly the extent) of dechlorination, reducing the time needed to reach clean-up goals and the need for an external electron donor, and as a result, the cost of remediation. Specific concentrations that are needed to achieve this effect need to be verified for each of these consortia individually and the toxicity of Cl-NOM generated in the manner described herein needs to be determined prior to its addition to the environment.

Results from operation of the CFR showed that there was a higher number of *Geobacter* ($P = 0.002$) and *Desulfitobacterium* ($P = 0.002$) during periods of good dechlorination. This could indicate that these bacteria can dechlorinate Cl-NOM and use it as a (dechlorinated) electron donor when no other electron donor is present or perhaps, as an alternative electron acceptor during facultative organohalide respiration. Since *Geobacter* has substrate versatility and can

utilize multiple electron donors, such as acetate, pyruvate, and hydrogen, and electron acceptors, such as PCE, nitrate, and fumarate (Sung et al., 2006), either possibility is reasonable. More work is needed, therefore, to determine what role, if any, *Geobacter* plays in the dechlorination of Cl-NOM. Increases in copy numbers of *Geobacter* and *Desulfitobacterium* could also be attributed to the NOM present in the CFR as well, which would have invariably been added when the Cl-NOM was added. Results from 16S Illumina sequencing showed that the community within the CFR became less diverse over time. This could indicate that Cl-NOM addition selected for specific organisms and was toxic or inhibitory to others. Results of qPCR for the PelicanRdh and Pelican-2-Haloacid Dh genes showed that both were present throughout reactor operation. The copy number of rdh genes was significantly higher than those of dh genes, suggesting that obligate OHRB were not enriched or grown; instead, facultative and hydrolytic dechlorinators seemed to be enriched in this reactor. Since rdh genes were lower during the good dechlorination phase and dh genes were higher, bacteria with dh genes were likely enriched during good dechlorination of Cl-NOM.

Previous studies have shown that non-reductive dechlorination processes and the dehalogenase genes that perform these processes can occur in anaerobic environments (Coleman et al., 2002), (Munro, 2017). Facultative OHRB bacteria such as *Geobacter, Desulfuromonas,* and *Desulfitobacterium* as well as hydrolytic bacteria such as *Pseudomonas* and *Acetobacterium* have all been identified as dechlorinators (Sung et al., 2006), (Löffler et al., 2000), (Suyama et al., 2002), (Seffernick, 2002). Few studies have been done to understand how Cl-NOM can enrich these organisms and act as a possible electron donor. During enrichment studies, Lim et al. found that *Geobacter* was not reproducibly enriched via Cl-NOM amendment (Lim et al., 2018). However, results from CFR operation in this study indicate that during good dechlorination of Cl-NOM, *Geobacter* and *Desulfitobacterium* appeared to be significantly enriched while

Desulfuromonas declined. Therefore, it appears that some facultative OHRB can be enriched with Cl-NOM, but more work is needed to understand this relationship.

Chapter 5 Conclusions

Together, Figure 2 and Figure 4 show that when obligate OHRB are amended with Cl-NOM, dechlorination of PCE is faster and formation of the PCE daughter product, cis-DCE, occurs. When amended with a higher concentration of Cl-NOM, PCE dechlorination is faster compared to amendment of low concentrations. These results suggest that addition of Cl-NOM at higher volumes to known obligate OHRB should be beneficial to dechlorination.

The picture is less clear for hydrolytic dechlorinators and their response to Cl-NOM amendment. CFR operation and analysis suggest that *Geobacter* and *Desulfitobacterium* grew during the period of dechlorination of Cl-NOM, while *Desulfuromonas* did not grow, indicating that Cl-NOM amendment selects for specific facultative OHRB and hydrolytic dechlorinators and may be toxic or inhibitory to others. Sequencing data showed that the estimated absolute abundance of bacteria within the *Proteobacteria* phylum was significantly higher during the good dechlorination phase compared to the startup phase. Indeed, no known obligate OHRB (specifically those within the *Chloroflexi* phylum) were detected in the sequencing performed on CFR samples.

More work is needed to determine a stronger relationship between these amendments and specific bacteria. For example, performing experiments with a known consortia of obligate and facultative OHRB and hydrolytic dechlorinators could better inform the effect of Cl-NOM addition on these organisms, particularly if performed with Cl-NOM as either the sole electron donor or acceptor. Finally, the toxicity of Cl-NOM to a variety of organisms needs to be determined, particularly because amendments of Cl-NOM to bioremediation systems bioaugmented with KB-1, and other obligate OHRB-containing consortia, do appear to be beneficial. Once the level of toxicity is determined, further experiments could combine obligate OHRB with facultative OHRB and hydrolytic dechlorinators that are not inhibited by Cl-NOM to

create a mixed culture. This mixed culture could then be amended with Cl-NOM as well as PCE and monitored to determine if/how the mixed culture shifts during specific dechlorination steps. More work is needed before use at contaminated sites or *in situ.*

Bibliography

Adrian, L., Hansen, S. K., Fung, J. M., Görisch, H., & Zinder, S. H. (2007). Growth of Dehalococcoides Strains with Chlorophenols as Electron Acceptors [Article]. *Environmental Science & Technology*, *41*(7), 2318–2323. https://doi.org/10.1021/es062076m

Ahn, Yo.-B., HÄGGBLOM, M. M., & FENNELL, D. E. (2005). CO-AMENDMENT WITH HALOGENATED COMPOUNDS ENHANCES ANAEROBIC MICROBIAL DECHLORINATION OF 1,2,3,4-TETRACHLORODIBENZO-P-DIOXIN AND 1,2,3,4-TETRACHLORODIBENZOFURAN IN ESTUARINE SEDIMENTS. *Environmental Toxicology and Chemistry*, *24*(11), 2775–2784.

Alfán-Guzmán, R., Ertan, H., Manefield, M., & Lee, M. (2017). Isolation and characterization of Dehalobacter sp. Strain TeCB1 including identification of TcbA: A novel tetra- and trichlorobenzene reductive Dehalogenase. *Frontiers in Microbiology*, *8*(APR). https://doi.org/10.3389/fmicb.2017.00558

Amos, B. K., Sung, Y., Fletcher, K. E., Gentry, T. J., Wu, W.-M., CRIDDLE, C. S., JIZHONG ZHOU, & LÖFFLER, F. E. (2007). Detection and Quantification of Geobacter lovleyi Strain SZ: Implications for Bioremediation at Tetrachloroethene- and Uranium-Impacted Sites [Article]. *Applied and Environmental Microbiology*, *73*(21), 6898–6904. https://doi.org/10.1128/AEM.01218-07

Ang, T. F., Maiangwa, J., Salleh, A. B., Normi, Y. M., & Leow, T. C. (2018). Dehalogenases: From improved performance to potential microbial dehalogenation applications. *Molecules*, *23*(5). https://doi.org/10.3390/MOLECULES23051100

ASTDR. (2017). *Toxicological profile of tetrachloroethylene.pdf*. https://www.atsdr.cdc.gov/ToxProfiles/tp18-c6.pdf

ATSDR. (2018). Production, Import/Export, Use, and Disposal. In *Toxicological Profile for Tetrachloroethylene*. https://www.atsdr.cdc.gov/ToxProfiles/tp18-c5.pdf

Bedard, D. L., Van Dort, H., & Deweerd, K. A. (1998). Brominated Biphenyls Prime Extensive Microbial Reductive Dehalogenation of Aroclor 1260 in Housatonic River Sediment. In *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* (Vol. 64, Issue 5, pp. 1786–1795). https://journals.asm.org/journal/aem

Coleman, N. V., Mattes, T. E., Gossett, J. M., & Spain, J. C. (2002). Phylogenetic and kinetic diversity of aerobic vinyl chloride-assimilating bacteria from contaminated sites. *Applied and Environmental Microbiology*, *68*(12), 6162–6171. https://doi.org/10.1128/AEM.68.12.6162-6171.2002

Duhamel, M., SD, W., L, Y., H, R., D, S., S, D., EE, C., & EA, E. (2002). Comparison of anaerobic dechlorinating enrichment cultures maintained on tetrachloroethene, trichloroethene, cis-dichloroethene and vinyl chloride. *Water Research*, *36*(17), 4193–4202. https://doi.org/10.1016/S0043-1354(02)00151-3

Edwards, E. A. (1994). *Anaerobic Degradation of Toluene and o-Xylene by a Methanogenic Consortiumt*.

Fennell, D. E., Carroll, A. B., Gossett, J. M., & Zinder, S. H. (2001). Assessment of indigenous reductive dechlorinating potential at a TCE-contaminated site using microcosms, polymerase chain reaction analysis, and site data. *Environmental Science and Technology*, *35*(9), 1830–1839. https://doi.org/10.1021/es0016203

Fetzner, S. (1998). Bacterial dehalogenation. *Applied Microbiology and Biotechnology*, *50*(6), 633–657. https://doi.org/10.1007/s002530051346

Gribble, G. W. (1998). *Naturally Occurring Organohalogen Compounds †*. https://pubs.acs.org/sharingguidelines

Grostern, A., & Edwards, E. A. (2006). Growth of Dehalobacter and Dehalococcoides spp. During degradation of chlorinated ethanes. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, *72*(1), 428–436. https://doi.org/10.1128/AEM.72.1.428–436.2006

Hug, L. A., Maphosa, F., Leys, D., Löffler, F. E., Smidt, H., Edwards, E. A., & Adrian, L. (2013). Overview of organohalide-respiring bacteria and a proposal for a classification system for reductive dehalogenases. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *368*(1616). https://doi.org/10.1098/RSTB.2012.0322

Jugder, B. E., Ertan, H., Bohl, S., Lee, M., Marquis, C. P., & Manefield, M. (2016). Organohalide respiring bacteria and reductive dehalogenases: Key tools in organohalide bioremediation. *Frontiers in Microbiology*, *7*(MAR). https://doi.org/10.3389/fmicb.2016.00249

Krzmarzick, M. J., mcnamara, patrick j., Crary, B. B., & Novak, P. J. (2013). *Abundance and diversity of organohalide-respiring bacteria in lake sediments across a geographical sulfur gradient*. *84*, 248–258. https://doi.org/10.1111/1574-6941.12059

Lim, M., Brooks, M., Boothe, M., & Krzmarzick, M. J. (2018). *Novel bacterial diversity is enriched with chloroperoxidase-reacted organic matter under anaerobic conditions*. *94*. https://doi.org/10.1093/femsec/fiy050

Löffler, F. E., Löffler, F., Sun, Q., Li, J., Tiedje, J. M., Pce, T., Löffler, ; F E, Li, J., Urbance, J. W., & Tiedje, J. M. (2000). 16S rRNA Gene-Based Detection of Tetrachloroethene-Dechlorinating Desulfuromonas and Dehalococcoides Species. In *Abstr. 98th Gen. Meet. Am. Soc. Microbiol* (Vol. 66, Issue 4, p. 450). http://www.dsmz.de

Major, D. W., McMaster, M. L., Cox, E. E., Edwards, E. A., Dworatzek, S. M., Hendrickson, E. R., Starr, M. G., Payne, J. A., & Buonamici, L. W. (2002). Field Demonstration of Successful Bioaugmentation To Achieve Dechlorination of Tetrachloroethene To Ethene [Article]. *Environmental Science & Technology*, *36*(23), 5106–5116. https://doi.org/10.1021/es0255711

Manchester, M., Hug, L. A., Zila, & Edwards, E. A. (2012). Discovery of a transdichloroethene-respiring Dehalogenimonas species in the 1,1,2,2-tetrachloroethane-dechlorinating WBC-2 consortium. *Applied and Environmental Microbiology*, *78*(15), 5280–5287.

Maphosa, F., Lieten, S. H., Dinkla, I., Stams, A. J., Smidt, H., & Fennell, D. E. (2012). Ecogenomics of microbial communities in bioremediation of chlorinated contaminated sites. *Frontiers in Microbiology*, *3*(OCT). https://doi.org/10.3389/fmicb.2012.00351

Mattes, T. E., Alexander, A. K., & Coleman, N. V. (2010). Aerobic biodegradation of the chloroethenes: Pathways, enzymes, ecology, and evolution. *FEMS Microbiology Reviews*, *34*(4), 445–475. https://doi.org/10.1111/j.1574-6976.2010.00210.x

McCarty, P. L., Criddle, C. S., & Vogel, T. M. (2020). Retrospective on microbial transformations of halogenated organics. *Environmental Science: Processes and Impacts*, *22*(3), 512–517. https://doi.org/10.1039/c9em00575g

Molenda, O., Quaile, A. T., & Edwards, E. A. (n.d.). *SUPPLEMENTAL INFORMATION Dehalogenimonas WBC-2 genome and identification of its trans-dichloroethene reductive dehalogenase, TdrA*.

Molenda, O., Quaile, A. T., & Edwards, E. A. (2015). *Dehalogenimonas sp. Strain WBC-2 Genome and Identification of Its trans-Dichloroethene Reductive Dehalogenase, TdrA*. https://doi.org/10.1128/AEM.02017-15

Munro, J. (2017). *Co-occurrence of genes for aerobic and anaerobic biodegradation of dichloroethane in organochlorine-contaminated groundwater*. *93*. https://doi.org/10.1093/femsec/fix133

Murray, A., Maillard, J., Rolle, M., Broholm, M., & Holliger, C. (2020). Impact of iron- A nd/or sulfate-reduction on a: Cis-1,2-dichloroethene and vinyl chloride respiring bacterial consortium: Experiments and model-based interpretation. *Environmental Science: Processes and Impacts*, *22*(3), 740–750. https://doi.org/10.1039/c9em00544g

Muyzer, G., De Waal, E. C., & Uitterlinden, A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA [Article]. *Applied and Environmental Microbiology*, *59*(3), 695–700. https://doi.org/10.1128/AEM.59.3.695-700.1993

Reina, R. G., Leri, A. C., & Myneni, S. C. B. (2004). Cl K-edge X-ray Spectroscopic Investigation of Enzymatic Formation of Organochlorines in Weathering Plant Material. *Environmental Science and Technology*, *38*(3), 783–789. https://doi.org/10.1021/es0347336

Rupakula, A., Kruse, T., Boeren, S., Holliger, C., Smidt, H., & Maillard, J. (2013). The restricted metabolism of the obligate organohalide respiring bacterium dehalobacter restrictus: Lessons from tiered functional genomics. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *368*(1616). https://doi.org/10.1098/rstb.2012.0325

Seffernick, J. (2002). Atrazine Chlorohydrolase from Pseudomonas Sp. Strain ADP Is a Metalloenzyme. *Biochemistry*, *41*, 14430–14437. https://doi.org/10.1021/bi020415s

Smits, T. H. M., Devenoges, C., Szynalski, K., Maillard, J., & Holliger, C. (2004). Development of a real-time PCR method for quantification of the three genera Dehalobacter, Dehalococcoides, and Desulfitobacterium in microbial communities [Article]. *Journal of Microbiological Methods*, *57*(3), 369–378. https://doi.org/10.1016/j.mimet.2004.02.003

Sung, Y., Kelly, ‡, Fletcher, E., Ritalahti, K. M., Apkarian, R. P., Ramos-Hernández, N., Sanford, R. A., Mesbah, N. M., & Löffler, F. E. (2006). Geobacter lovleyi sp. Nov. Strain SZ, a Novel Metal-Reducing and Tetrachloroethene-Dechlorinating Bacterium †. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, *72*(4), 2775–2782. https://doi.org/10.1128/AEM.72.4.2775-2782.2006

Suyama, A., Yamashita, M., Yoshino, S., & Furukawa, K. (2002). Molecular Characterization of the PceA Reductive Dehalogenase of Desulfitobacterium sp. Strain Y51. *JOURNAL OF BACTERIOLOGY*, *184*(13), 3419–3425. https://doi.org/10.1128/JB.184.13.3419-3425.2002

Tang, S., & Edwards, E. A. (2013). *Identification of Dehalobacter reductive dehalogenases that catalyse dechlorination of chloroform, 1,1,1-trichloroethane and 1,1-dichloroethane*. https://doi.org/10.1098/rstb.2012.0318

Temme, H. R., Carlson, A., & Novak, P. J. (2019). Presence, diversity, and enrichment of respiratory reductive dehalogenase and non-respiratory hydrolytic and oxidative dehalogenase genes in terrestrial environments. *Frontiers in Microbiology*, *10*(JUN). https://doi.org/10.3389/fmicb.2019.01258

Temme, H. R., & Novak, P. J. (2020). Diverse dechlorinators and dechlorination genes enriched through amendment of chlorinated natural organic matter fractions. *Environmental Science: Processes and Impacts*, *22*(3), 595–605. https://doi.org/10.1039/c9em00499h

United States. Environmental Protection Agency. Office of Solid Waste and Emergency Response. (2004). *Cleaning up the nation's waste sites: Markets and technology trends.* [Document]. U.S. Environmental Protection Agency, Office of Solid Waste and Emergency Response.

U.S. EPA. (2004). *Cleaning up the nation's waste sites: Markets and technology trends.* [Document]. U.S. Environmental Protection Agency, Office of Solid Waste and Emergency Response.

Waller, A. S., Krajmalnik-Brown, R., Löffler, F. E., & Edwards, E. A. (2005). Multiple reductive-dehalogenase-homologous genes are simultaneously transcribed during dechlorination by dehalococcoides-containing cultures. *Applied and Environmental Microbiology*, *71*(12), 8257–8264. https://doi.org/10.1128/AEM.71.12.8257-8264.2005

Appendices

Appendix A Results from analysis of Pelican Lake soil

18 grams of Pelican Lake sediment was placed into 100 mL of DI water. The mixture was shaken at room temperature for 4 days and aqueous samples were taken and measured via ion chromatography. The sediment was found to have an average chloride concentration of 0.0711 ± 0.008 mM.

Appendix B Pelican Lake sediment dechlorination reactors

Batch reactors were seeded with biomass from the continuous flow reactor after continuous amendment with low Cl-NOM (0.03% v/v) and spiked with 100 µM. Results from these experiments showed no decline in PCE over time and no formation of degradation products.

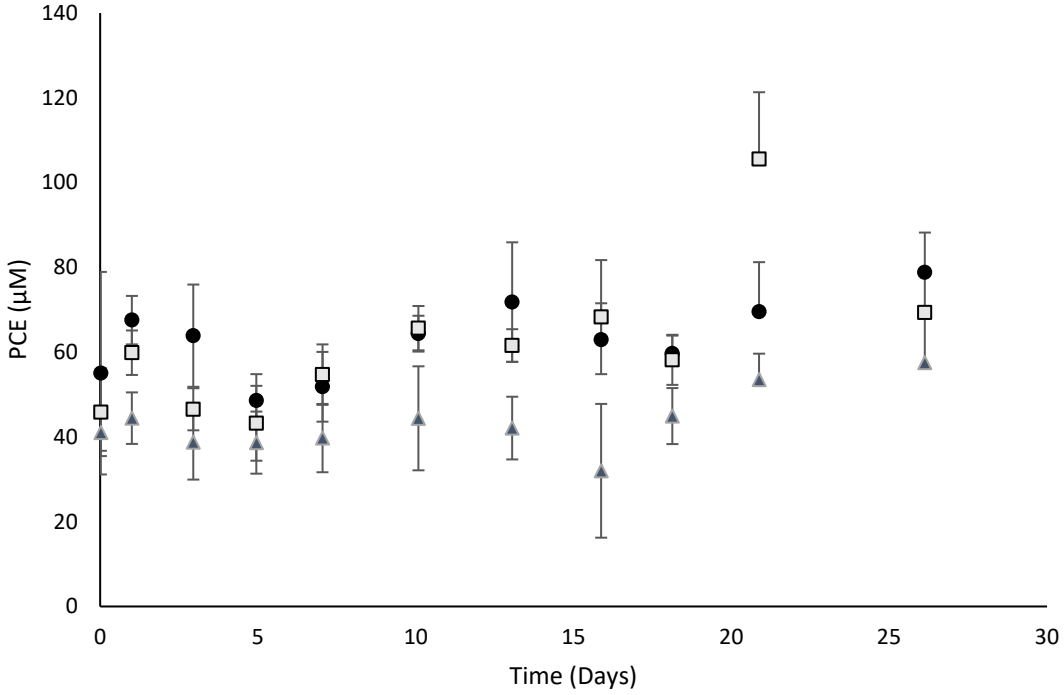

Figure 11 Experiment testing dechlorination of PCE in reactors amended with low Cl-NOM (●), high Cl-NOM (□) and autoclaved control (▲). All reactors were seeded from the CFR, which was hypothesized to contain facultative OHRB and hydrolytic dechlorinators. No PCE dechlorination was seen over the 30-day experiment.

Additional experiments were carried out after amending the CFR with high Cl-NOM for two weeks. PCE declined over time, however, this decline could not be directly attributed to biological dechlorination, as the decline was present in the autoclaved control as well.

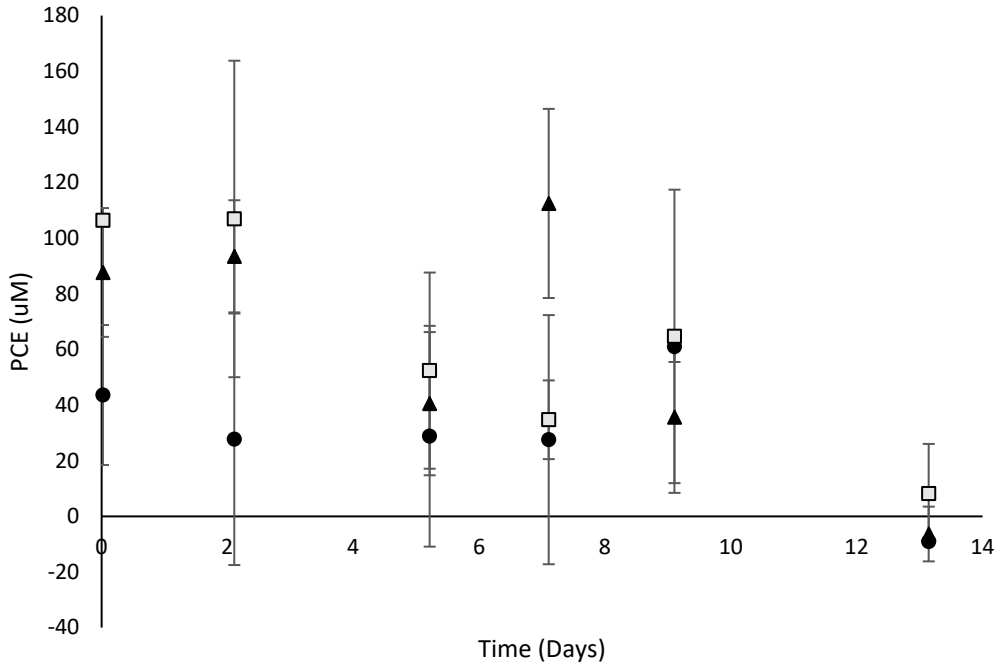

Figure 12 Biomass was taken from the CFR to seed three triplicate reactors which were either not amended (●) or amended with additional Cl-NOM (□) or autoclaved (▲).

Appendix C Additional information for Chapter 3

Chloride samples were collected from batch reactors in Groups A, B, and C. Samples were measured via ion chromatography. However, concentrations were inaccurate due to machine malfunction. Instead, estimated chloride concentration was calculated based on concentrations of chlorinated compounds with the following equation.

$$\frac{4\ mol\ Chloride}{1 mole\ PCE}\left(PCE_i - PCE_f\right) + \frac{3\ mol\ Chloride}{1\ mole\ TCE}\left(TCE\right) + \frac{2\ mol\ Chloride}{1\ mole\ TCE}\left(DCE\right)$$

Appendix D Additional Sequencing Information

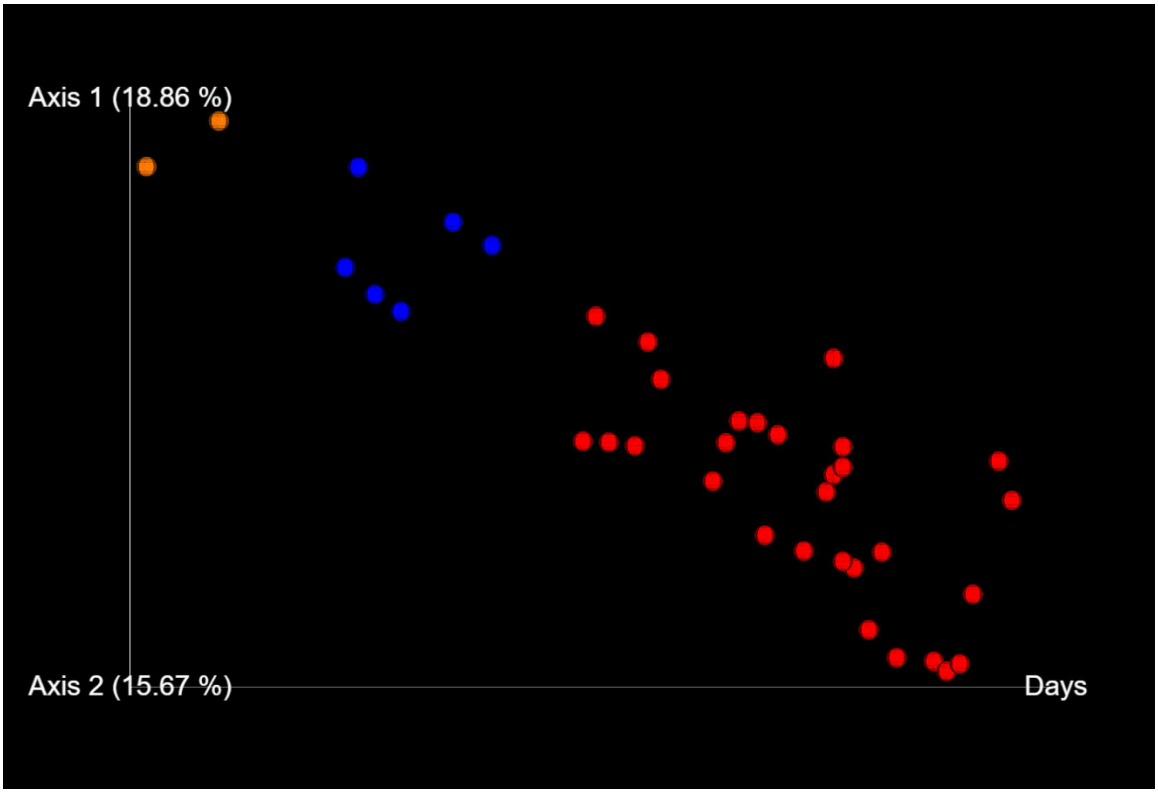

Figure 13 Bray-Curtis distance of CFR operation phases: startup (yellow), poor dechlorination(blue) and good dechlorination (red)