Connecting Dominant Bacterial Dechlorination Pathways to Environmental Drivers

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Dedication

This dissertation is dedicated to my parents, James and Joy Miller, who first encouraged my love of the outdoors and taught me the importance of preserving it; and to my husband Andrew Temme, with whom I continue to enjoy the outdoors and who has always been there to encourage and support me.

Abstract

Bacterial dechlorination of chlorinated contaminants has been well studied as a method for bioremediation of common chlorinated contaminants yet efforts can still be made to improve bioremediation and help it become a more robust technique. The work presented in this dissertation analyzes microbial communities in uncontaminated environments to better understand what promotes dechlorination in uncontaminated environments and which dechlorination processes are dominant in various environments. An understanding of the capabilities of these bacteria in uncontaminated sites can be used to improve bioremediation techniques.

Organohalide respiring bacteria are slow growing and can be difficult to enrich and isolate for further study. A method was developed to rapidly select for putative organohalide respiring bacteria. Organohalide respiring bacteria were found to be at higher ratios than other bacteria at the interface between aqueous media and a hydrophobic liquid. Trichloroethene and hexadecane were both tested, and the fraction of *Dehalococcoides*-like bacteria was found to increase up 20 times at the interface in just 20 minutes. This shows that the selection was a result of physical interactions and not growth. After the selection process, the bacteria were still viable and capable of dechlorinating TCE. This method was verified with a PCB enrichment culture, uncontaminated sediment, and anaerobic digester sludge.

The metagenomes of urban lakes with no known chlorinated contaminants and of bacteria enriched with chlorinated natural organic matter (Cl-NOM) were analyzed for genes of enzymes capable of dechlorination. The lake sediments had varying degrees of urban impact. Both reductive dehalogenase (rdh) genes and non-respiratory

dehalogenase (dh) genes were found in the metagenomes with the dh genes typically at higher frequencies. The one exception was the uncontaminated lake sediment enriched with chlorinated natural organic matter. This showed an increase in the rdh genes compared to the bacteria enriched with only natural organic matter. These findings show that rdh genes can be enriched for with high concentrations of chlorinated natural organic matter; but in uncontaminated lake sediments with lower concentrations of organochlorines, dh's are the dominant dechlorination mechanism. qPCR primers were developed to target specific rdh and dh genes to track them through the enrichment period with Cl-NOM and in more lakes with varying degrees of urban impact. Some of the dh and rdh genes were found in multiple lakes while others were specific to one or two, indicating that some enzymes capable of dechlorination are more widely distributed in the environment than others. The presence and concentrations of specific genes was not an indication of the ability of the lake sediment to dechlorinate trichloroethene, a common environmental contaminant.

The types of Cl-NOM that are preferentially dechlorinated and how enrichment with different types of Cl-NOM affect the ability to dechlorinate contaminants was investigated with PCB contaminated soil. The Cl-NOM was fractionated into three broad groups based on hydrophobicity. The least hydrophobic fraction was found to be dechlorinated more readily followed by the moderate hydrophobicity; however, it was the moderate hydrophobic fraction that promoted the dechlorination of trichloroethene and tetrachlorobenzene. During the enrichment period, known organohalide respiring bacteria were enriched as well as several other bacteria that have unknown dehalogenation potential. Additionally, dh genes and not rdh genes were shown to increase in

concentration demonstrating that bacteria utilizing other dechlorination mechanisms could be important for bioremediation and chlorine cycling as well.

The dechlorination potential of bacteria in the Soudan Mine, a carbon limited system with higher concentrations of iron and chloride, was analyzed. The dh and rdh genes in metagenomes from groundwater in three boreholes were analyzed. Dh genes were found at higher frequencies than rdh genes. Bacteria capable of dechlorination may have a metabolic advantage in this carbon limited system because it allows them to utilize Cl-NOM.

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Chapter 1

Introduction

Chlorinated organics have been used in a variety of industrial applications that have contributed to widespread contamination of surface water, groundwater, and sediment (1). Common contaminants include polychlorinated biphenyls (PCBs), trichloroethene (TCE), tetrachloroethene (PCE), chlorinated benzenes, and chlorinated phenols (2–6). Excluding Department of Energy and Department of Defense sites, in 2004 it was estimated that 350,000 contaminated sites in the United States will require remediation in the next 20 years (1). Of these, The U.S. EPA predicts that the Superfund sites alone (1317 total) will cost $19 billion to remediate, of which 69% are contaminated with halogenated volatile organics, 27% with PCBs, and 26% with other halogenated semi-volatile chemicals (1). Many these chlorinated organic pollutants are hazardous and potential carcinogens (7). Improvements in remediation techniques are needed to improve the effectiveness of remediation techniques while reducing the cost and time required for remediation.

In contaminated environments, multiple bacteria and microbial processes have been identified that are capable of dechlorinating toxic chlorinated organic pollutants. The first group of bacteria, organohalide respiring bacteria, use the chlorinated contaminants as electron acceptors for growth and have the potential to produce less- or non-toxic compounds as byproducts (8–13). This process requires reductive dehalogenase enzymes (rdhs) to remove the chlorine atom. Organohalide respiring bacteria are found in many phylogenetic groups (13, 14), with those in the genus *Dehalococcoides* receiving the most attention because of their strict requirement of halogenated compounds for

growth (8, 15–17). Another broader group of bacteria use dehalogenases (dhs) to dechlorinate contaminants for non-respiratory reasons, such as the use of the contaminant's carbon backbone as a carbon source or electron donor (18). Examples of these enzymes include haloalkane dehalogenases, 2-haloacid dehalogenases, and haloacetate dehalogenases (18).

Bioremediation utilizing both dehalogenation processes, organohalide respiration and non-respiratory dehalogenation, have been shown to be effective in the remediation of chlorinated chemicals including chlorinated ethenes and PCBs (16, 19–22). While bioremediation is a promising technique and has been shown to be effective at some sites, there are also instances where it is not successful. Sometimes the bacteria present are not capable of dechlorinating a given contaminant/contaminants, or in other cases, the bacteria stall or stop dechlorinating before the contaminant has been remediated to acceptable safe concentrations (23–27). Bioremediation efforts have been improved by adding electron donors and carbon sources to the contaminated site to specifically stimulate organohalide respiration, although this may not completely solve either problem (28, 29). Stalling could occur because the chlorinated contaminant concentration becomes too low to support organohalide respiring bacteria that use the chlorinated contaminant(s) as their electron acceptor (27). In this case, the contaminant concentration remains above safe levels but below that needed by the bacteria to sustain an active dechlorinating population. A potential solution to this is to add more electron acceptor, (presumably non-toxic) chlorinated or brominated organic chemicals, to "prime" the microbial population (30). Since most chlorinated and brominated organic chemicals are hazardous, this solution has not been widely adopted (27). With respect to non-respiratory dehalogenation, dhs have been found at anaerobic contaminated sites but their role in

bioremediation compared to rdhs is unclear and under-investigated (31). Analysis of the role of dhs in contaminant dechlorination, particularly at low contaminant concentrations, could provide insight into which dechlorination processes and bacteria are dominant and offer the greatest potential for stimulation under different conditions.

In uncontaminated sites, bacteria are naturally exposed to low concentrations of organochlorine compounds. Several thousand different types of chlorinated chemicals are produced naturally by fungi, plants, insects, and marine organisms (32, 33). Bacteria capable of dechlorination are thought to have developed the ability to dechlorinate contaminants from an ability to dechlorinate this chlorinated natural organic matter (Cl-NOM) (9, 34, 35). Organohalide respiring bacteria, including *Dehalococcoides*-like organisms, in addition to rdh and dh genes have been found in uncontaminated ecosystems (9, 34, 36–40). The dechlorination capabilities of bacteria in uncontaminated soils and sediments has yet to be fully explored, however. It is unknown how similar these bacteria are to those found in contaminated sites, both phylogenetically and with respect to their ability to degrade various chlorinated compounds. Expanding our knowledge of how the bacteria capable of dechlorination in uncontaminated soils and sediments degrade Cl-NOM, and which processes (respiratory versus non-respiratory) dominate under different conditions, may enable improvement of engineered bioremediation systems. Additionally, the Cl-NOM present at a site could also act as a primer to promote respiratory bioremediation at lower contaminant concentrations. Further research into these bacteria will not only improve our understanding of the chlorine biogeochemical cycle but will also provide information that could be applied to the development of new biostimulation tools.

1.1 Objectives

The objectives of this research were to 1) develop a technique to quickly select for organohalide respiring bacteria, improving our ability to study them; 2) analyze the dechlorination ability and types of reductive dehalogenases and non-respiratory dehalogenases present in sediments and soils with varying prior exposure to pollutants; 3) determine what type(s) of Cl-NOM are preferentially dechlorinated and whether Cl-NOM could be used as a primer in bioremediation efforts; and 4) analyze whether rdhs and dhs are present in the metagenome of an uncontaminated, low carbon, anaerobic system, providing insight into the chlorine cycle and the ways in which low concentrations of Cl-NOM may be transformed under nutrient-limited conditions. These objectives are addressed in the following chapters, which are summarized below.

1.2 Rapid Enrichment of Dehalococcoides-like Bacteria by Partial Hydrophobic Separation

A technique was developed to rapidly enrich putative organohalide respiring bacteria. Putative organohalide respiring bacteria were enriched >20x in approximately 20 minutes a result of physical-chemical interactions between the cell surface and hydrophobic solvent. This method was verified with bacteria from a PCB-enriched culture, uncontaminated lake sediment, and sludge from an anaerobic digester. After separation, the bacteria were found to still be capable of dechlorinating TCE to *cis*-dichloroethene, indicating that the separation/enrichment technique did not inactivate the bacteria. Organohalide respiring bacteria can be difficult to study because they are difficult to enrich; this method addresses this problem, allowing for rapid enrichment, which could facilitate their study through further enrichment or the use of techniques such as metagenomics. This work has been published in *Applied and Environmental Microbiology* (41). I am the

first author, and as such planned and conducted the experiments, analyzed the results, and wrote the paper. Kipp Sande is a second author. Kipp was an undergraduate working with me at the time and conducted experiments on the enrichment from sediment. Tao Yan is a third author. Tao worked with my advisor, Paige Novak, to initially conceive of these experiments.

1.3 Presence, Diversity, and Enrichment of Reductive Dehalogenases and Dehalogenases in a Variety of Environments

Rdh and dh genes were identified in metagenomes from enrichments and urban lakes experiencing a variety of urban impact; some of these genes were enriched as Cl-NOM dechlorination occurred but none could be correlated to urban impact. The dhs were found to occur at a higher frequency than the rdhs in all metagenomes, showing that dhs may be more widespread and important than previously considered. Haloalkane dehalogenases were the most common dhs sequenced in all samples. The rdhs detected in all samples were similar to those found in non-obligate organohalide respiring bacteria commonly found in soil, such as *Burkholderia* and *Bradyrhizobium*. In enrichments, the frequency of rdhs detected in metagenomic data increased with enrichment of Cl-NOM in uncontaminated lake sediment samples; dhs detected via a qPCR method developed from the metagenomic data also significantly increased in the enrichments during Cl-NOM dechlorination. With respect to the urban lakes, although all showed potential to dechlorinate in the fact that rdhs and dhs were present, only two of the lakes had the ability to dechlorinate TCE. TCE dechlorination ability was not predictable by the number or type of rdhs or dhs present. None of the genes analyzed in the lake samples were correlated with urban impact. This work demonstrates that the potential to dechlorinate is widespread, though not predictive of the ability to dechlorinate a common contaminant.

This variability could not be predicted by either the type or concentration of rdh or dh genes. This work suggests that both rdh and dh enzymes are responsible for the natural cycling of chlorine, can be enriched through the addition of Cl-NOM, and may have the potential to be used in bioremediation. This work will be submitted to *FEMS Microbiology Ecology*. I am the first author, and as such planned and conducted the experiments, analyzed the results, and wrote the paper. Aaron Carlson is a second author. Aaron is an undergraduate working with me and conducted the dechlorination experiments with the lake sediments amended with TCE. My advisor, Paige Novak, will serve as the final author, having helped generate the concept of the study and assisted with writing the paper.

1.4 Differential Priming of Contaminant Dechlorination through Amendment of Different Cl-NOM Fractions

When Cl-NOM was fractionated based on hydrophobicity and added to sediment, differences in gene and bacterial enrichment and Cl-NOM, and subsequent contaminant, dechlorination were observed. In initial Cl-NOM enrichments, the least hydrophobic fraction of Cl-NOM was dechlorinated to the greatest extent, followed by the moderate hydrophobic Cl-NOM fraction. No dechlorination was detected in the most hydrophobic fraction. After enrichment, the ability of each fraction to dechlorinate TCE and tetrachlorobenzene (TeCB) was then assessed, with the community enriched on the moderately hydrophobic Cl-NOM dechlorinating the contaminants more rapidly. This supports the idea that the dechlorination of contaminants can be primed by Cl-NOM. Community analysis was completed during the enrichment period and it showed that known organohalide respiring bacteria increased in concentration, as well as several other bacteria with no known organohalide respiration ability. These bacteria could be dechlorinating via a different mechanism or could be benefiting in some other way as a

result of other community members dechlorinating the Cl-NOM. Haloalkane dehalogenase genes were shown to increase in concentration over enrichment with and degradation of Cl-NOM. This work shows that different types of Cl-NOM could be used to prime the bioremediation of chlorinated contaminants and that non-respiratory dechlorination processes may be important in the dechlorination of both Cl-NOM and contaminants. This work will be submitted to *Environmental Science and Technology*. As the first of two authors I planned and conducted the experiments, analyzed the results, and wrote the paper. My advisor, Paige Novak, will serve as the other author, having helped generate the concept of the study and assisted with writing the paper.

1.5 Presence of Dehalogenase and Reductive Dehalogenase Enzymes in Very Low Nutrient Boreholes in the Soudan Mine, Minnesota

The Soudan Mine in northern Minnesota is a unique environment because it contains high chloride and iron concentrations but very low organic carbon and oxygen concentrations. As a part of a larger study focused on monitoring the microbial communities and geochemical processes in the mine for over a decade, bacterial metagenomes from the water of three boreholes in the deepest level of the Soudan Mine were analyzed to assess the potential for dechlorination in this environment. Rdhs and dhs were found in all three boreholes with dhs at higher frequencies. Cl-NOM could form through reactions with the iron, chloride, and small amounts of organic carbon present. The ability to dehalogenate such compounds could provide a metabolic advantage to bacteria by allowing them to utilize the dechlorinated Cl-NOM as a carbon source in an environment where organic carbon could be a limiting nutrient. This work shows that even in low carbon systems, bacteria have the potential to dechlorinate and that dh enzymes could provide an ecological advantage. This work requires additional input from others on the chemistry

and the generation of the original metagenomic dataset that I mined, as that work has not yet been published. It is likely that this work will be submitted as a communication or technical note to *Applied and Environmental Microbiology*. I will be the first author; I conceived of the study, performed the analysis, and wrote the paper. It is anticipated that Jon Badalamenti and Daniel Bond will be co-authors, as they generated the metagenomic dataset that I mined. It is anticipated that Brandy Toner will submit a paper on the chemical data related to these boreholes as well, and that paper can simply be referenced. My advisor, Paige Novak, will serve as the final author, having helped generate the concept of the study and assisted with writing the paper.

Chapter 2

Literature Review

2.1 Bioremediation

Chlorinated chemicals are common environmental contaminants that represent a public health risk, as many are toxic and carcinogenic (42, 43). The process of bioremediation uses the metabolic capabilities of bacteria to degrade or detoxify harmful chemicals in soil, sediment, and groundwater. Compared to other remediation technologies, bioremediation can be a less expensive approach. It has also been shown to be effective at sites contaminated with chlorinated chemicals (29, 44–47). Organohalide respiring bacteria are thought to be particularly important for the bioremediation of chlorinated contaminants because they gain energy by anaerobically dechlorinating the contaminants; therefore, they dechlorinate and detoxify contaminants as a way to survive and grow (6, 8, 14, 16, 48, 49). This process is called reductive dehalogenation. There are other bacteria that are also capable of dechlorinating contaminants via non-energy yielding mechanisms to release organic carbon for use as an electron donor (50).

Bioremediation of contaminated sites can vary from heavily engineered systems to systems that consist simply of monitoring. Natural attenuation is the biodegradation of contaminants by indigenous microbial populations without intervention in the biodegradation process. Plumes of chlorinated ethenes (29, 45, 46, 51, 52) and PCBs (53, 54) have been remediated by natural attenuation, with growth of bacteria capable of dechlorination observed (2). Natural attenuation can be very slow and may not work everywhere, however, because the indigenous microbial community may not be capable

of fully degrading or dechlorinating the contaminant. This could possibly result in, for example, the formation of toxic less-chlorinated byproducts (24, 25) or the biodegradation process stalling before remediation is complete (26, 27). Biostimulation can help by providing additional nutrients required by indigenous bacteria to continue degradation. Nutrients, electron acceptors, or—in the case of chlorinated contaminants—electron donors, are added to the contaminated site to promote the activity and growth of specific contaminant-degrading bacteria. The addition of carbon (56) or electron donors such as butyrate, lactate, propionate, and acetate are typically added to stimulate dechlorination (27, 57, 58). This has been successful in promoting the bioremediation of chlorinated ethenes (29, 45, 51, 52) and polychlorinated biphenyls (59). The application of biostimulation can also result in insufficient degradation, however. It can be difficult to predict which sites contain microbial communities capable of dechlorinating specific chlorinated contaminants and how to stimulate these communities to prevent dechlorination from stalling (26). While efforts have been made to better understand the bacteria and functional genes required for predictable stimulation, better prediction methods are still needed (51, 60). In cases where bacteria are incapable of degrading the specific contaminants present, bioaugmentation may be used. Bioaugmentation is the addition of bacteria that can degrade a specific contaminant to a site. While bioaugmentation has been effective in the remediation of chlorinated solvents (4, 61–63), there are many other chlorinated contaminants where an effective microbial consortium has yet to be developed. It is also subject to failure because the introduced bacteria may not effectively compete with native bacteria or grow well in their new environment (27, 64). All three of these bioremediation strategies—natural attenuation, biostimulation, and bioaugmentation—are less effective at low contaminant concentrations when the

contaminant serves a metabolic purpose (for example, as the electron acceptor for reductive dechlorination) because in such cases it may be impossible to maintain adequate populations of contaminant degraders.

For chlorinated contaminants, one solution to the problem of maintaining adequate numbers of dechlorinating bacteria at a contaminated site is the addition of so-called "primers." Priming, a specific type of biostimulation, is the addition of a chemical, often chlorinated or brominated, that can be co-degraded with the chlorinated contaminant and serves the same metabolic need. This allows the population to thrive even as the concentration of one energy source, the contaminant, is reduced. Priming relies upon the activity of native communities, and therefore avoids problems often encountered with bioaugmentation and the addition of exogenous bacteria to a given site. As mentioned above, previous work has found that other chlorinated and brominated organic chemicals can be used to stimulate dechlorination of a contaminant (65–67). Brominated biphenyls are more readily biodegraded than PCBs and have been found to stimulate the dechlorination of existing weathered PCBs in contaminated sediment (30, 66). Different bromination patterns on the biphenyl stimulated different PCB dechlorination pathways with varying effectiveness (30). PCB dechlorination has also been successfully primed by other aromatic compounds, including brominated congeners of benzoate, benzonitrile, nitrobenzene, benzamide, benzophenone, benzoic hydrazide, benzene sulfonate, and benzoic methyl ester; brominated acetophenones, phenols, or toluenes were unable to prime PCB dechlorination (67). Pentachloronitrobenzene was also found to enhance the dechlorination of PCBs and increase the concentrations of putative reductive dehalogenase genes and of *Dehalococcoides mccartyi*, a known organohalide respiring bacteria (27, 65). Although effective, the previously mentioned primers are also toxic.

Different chemical primers are needed to help improve remediation efforts without augmenting the toxicity and contaminant levels at a given site.

2.2 Organohalide Respiring Bacteria

Organohalide respiring bacteria use halogenated compounds as their physiological electron acceptors for growth, obtaining energy when these compounds are dehalogenated (6, 14, 49, 68). Bacteria from both contaminated and uncontaminated environments have been found that are capable of organohalide respiration (68–70). Organohalide respiring bacteria can be difficult to culture and isolate, which has limited the number of these bacteria available for study (14, 24). With respect to understanding organohalide respiring bacteria, the focus has been on *Dehalococcoides mccartyi* in the *Chloroflexi* phylum because this organism's sole metabolic scheme is organohalide respiration. Organohalide respiring bacteria have also been isolated from the *Firmicutes* and *Proteobacteria* phyla (17, 71–74). There are many uncultivated bacteria, however, that are suspected to have this metabolic capability. These include those in the *Anaerolineae* family of the *Chloroflexi* phylum and in the *Firmicutes* phylum, but this function has not been confirmed because these organisms have yet to be isolated or highly enriched (75, 76).

2.2.1 Chloroflexi

The *Chloroflexi* phylum is a diverse group of bacteria that contains several obligate organohalide respiring bacteria (8, 14, 49, 68). *Dehalococcoides mccartyi* is the most commonly studied organohalide respiring bacteria and multiple *D. mccartyi* strains have been isolated (14, 16). A summary of the isolated strains and their known degradation capabilities is presented in Table 2.1. *D. mccartyi* are small, non-motile cells with small genomes ranging from 1.34 to 1.47 Mbp and limited metabolic capabilities (14). *D.*

mccartyi uses hydrogen as an electron donor, acetate as a carbon source, and requires various forms of cobalt-containing porphyrins as cofactors for its reductive dehalogenase gene (14, 77). It is a strict anaerobe and even small amounts of or brief exposure to oxygen can cause irreversible loss of dechlorination ability (78). The strains have a diverse substrate range that is not predicted by its phylogeny (14). Chlorinated ethenes have predominantly been used for enrichment and isolation of *D. mccartyi*, as these organisms typically grow faster with chlorinated ethenes compared to larger and more complex chemicals like PCBs (79). This slow growth has limited the study of PCB-dechlorinating strains; strains JNA, CG1, CG4, and CG5 were enriched with PCBs, however (79, 80). Some strains are only capable of dechlorination at specific molecular positions, leaving behind less chlorinated chemicals (6, 48, 79). Several *D. mccartyi* strains, including 195, BAV1, and FL2, are capable of co-dechlorinating additional contaminants, but are unable to respire and grow with them as sole electron acceptors (Table 2.1) (14, 69, 81). The variations in isolated strains shows that *D. mccartyi* has a diverse dechlorinating metabolism that likely has yet to be fully understood.

Table 2.1 Characteristics of isolated D. mccartyi strains.

Strain	Isolated From	Degradation Capabilities	Other	Ref
195	Digester sludge, Ithaca, NY	PCE[a], TCE[b], *cis*-DCE[c], 1,1-DCE, 1,2-DCA[d], DBM[e], PCDDS[f], chlorinated naphthalenes, 2,3-DCP[g], 2,3,4-TCP[h], 2,3,6-TCP, CB[i], (cometabolize: *trans*-DCE, VC[j])	Dechlorinates only the *ortho* position for chlorinated phenols	(6, 16)
CBDB1	Saale River sediment, Germany (check)	1,2,3-TCB[k], 1,2,4-TCB, PCE, TCE, 2,3-DCP, all TCP, all TeCP[l], PCP[m], HCB[n], PCBZ[o], TeCB[p], 1,2,4-TCB, PCDDs, PCBs[q]	Preferentially dechlorinates in the *ortho* position	(6, 48)
VS	Contaminated aquifer, Victoria, TX	1,1-DCE, VC, *cis*-DCE, *trans*-DCE, 1,1-DCE, VC,		(82)
BAV1	Chlorinated ethene contaminate aquifer, Oscoda, MI	*cis*-DCE, t*rans*-DCE, VC, 1,1-DCE, VB[r], 1,2-DCA (cometabolize: PCE, TCE)	Can use VC as sole electron acceptor (other strains cometabolize)	(24, 81)
FL2	Red Cedar River, Okemos, MI	TCE, *cis*-DCE, *trans*-DCE (cometabolize: PCE, VC)	16S rRNA gene 99% similar to BAV1 but has a much more limited growth.	(69)
GT	Contaminate aquifer, Cottage Grove, WI	TCE, *cis*-DCE, 1,1-DCE, VC		(83)
MB	San Francisco Bay	PCE, TCE	Predominantly produces *trans*-DCE	(84)
JNA	Housatonic River	Most hexa- and heptachlorobiphenyls in Arocolor 1260 Predominantly dechlorinates *meta* position	85 distinct dechlorination reactions and 56 pathways identified	(20, 85)
DCMB5	Dioxin-polluted river sediment	HCB, PCBZ, TeCB, 1,2,3-TCB, 1,2,3,4-TCDD, 1,2,4-TCDD, 1,3-1,2- and 1,4-TCDD, PCP, TeCP, TCP, TCE	Gene CbrA was most commonly used rdh gene in dechlorination	(86)
ANAS1	Long enrichment in ANAS culture, originally from Alameda Naval Air Station, CA	TCE, DCE	Genome differences between ANAS1 and ANAS2 were predominantly in hyper variable regions	(87, 88)

Strain	Isolated From	Degradation Capabilities	Other	Ref
ANAS2	Long enrichment in ANAS culture, originally from Alameda Naval Air Station, CA	VC	Genome most similar to strain 195, ANAS1 and ANAS2 were two strains identified in ANAS enrichment culture	(87, 88)
CG1, CG4, CG5	Enrichment culture maintained with PCBs	Various PCB congeners in Aroclor 1260 Predominantly dechlorinate *meta*- and *para*- positions	Three strains do have differences in the specific congeners they are capable of dechlorinating	(79)
[a]tetrachloroethene, [b]trichloroethene, [c]dichloroethene, [d]dichloroethane, [e]dibromomethane, [f]polychlorinated dibenzodioxins, [g]dichlorophenol [h]trichlorophenol [i]chlorinated benzene [j]vinyl chloride [k]trichlorobenzene [l]tetrachlorophenol [m]pentachlorophenol [n]hexachlorobenzene [o] pentachlorobenzene, [p]tetrachlorobenzene, [q]polychlorinated biphenyls, [r]vinyl bromide, [s]tetrachlorodibenzo-*p*-dioxin				

Other bacteria phylogenetically related to *D. mccartyi*, known as *Dehalococcoides*-like bacteria, are also suspected to be capable of reductive dehalogenation. The majority of these bacteria fall within the *Dehalococcoidia* family. *Dehalogenimonas* was isolated from groundwater from a Superfund site near Baton Rouge, LA. It is capable of organohalide respiration of chlorinated ethanes (68). *Dehalobium chlorocoercia* strain DF-1 is capable of dechlorinating PCBs that contain double-flanked chlorine atoms, chlorinated benzenes, and chlorinated ethenes. *Dehalobium* could be useful in the bioremediation of PCBs because it can dechlorinate PCBs to an extent that then allows them to be more readily degraded by other pathways (21). Like *Dehalococcoides*, this strain is also easier to maintain in a co-culture (49).

Dehalococcoides spp. and *Dehalococcoides*-like bacteria have also been enriched for in laboratory cultures from contaminated and uncontaminated sediments, soils, anaerobic digesters, and aquifer material using a variety of chlorinated compounds (6, 11, 16, 48, 57, 69, 85, 88–90). For example, the KB-1 culture is highly enriched, containing at least two *Dehalococcoides* phylotypes and is capable of dechlorinating TCE to ethene

(61). Strain *o*-17 in the *Dehalococcoidia* family has been enriched but not isolated, and is capable of *ortho* dechlorination of PCBs (12, 91). The Lahn cluster was enriched with PCE from uncontaminated river sediment (70). Finally, some *Anaerolinea* are grouped as *Dehalococcoides*-like, and, while putative reductive dehalogenase genes were found in deep sea *Anaerolineales* genomes (76), it is unknown whether they, or other bacteria within the *Chloroflexi* phylum but outside the *Dehalococcoidia* family, are capable of organohalide respiration.

Dehalococcoides-like bacteria, including *Dehalococcoides mccartyi*, have been detected extensively in the environment, especially in contaminated sites (92–94). In a survey of 21 aquifers contaminated with chlorinated ethenes in North America and Europe, *Dehalococcoides* was detected in 17 of them, all of which demonstrated active dechlorination (95). At other sites, however, *Dehalococcoides* was detected but biostimulation was not effective—further emphasizing that the presence of these bacteria alone cannot guarantee particular biodegradation capabilities (96). Efforts have been made to understand why some sites have more *Dehalococcoides* bacteria than other sites and what impacts the dechlorination of specific contaminants. Indeed, a study of a river contaminated with hexachlorobenzene found that *Dehalococcoides* quantities did not correlate with hexachlorobenzene or any of the other measured geochemical parameters, indicating that there might be different or multiple parameters affecting *Dehalococcoides* numbers (97). Finally, *Dehalococcoides*-like bacteria are also thought to be common in uncontaminated soil and sediment, further clouding their link to active de-chlorination (34, 36).

2.2.2 Non-*Chloroflexi* Organohalide Respiring Bacteria

Organohalide respiring bacteria have also been found in the *Proteobacteria* and *Firmicutes* phyla (71, 83, 98, 99). The majority of these are not obligate organohalide respiring bacteria and have more diversified metabolic capabilities (17, 71, 74, 99). These bacteria use a broader range of electron donors and acceptors, including organic acids and metals (17, 71). The presence of other electron acceptors besides organochlorines has not been found to inhibit dechlorination by these organisms (17, 71). The *Proteobacteria* that can respire organochlorines are *Geobacter, Anaeromyxobacter, Desulfomonile, Desulfuromonas* and *Sulfurospirillum* (17, 71, 73, 99, 100). Compared to *Dehalococcoides*, these bacteria tend to have far fewer reductive dehalogenase genes in their genomes and may have more limited dechlorination capabilities (101). The known *Firmicutes* organohalide respiring bacteria are *Dehalobacter* and *Desulfitobacterium*, with the known metabolism of *Dehalobacter* being limited (102). There is also evidence that other *Firmicutes* may be capable of anaerobic dechlorination, though it is unknown if this dechlorination is tied to a respiratory process (75). Table 2.2 lists characteristics of other isolated organohalide respiring bacteria.

Table 2.2 Characteristics of other organohalide respiring bacteria outside the Chloroflexi phylum.

	Bacteria (Genus)	Isolated From	Degradation Capabilities	Other Information	Ref
Proteobacteria	*Geobacter*	Uncontaminated sediment	PCE, TCE, TCA, carbon tetrachloride, PCP	Electron donors: hydrogen and pyruvate Electron acceptors: nitrate, fumarate, Fe(III), malate, Mn(IV), U(VI), sulfur	(17, 103, 104)
	Anaeromyxobacter	Various sediment and soil, originally enriched with 2-chlorophenol	Chlorinated phenols	Acetate is best electron donor, but can also use hydrogen; Also use nitrate, oxygen, and fumarate as electron acceptors	(73)
	Desulfomonile	Marine sediment enriched with 3-chlorobenzoate	3-chlorobenzoate 3-bromobenzoate 2,3-dichlorobenzoate 2,5-dichlorobenzoate 3,5-dichlorobenzoate 2,3,5-trichlorobenzoate	Electron donor: lactate Electron acceptor: fumarate, sulfate, sulfite, thiosulfate, nitrate Requires sodium chloride concentration above 0.32% (w/v) but optimal at half salt water concentration	(71)
	Desulfuromonas	Pristine river sediment and chloroethene contaminated aquifer	PCE and TCE to *cis*-DCE	Electron donor: acetate, lactate, pyruvate, succinate, malate, fumarate (not hydrogen) Electron acceptor: fumarate, malate, ferric iron, sulfur, PCE, TCE	(100)
	Sulfurosprillum	Contaminated soil	PCE to *cis*-DCE with small amounts of VC	Electron donor: hydrogen and lactate Electron acceptor: sulfur, nitrate, nitrite, fumarate	(99)
Firmicutes	*Desulfitobacterium*	Chlorinate phenols (except monochlorinated)	Chlorinated phenols, TCA	Electron donors: lactate, pyruvate, formate, butyrate, crotonate, and hydrogen Other electron acceptors: sulfite, thiosulfate, sulfur, various metals	(74, 105)
	Dehalobacter	Contaminated groundwater	TeCB, TCP, TCA, TCM, Dichlorotoluenes	Hydrogen only electron donor Only other metabolic capability is the fermentation of dichloromethane	(72, 106, 107)

2.3 Chlorine Cycling in the Environment

The primary focus of organohalide respiring bacteria has been on the bioremediation of contaminated sites and the degradation of anthropogenic contaminants. Organohalide respiring bacteria, however, may also play important roles in the cycling of chlorine and carbon in the environment. Thousands of different chlorinated chemicals are produced naturally and are broadly classified as chlorinated natural organic matter (Cl-NOM) (32). Cl-NOM includes a range of aromatic and aliphatic chemicals with varying functional groups, leading in turn to a range of chemical characteristics (solubility, bioavailability, and hydrophobicity) (33, 108, 109). Cl-NOM has been found in marine, soil, and freshwater environments (reviewed in 85). Multiple studies have shown that over 14% of the chlorine that enters soil or sediment as chloride is converted to organochlorine, with coniferous soil having higher chlorine retention rates, at greater than 25% (110–112). This equates to about 0.15 to 2.8 mg organochlorine/g organic carbon in forest soil (113, 114) and 11-185 µg organochlorine/L in freshwater lakes (114).

2.3.1 Halogenation of Natural Organic Matter

Abiotic and biological sources of Cl-NOM exist, but evidence suggests that biological sources of Cl-NOM are by far the greater of the two (115, 116). Marine sponges, bacteria, and algae have been found to produce over 100 halogenated chemicals including TCE (32, 117). Terrestrial plants produce chlorinated growth hormones and insect repellents (32). Insects produce chlorinated pheromones and defense chemicals (32). In freshwater and terrestrial systems fungi and bacteria are thought to produce the majority of the Cl-NOM present (32, 118). Fungal chloroperoxidase enzymes nonspecifically chlorinate organic matter by oxidizing chloride with hydrogen peroxide to form the reactive chlorine species HOCl. Reactions of this HOCl with natural organic matter produces a range of Cl-

NOM as a function of the organic matter present in the soil or sediment (108). Laboratory studies with chloroperoxidase isolated from the fungi *Caldariomyces fumagao* show that chlorination in the presence of this enzyme results in similar Cl-NOM as that found in soil (119). Many bacteria and some fungi also produce flavin-dependent halogenases (110). These enzymes primarily halogenate aromatic compounds and synthesize specific products, resulting in a much smaller range of Cl-NOM produced by flavin-dependent halogenases compared to that produced by chloroperoxidases (110, 120). Other enzymes involved in halogenation in terrestrial and freshwater environments, albeit to a lesser extent, include vanadium-dependent haloperoxidases, SAM-dependent chlorinases, and methyl halide transferases. Metagenomic analysis has continued to uncover new putative halogenase genes (121).

The amount of Cl-NOM in soil is dependent on the amounts of both organic matter and chloride in the soil (113). Sources of chloride are either anthropogenic (*e.g.*, road salt input) or natural (impacts and input from sea salt, forest fires, and volcanic activity) (50). Distance from sources of chloride, such as the ocean, roads, or combustion sites, are therefore likely to influence the amount of Cl-NOM present in a given soil or sediment (122). For example, one study found that the amount of chloride and Cl-NOM in soil decreased exponentially as a function of distance from the ocean (123). It is unknown if a similar spatial trend exists with lakes contaminated with road salt. There is also the possibility of strong temporal patterns of Cl-NOM production, as some lakes near large metropolitan areas in northern portions of the United States can see dramatic changes in chloride concentration throughout the year (124). Oxygen, pH, water content, and total organic content may also impact the quantity and quality of Cl-NOM present (111, 113, 125). With the variety of synthesis pathways for Cl-NOM, it is expected that that the

biogeochemistry of a location will lead to variability in both concentration and chemical structure of Cl-NOM, which could in turn lead to variability with respect to lability to dechlorination and further chlorine cycling.

2.3.2 Dechlorination of Chlorinated Natural Organic Matter

Dechlorination of Cl-NOM could provide a role for organohalide respiring bacteria, or more generally dechlorinating bacteria, in the environment. While some of these bacteria do have other metabolic capabilities besides dechlorination, their ability to respire chlorinated contaminants could have developed from respiring Cl-NOM. *Dehalococcoides mccartyi* and other organohalide respiring bacteria have been found to be widespread in uncontaminated soils and sediments (34, 36). *Dehalococcoides*-like bacteria were found in 103 out of 116 uncontaminated soil samples and 67 out of 68 uncontaminated upper Midwest lake sediment samples (34, 36). *Dehalococcoides*-like bacteria were found to grow while dechlorinating Cl-NOM and their numbers also correlated with Cl-NOM concentrations in soil profiles (34). Other organohalide respiring bacteria including *Dehalobacter, Geobacter,* and *Desulfitobacterium* have also been found in uncontaminated sediments and soils (36, 39) and novel dechlorinating bacteria belonging to the *Firmicutes* phylum were enriched with chlorinated xanthones, a naturally produced chemical with a similar chemical structure to dioxins and PCBs (75). Bacteria in uncontaminated environments have also been found to dechlorinate anthropogenic chemicals. Organisms in deep sea sediments were capable of dechlorinating chlorinated phenols and trichloroethene (35). Other marine bacteria have been found capable of dechlorinating TCE and brominated phenols (9, 89). PCBs were dechlorinated by microbial communities from uncontaminated rice paddy soil (126). Different types of Cl-NOM could be the substrate for these dechlorination reactions in uncontaminated

environments. Nevertheless, while suspected organohalide respiring bacteria have been found in uncontaminated environments, the presence of these organisms does not necessarily indicate the ability to respire Cl-NOM. Indeed, the sequencing of three single *Dehalococcoidia* cells from deep sea sediments revealed that although these bacteria are similar to known organohalide respiring bacteria, they did not contain reductive dehalogenase genes (127). A better understanding of the factors that contribute to a bacterium's ability to dechlorinate both natural and anthropogenic chlorinated chemicals will help create more targeted bioremediation strategies and expand the understanding of the dechlorination capabilities of bacteria in different sites.

2.4 Reductive Dehalogenases in Organohalide Respiration

Energy conservation in organohalide respiration occurs via the activity of the reductive dehalogenase (rdh) enzyme. Rdhs are membrane-bound enzymes that function as part of the electron transport chain of organohalide respiring bacteria (128). Electrons that originate from H_2 are passed down a chain of electron carriers to the rdh, where they are then used to reduce a chlorinated electron acceptor, such as a contaminant, and in the process, release the chlorine atom as chloride (129, 130). The enzyme is located on the outside of the cell membrane so the chlorinated electron acceptors do not have to be transferred into the cell (129, 131). There are two components to this enzyme. The RdhA enzyme associates with the membrane anchoring protein RdhB (128). RdhA is the active protein and contains two iron-sulfur cluster-binding motifs, a twin-arginine signal motif to translocate the enzyme across the cell membrane, and a corrinoid co-factor (128, 132, 133). The corrinoid co-factor plays a large role in the active site and catalytic activity of RdhA. Specific corrinoid co-factors are required to create an active enzyme (134). Replacing a corrinoid co-factor with a similar one produced by a different bacteria will

result in an inactive enzyme (135). In addition, these enzymes are deactivated by even low concentrations of oxygen (133, 136, 137). Finally, the structure and gene sequence of rdh enzymes are extremely variable, but these variations are not predictive of the specificity of the enzyme (128).

Biochemical characterization of rdh enzymes through enzyme purification or cell assays have identified the degradation ability of a few of these enzymes. A limited number of rdh enzymes have been purified because of difficulties in obtaining enough biomass from which to isolate the enzymes, limiting our knowledge of them (128, 138). Table 2.3 shows existing purified rdh enzymes and their specificities. In some cases, such as that of CprA, multiple variants of an enzyme have been biochemically analyzed and the small variations in the enzyme cause slight variability in the dechlorination specificity and dechlorination pattern (139).

Table 2.3 Reductive dehalogenase genes have been biochemically characterized.

Enzyme	Genera found in	Specificity	Reference
PceA	*Sulfuospirillum, Desulfitobacterium, Dehalobacter, Dehalococcoides*	PCE to *cis*-DCE PCE to TCE in *Dehalococcoides*	(132, 133, 140, 141)
TceA	*Dehalococcoides*	TCE to ethene 1,2-dichloroethane to ethene 1,2-dibromoethane to ethene Lower dechlorination rates of various haloalkanes and haloalkenes	(141, 142)
VcrA	*Dehalococcoides*	all DCE isomers and VC to ethene, 1,2-DCA to ethene	(82)
BvcA	*Dehalococcoides*	TCE, DCE isomers, VC, and 1,2-DCA	(143, 144)
CbrA	*Dehalococcoides*	1,2,3,4-tetraCB 1,2,3-triCB	(138)
CrdA	*Desulfitobacterium*	Polychlorophenols in *ortho* position	(137)
CprA (several variations)	*Desulfitobacterium*	Polychlorinatedphenols (different purified enzymes dechlorinated at different positions)	(139, 145, 146)

While very few rdh enzymes have been purified, many more putative rdh DNA gene sequences have been found (39, 147, 148). Sequencing of *D. mccartyi* genomes

has revealed that these bacteria have 10 to 36 *rdhA* genes, some of which have a known function while several have an as yet undetermined one (14, 84, 128, 141, 144). These rdh genes are often located in highly plastic regions of the genome that show signs of horizontal gene transfer (149). Transcriptional analysis in combination with dechlorination data has also led to the prediction of novel rdh genes and their function. For example, the *mbrA* gene was shown to be upregulated 10-fold during the dechlorination of *trans*-DCE and a *dcaA* gene was identified as capable of dechlorinating dichloroethane (150, 151). To date, no rdh genes specific to PCB dechlorination have been confirmed, though several *Dehalococcoides*-like bacteria are capable of dechlorinating PCBs (91); a novel rdh gene, however, was shown to have increased transcription during PCB degradation (152).

Metagenomic sequencing and sequencing of the amplicons of degenerate rdh primers has further expanded the number of putative rdh genes identified (39, 147, 152–156). These molecular biology techniques have been especially important in starting to uncover the diversity and abundance of rdh genes in uncontaminated environments (39, 147). A survey using a suite of PCR primers for rdh genes found that even uncontaminated systems had a diversity of *rdhA* genes, including novel genes and ones similar to those found in contaminated sites (147). Novel putative rdh genes similar to those previously found in the *Chloroflexi* phylum have been identified in deep sea sediments (35, 38). Metagenomic analyses have also yielded novel rdh genes in meromictic lakes and soil (39, 157). The increased ability to deeply sequence the DNA of an environmental sample or enrichment culture will most likely continue to uncover novel rdh genes.

2.5 Dehalogenases

Dehalogenation is not always linked to respiration, but instead, to the removal of the halogen atom so that the dechlorinated organic molecule can be used as a carbon

source (reviewed in 14). The broad range of dehalogenase (as opposed to rdh) enzymes currently known make use of diverse reaction mechanisms, including hydrolytic dehalogenation, intramolecular substitution, and methyl transfers. They are found in several common soil bacteria, including *Pseudomonas, Burkholderia, Sphingomonas, Mycobacterium,* and *Rhizobium* (50, 158–165) and this process of non-respiratory reductive dehalogenation has been demonstrated in *Rhodospirillum*, *Azotobacter*, *Pseudomonas*, and *Shewanella* to remove the halogen so the carbon backbone can be aerobically used (50). Many of these genes are found on plasmids or transposable elements, making it easier for the genes to be transferred between different bacteria (166, 167). Table 2.4 summarizes commonly found dehalogenase enzymes in soil and sediment. Of these, the haloalkane dehalogenase has the most diverse substrate range, with variants having preferences towards different haloalkane chain lengths (50, 163, 168). Indeed, bacteria with haloalkane dehalogenases have been found to be capable of using over 24 chlorinated chemicals as their sole carbon sources (50). Dehalogenation can also occur cometabolically or via enzymes whose typical substrate is non-chlorinated. These include methane, ammonia, toluene, and phenol monooxygenase enzymes (50, 163, 169–173).

Non-respiratory dehalogenase enzymes have been shown to dechlorinate contaminants, including the pesticide lindane (174), herbicide atrazine (175), dichloroethane (163), and haloacetic acids (158). Bacteria with haloacid dehalogenases have been isolated from drinking water distribution systems where they can use the disinfection byproduct haloacetic acid as their sole carbon source (158–160, 176). The haloacid dehalogenase also play a role in the biodegradation of other contaminants, including chlorinated ethanes and cyclohexanes (177). The contaminant

hexachlorobenzene, a pesticide known as lindane, is dechlorinated by the haloalkane dehalognease LinB and a dehydrochlorinase LinA through different pathways (reviewed in 151). The majority of the biodegradation studies involving dehalogenase enzymes have analyzed these genes in aerobic systems; nevertheless haloalkanoates (178, 179) and chlorothalonil (180) have been found to degrade chlorinated contaminants anaerobically and other dehalogenase enzymes have been found in facultative anaerobic bacteria (50).

In uncontaminated sites, it is hypothesized that dehalogenases are capable of dechlorinating chlorinated natural organic matter (Cl-NOM) (157, 181). A metagenomic study of forest soil found the relative abundance of dehalogenases was similar to that of the nitrous oxide reductases and nitrogenase genes used in nitrogen cycling. In uncontaminated environments the most common dehalogenases were found to be haloalkane monooxygenase, 2-haloacid dehalogenase, and haloacetate dehalogenase, all of which far outnumbered the number of detected reductive dehalogenase genes, showing the importance of these enzymes in the natural chlorine cycle (157). Two-haloacid dehalogenases have also been found in the Arctic in an isolated psychrotrophic *Psudoalteromonas* sp. This enzyme was active at much lower temperatures than typically observed and was capable of dehalogenating monobromoacetic acid and 2-bromopropionic acid (182). Dehalogenases have been widely found in marine environments as well, with a haloalkane dehalogenase isolated from *Rhodobacteraceae* that further expanded on the substrate range of this enzyme to include longer haloalkanes (168). The diversity of the genes in uncontaminated environments shows that these enzymes have a broad, and largely yet to be understood, substrate range.

Table 2.4 Characteristics of dehalogenase enzymes.

Dehalogenase	Mechanism	Dechlorination Capabilities	Examples of Genera with enzymes	Reference
2-haloacid dehalogenase	Hydrolytic dehalogenation (are stereospecific)	Chlorinated carboxylic acids including haloacetic acids, 2-chloropropionate, 2-chloroacetate	*Xanthobacter* *Delftia* *Rhizobium* *Afipia* *Burkholderia* *Bradyrhizobium* *Pseduomonas*	(158–160)
Haloacetate dehalogenase	Hydrolytic dehalogenation	Halogenated acetates to produce glycolate	*Burkohlderia* *Ralstonia* *Delftia*	(167, 183, 184)
Haloalkane dehalogenase	Hydrolytic dehalogenation	Multiple chlorinated halkoalkanes ranging in length from chlorinated ethanes to octanes to corresponding alcohol Including 1,2-dichloroethane and lindane	*Xanthobacter* *Ancylobacter* *Delftia* *Rhodobacteraceae* *Sphingomonas* *Bradyrhizobium* *Mycobacterium* *Plesiocystis* *Agrobacterium*	(50, 161–165)
Chloromethane dehalogenase	Methyl transfer	Halogenated methanes	*Acetobacterium* *Dehalobium* *Methylobacterium* *Hyphomicrobium*	(185, 186)
Halohydrin dehalogenase	Intramolecular substitution	Haloalcohols and haloketones to form epoxides Including chloropranols, chloroethanol, and chloroacetone	*Pseudomonas* *Arthrobacter* *Flavobacterium* *Corynebacterium*	(187)
Haloaromatic di- and monooxygenases	Oxidative dehalogenation	Chlorinated phenols, benzoates, benzenes, PCBs, and hydroquinones	*Pseudomonas* *Burkholderia* *Alcaligenes* *Sphingomonas* *Mycobacterium*	(reviewed in 14)
Atrazine chlorohydrolase	Hydrolytic dehalogenation	Atrazine to hydroxyatrazine	*Pseduomonas* *Arthrobacter* *Nocardioides* *Ancylobacter*	(175, 188)
4-chlorobenzoyl-CoA dehalogenase	Hydrolytic dehalogenation	chlorobenzoate to hydroxybenzoate	*Pseudomonas* *Burkholderia* *Ralstonia*	(179)

2.7 Research Needs and Dissertation Summary

Based on the literature, it is clear that bacteria can dechlorinate a variety of chlorinated contaminants using both dehalogenase and reductive dehalogenase enzymes. It is also known that Cl-NOM is widely produced in uncontaminated environments and that it can serve as a substrate for dehalogenation in these environments. It is unknown, however, which dechlorination processes are dominant in contaminated and uncontaminated sites, and how these processes compare to one another. The research presented in this dissertation is intended to fill this knowledge gap. Specifically, this work compares the dechlorination capabilities and processes involved in the dechlorination of Cl-NOM in uncontaminated and contaminated sediment. It also compares the dehalogenation processes performed by bacteria exposed to different concentrations and types of Cl-NOM to understand how this impacts the ability of these bacteria to dechlorinate anthropogenic contaminants. Improved understanding of the dominant dechlorination processes under different biogeochemical conditions will help improve targeted bioremediation approaches and better predict a site's ability to dechlorinate specific contaminants. Improving our understanding of this connection between the dechlorination of Cl-NOM and contaminants can also help us better use Cl-NOM as a primer to improve biostimulation. To aid in this research and in the expansion of our knowledge of dechlorinating bacteria, a new method was developed to quickly select for organohalide respiring bacteria based on their membrane properties.

Chapter 3

Rapid Enrichment of *Dehalococcoides*-like Bacteria by Partial Hydrophobic Separation

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3.1 Chapter Summary

Organohalide respiring bacteria can be difficult to enrich and isolate, which can limit research on these important organisms. The goal of this research was to develop a method to rapidly (minutes to days) enrich these organisms from a mixed community. The method presented is based on the hypothesis that organohalide respiring bacteria would be more hydrophobic than other bacteria as they dehalogenate hydrophobic compounds. The developed method tests this hypothesis by separating a portion of putative organohalide respiring bacteria, those phylogenetically related to *Dehalococcoides mccartyi*, at the interface between a hydrophobic organic solvent and aqueous media. This novel partial separation technique was tested with a polychlorinated biphenyl-enriched sediment-free culture, a tetrachloroethene-enriched digester sludge culture, and uncontaminated lake sediment. Significantly higher fractions, up to 20.4 times higher, of

putative organohalide respiring bacteria were enriched at the interface between media and either hexadecane or trichloroethene. The selective partial separation of these putative organohalide respiring bacteria occurred after twenty minutes, strongly suggesting that the separation was a result of physical-chemical interactions between the cell surface and hydrophobic solvent. Dechlorination activity post-separation was verified by the production of *cis*-dichloroethene when amended with tetrachloroethene. A longer incubation time of 6 d prior to separation with trichloroethene increased the total number of putative organohalide respiring bacteria. This method provides a way to quickly separate some of the putative organohalide respiring bacteria from other bacteria, thereby improving our ability to study multiple and different bacteria of potential interest and improving knowledge of putative organohalide respiring bacteria.

3.2 Importance

Organohalide respiring bacteria, bacteria capable of respiring chlorinated contaminants, can be difficult to enrich, which can limit their predictable use for the bioremediation of contaminated sites. This paper describes a method to quickly separate *Dehalococcoides*-like bacteria, a group of organisms containing organohalide respiring bacteria, from other bacteria in a mixed community. From this work, *Dehalococcoides*-like bacteria appear to have a hydrophobic cell surface, facilitating a rapid (20 minute) partial separation from a mixed culture at the surface of a hydrophobic liquid. This method was verified in a polychlorinated biphenyl-enriched sediment-free culture, anaerobic digester sludge, and uncontaminated sediment. The described method can drastically reduce the amount of time required to partially separate *Dehalococcoides*-like bacteria from a complex mixed culture, improving researchers' ability to study these important bacteria.

3.3 Introduction

Incredible advances in culture-independent methods have provided a glimpse of the microbial diversity and capabilities that exist in the environment (147, 189, 190); nevertheless, microbial enrichments and isolates are still critical for advancing knowledge. Enrichments or isolates facilitate better assessment of the physiology, structure, and function of populations within a complex microbial community. In fact, the study of enrichments and isolates has resulted in discoveries as diverse as determining the enzymes responsible for degradation of the pollutant vinyl chloride (82), isolating possible anti-cancer agents (191), and understanding the cell structures of bacteria within candidate phyla with no cultured representatives (192). Unfortunately, while culturing techniques have improved, they still rely heavily on knowledge of an organism's ideal growth conditions and tend to enrich the fastest growing organisms in a sample (193). Better enrichment methods, which may combine physical and metabolic enrichment techniques, are therefore needed to facilitate the progress of biotechnological applications and enhance basic knowledge.

Differences in cell surface properties may be one way to distinguish among, and therefore separate bacteria with specific capabilities from a community. Indeed, bacteria have evolved varying cell surface characteristics to allow them to better exploit a particular niche. For example, in the medical field, bacteria that colonized implants were found to have positively charged cell surfaces to promote adhesion to negatively charged implant materials (194). Bacteria capable of degrading hydrophobic polycyclic aromatic hydrocarbons (PAHs) and petroleum compounds have been observed to be more hydrophobic than other bacteria, allowing them to increase uptake of, and promote adhesion to, PAHs (195). Although not all hydrophobic bacteria are capable of degrading

hydrophobic chemicals (196), those that are exhibit increased biodegradation rates compared to less hydrophobic strains as a result of stronger adhesion to non-aqueous phase liquids (NAPLs) (195, 197). Although the use of cell surface properties for the separation of bacteria has been practiced (198–200), these properties could be used more extensively to separate additional organisms of interest.

Organohalide respiring bacteria are one example of important organisms that can be difficult to enrich and isolate (*e.g*., (11)), and yet have a great deal of potential in biotechnological applications. Further characterization of the biochemistry and physiology of organohalide respiring bacteria should facilitate improved bioremediation of hazardous chlorinated chemicals in sediments and groundwater (201). Putative organohalide respiring bacteria have also been found in uncontaminated environments (34). Though little is known about the biochemistry and physiology of these organisms, they may also have utility in remediation applications.

Because chlorinated compounds are hydrophobic and often present in the dissolved phase at very low concentrations (34, 202), it is possible that organohalide respiring bacteria could have developed cell surface characteristics, such as hydrophobic surface moieties, that thermodynamically encourage their adhesion to hydrophobic electron acceptors. *Dehalococcoides mccartyi*, a well-studied organohalide respiring bacterium, has been found to grow at dense non-aqueous phase liquid (DNAPL) interfaces (203–205), suggesting that there might be some physiological mechanism by which this interaction occurs. In addition, in uncontaminated environments where concentrations of chlorinated compounds are very low (34, 206), a hydrophobic surface could provide an advantage, promoting the adhesion of organohalide respiring bacteria to chlorinated electron acceptors. The objective of this research was to test the hypothesis

that putative organohalide respiring bacteria have hydrophobic cell surfaces, facilitating the rapid partial separation of *Dehalococcoides*-like bacteria at an interface between the cell suspension and a hydrophobic liquid. If this hypothesis is correct and can be exploited, it would provide a method for faster separation and improved study of these bacteria.

3.4 Methods

3.4.1 Microbial Culture and Sediment

The polychlorinated biphenyl (PCB)-enriched sediment-free culture used for separation experiments was originally inoculated with sediment from the Raisin River, Michigan (28), and enriched over time with 2,3,4,5-tetrachlorobiphenyl; 2,3,4-trichlorobiphenyl; 3,4,5-trichlorobiphenyl; and 3,4-dichlorobiphenyl to produce a sediment-free culture in reduced anaerobic mineral media (RAMM) (207). Acetate (20 mM) was amended approximately every 4 months. The culture headspace was 4% H_2, which was also replaced approximately every 4 months. The culture was stored in an anaerobic chamber containing 96% N_2 and 4% H_2 (Coy Laboratory Products).

Another culture was enriched from anaerobic digester material obtained from the Empire Wastewater Treatment Plant in Minnesota. In an anaerobic chamber, digester material (5 ml) was diluted into RAMM media (65 ml) in six 100-mL serum bottles. The bottles were amended twice with 100 µM tetrachloroethene (PCE) over 2 months to enrich for organisms capable of dechlorination (presumably organohalide respiring bacteria).

Uncontaminated sediment samples were collected from Pelican Lake, MN (46° 36’ 42.7566’’N, 94° 9’ 26.4024’’W). Samples were taken at an approximate depth of 0.3 m, placed in sterile bottles, and transported to the laboratory within 24 hours for storage in an anaerobic chamber.

3.4.2 Experimental Set-up

Hydrophobic separation experiments were performed as described below with the PCB-enriched sediment-free culture, the anaerobic digester sludge, or Pelican Lake sediment. All experiments were performed in replicate, as stated below.

3.4.2.1 Separation Only

Separation experiments were performed to determine whether a variety of organohalide respiring bacteria, including *Dehalococcoides*-like bacteria, in the PCB-enriched sediment-free culture, digester enrichment culture, or uncontaminated sediment possessed a more hydrophobic cell surface than other organisms present in the sample and could therefore be partially separated through mixing with a hydrophobic solvent. For experiments with the PCB-enriched sediment-free culture, an initial sample (1 ml) was taken and centrifuged for microbial analysis (see below). A second aliquot of the culture (3 ml) was placed in a sterile 10-ml glass vial. Approximately 500 µl of a hydrophobic, non-aqueous phase liquid (NAPL), either hexadecane (HD) (log octanol-water coefficient ($\log K_{ow}$) =8.2) or trichloroethene (TCE) ($\log K_{ow}$=2.42), was added to the vial to form a continuous layer of NAPL in contact with the cell culture. TCE is denser than water and therefore formed a layer on the bottom of the vial, whereas HD is less dense and formed a layer on top of the culture (Figure A.1). The vials were shaken vigorously for 30 s and then allowed to stand for five min to allow the NAPL and aqueous layer to separate. After this initial mixing period, three cycles of gentle mixing via rolling or swirling the vials for approximately 30 s followed by standing for 3 min were performed. After the last gentle mixing period, the vials were allowed to stand an additional 5 min to allow the NAPL and aqueous layers to completely separate. A 200-µl sample of the cell culture was carefully removed via pipette from the aqueous phase at the interface of the NAPL phase

("interface") for microbial analysis (Figure A.1). In some samples the cell culture was also sampled (1 ml) at a location away from the interface ("supernatant") for DNA analysis as well (Figure A.1). When initial, interface, and supernatant samples were obtained, a mass balance over the 16S rRNA gene for *Bacteria* and *Dehalococcoides*-like bacteria was calculated. Both the interface and supernatant samples were centrifuged at 7000 rpm for 10 min to concentrate the cells present and remove any NAPL that might remain in the sample and interfere with DNA extraction. The experiment was repeated 12 times. In one set of triplicate vials, the HD layer was sampled for microbial analysis.

To verify that the volume of culture used in the separation experiment did not affect the outcome, the experiment was repeated (in triplicate) with volumes of 1, 3, and 6 ml PCB-enriched sediment-free culture, instead of the 3 ml described above, and placed into 3, 10, and 20-ml vials, respectively. These experiments were performed identically to those described above except that 0.2, 0.5, and 1-ml of NAPL were added to the vials, respectively.

Experiments were also performed in the same manner with the PCE-enriched digester culture using TCE and HD as the NAPL phase. In these experiments the viability of the bacteria present in the interface samples after performing this separation procedure was verified by repeating the experiment and resuspending the interface (approximately 200 µl) in RAMM (10 ml) and amending with 100 µM PCE. The dechlorination of the PCE and formation of the daughter products TCE and *cis*-dichloroethylene (*cis*-DCE) were then monitored with time by gas chromatography (GC). A positive control (1 ml enrichment culture in 9 ml RAMM) and negative control (10 ml sterile RAMM) were also amended with 100 µM PCE and monitored for PCE dechlorination and daughter product formation. Experiments were performed in quadruplicate in the anaerobic chamber.

For experiments with Pelican Lake sediment, bacteria were removed from the sediment prior to subjecting them to the separation procedure. Briefly, 5 ml sediment and 5 ml RAMM were mixed in a blender (Waring Commercial) for 3 min on the low speed setting. Soil particles were allowed to settle for 5 min and the liquid fraction was removed. The separation method as described above for the PCB-enriched sediment-free cell culture was performed with 3 ml of this liquid fraction. The DNA from these samples was processed using the bead beating method, as described below; therefore, the Pelican Lake samples were not centrifuged prior to DNA extraction. Only TCE was tested as the NAPL phase in experiments with sediment. The experiment was performed in triplicate.

3.4.2.2 Incubation Plus Separation

Experiments were also performed in which the PCB-enriched sediment-free culture was left in contact with the NAPL phase (TCE, HD, or HD containing 200 µM TCE (HD+TCE)) for up to one week prior to sampling and analyzing the interface. The culture contained 20 mM acetate, H_2 (4%) in the headspace, and titanium nitrilotriacetic acid (100 µM, final concentration) (208) to maintain reduced conditions in the vials, based on the color indicator resazurin (1 mg/ml).

The procedure was performed as described above for the separation only experiments, except that instead of removing the interface for analysis after 20 min, the 10-ml vials were crimped closed in the anaerobic chamber and placed on a rotator at 20 rpm for varying periods of time. All vials were started with a common initial culture that was also sampled for microbial analysis. At specified time points, triplicate vials were removed from the rotator and allowed to stand for 5 min before they were sacrificed for sampling of the interface and supernatant as described above. Experiments with HD or

HD+TCE as the NAPL phase were sampled after 1 and 7 days of incubation. Experiments with TCE as the NAPL phase were sampled after 1, 2, 3, 4, 5, 6, and 7 days of incubation.

3.4.2.3 Cycles of Incubation and Separation

Multiple incubation and separation cycles were also tested to determine whether further concentration and enrichment of putative organohalide respiring bacteria could be achieved if multiple partial separation events were combined with incubation. This procedure allowed for a longer incubation period but also prevented the build-up of byproducts by providing fresh media to the organisms present. The vials were prepared as described in the incubation plus separation experiments above. Only TCE was used as the hydrophobic phase for these experiments because it is a possible electron acceptor for some *Dehalococcoides*-like bacteria. Three ml of PCB-enriched sediment-free culture (containing 20 mM acetate) were mixed with 500 µl of TCE in a crimp top vial (10-ml) in an anaerobic chamber. The headspace of the vials contained 4% H_2. As in the week-long experiment, the total number of vials required for sacrificial analysis throughout the experiment were started with the same initial culture. The vials were mixed on a rotator in the anaerobic chamber at 20 rpm for 4 days. After the initial incubation, one set of triplicate vials underwent the separation procedure (described above) and the interface was analyzed. The remaining four sets of triplicate vials were also subjected to the separation procedure, performed in the anaerobic chamber, and each interface sample (200 µl) was transferred to a 10-ml vial containing 3 ml of sterile RAMM media and 500 µl fresh TCE. Mixing of the vials was then continued on a rotator in the anaerobic chamber for another 4 days. This 4-day incubation cycle followed by a separation step was performed 1-5 times, with all vials sacrificed for microbial analysis or transferred in triplicate.

3.4.3 Microbial Analysis

DNA from the interface, supernatant, and initial culture samples was extracted for further analysis. Samples from experiments performed with the PCB-enriched sediment-free culture or digester sludge were extracted using the FastDNA extraction kit (MP Bio); samples from experiments performed with sediment were extracted and purified using the Soil DNA extraction kit (MoBio). The HD phase was also extracted from triplicate vials in one preliminary experiment, but no DNA was detected (data not shown); therefore, subsequent sampling of the hydrophobic phases was not continued.

Previously developed qPCR primers were used to quantify the relative number of *Dehalococcoides*-like bacteria (34), *Dehalogenimonas* (68), *Desulfitobacterium* (209), and *Dehalobacter* (209) in the samples. The *Dehalococcoides*-like primers target bacteria that are phylogenetically similar to *Dehalococcoides mccartyi*, but also include *Dehalogenimonas* spp. and a broader group of the *Chloroflexi*, including organisms found in uncontaminated environments (34). The organisms targeted by these primers are almost certainly not all organohalide respiring bacteria but contain known organohalide respiring bacteria and target phylogenetically related unknown and less-studied bacteria that may in fact be organohalide respiring (210, 211). The total number of 16S rRNA genes was quantified with general bacterial primers targeting the V3 region of the 16S rRNA gene (212). The qPCR mixture (15 µl) contained 1X SYBR green MasterMix (Bio-Rad Laboratories), 100 nM of each primer, 1 mg/L of BSA, and 1 µl of undiluted template. The general qPCR cycle was a 95ºC initial denaturation for 10 min followed by 40 cycles of 95ºC denaturation for 15 s and 1 min anneal/extension at the specific annealing temperature for each primer set. A melting curve analysis was completed at the end of each run for quality control/assurance. Additionally, DNA from an interface, supernatant,

and initial culture sample were diluted (1:5, 1:10, and 1:100) and quantified to insure that PCR inhibitors did not affect quantification; there was no evidence of PCR inhibitors in the undiluted template. The number of gene copies in each sample was determined with a standard curve of tenfold dilution standards ranging from 10^9 to 10^2 for total *Bacteria* 16S rRNA genes and 10^8 to 10^0 for all other target organisms. Standards were made by ligating the 16S rRNA gene from the target organism into pGEM-T Easy vectors (Promega) according to the manufacturer instructions. This was transformed into *E. coli* JM109. Plasmids were purified using a MiniPrep Kit (Qiagen). Hoechst 33258 dye was used to stain the DNA for quantification on a TD-700 fluorometer using calf thymus as the DNA standard. Limits of quantification for *Dehalococcoides*-like bacteria, *Dehalogenimonas, Desulfitobacterium*, *Dehalobacter*, and total *Bacteria* 16S rRNA genes with these methods were $1x10^3$, $5x10^2$, $5x10^2$, $5x10^2$, and $5x10^4$ copies/ml of sample, respectively.

Illumina sequencing was also performed to determine whether specific organisms were partially selected for or against at the HD or TCE interface. Briefly, the microbial community of the initial culture, interface, and supernatant were compared using Illumina sequencing technology. The same primer sequences used to quantify the total number of 16S rRNA genes were used to sequence the V3 region of the 16S rRNA genes (212). Additionally, the primers contained the sequences required to bind to the Illumina platform, a 6 base pair tag for multiplexing, and a random sequence ranging from 1 to 6 base pairs to limit PCR bias due to the different tags (213). PCR was used to amplify the 16S rRNA gene fragment following the method outlined in Bartram et al. (213). Illumina MiSeq paired end sequencing (2x150) was completed by the University of Minnesota Genomics Center (UMGC). Analysis of the sequencing reads was completed with Quantitative Insights into Microbial Ecology (QIIME) through the Minnesota Supercomputing Institute (MSI) (214).

In summary, reads were trimmed to 125 base pairs before the forward and reverse reads were paired. All sequences with a Q score below 35 were removed. De novo OTU picking was performed using the UCLUST algorithm (215). Taxonomy was assigned to each OTU with the RDP classifier based on the most abundant sequence in the OTU (216). Spurious Illumina results were observed when the initial quantity of 16S rRNA gene copies was less than about $5x10^7$ total copies/ml of sample and the initial quantity of *Dehalococcoides*-like 16S rRNA gene copies was less than about $5x10^6$ copies/ml of sample. This was thought to be a result of background signal (36, 37) or PCR bias (213, 217, 218).

The raw sequences from the amplicon libraries were deposited in the Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/sra) under accession numbers SRR4436127-SRR4436134.

3.4.4 Chemical Analysis

Headspace samples (20 µl) were taken to quantify PCE, TCE, 1,1-DCE, *cis*-DCE, and *trans*-DCE in vials by GC with an electron capture detector. The oven temperature was maintained at 40ºC, the inlet at 300ºC, and detector at 325ºC. The nitrogen gas flow rate was 2 ml/min. Method detection limits were <100 nM for PCE and TCE and 5 µM 1,1-DCE, *cis*-DCE, and *trans*-DCE. Vinyl chloride and ethene were not quantified.

3.4.5 Data Analysis

The fraction or relative abundance of *Dehalococcoides*-like 16S rRNA genes was defined as the quantity of *Dehalococcoides*-like 16S rRNA genes divided by the quantity of total *Bacteria* 16S rRNA genes. This is generally expressed as the percentage of *Dehalococcoides*-like 16S rRNA genes out of the number of quantified *Bacteria* 16S rRNA genes. Two-tailed Student's *t*-tests were performed with Microsoft Excel to determine significance. A P value < 0.05 was considered significant.

3.5 Results

3.5.1 Separation Only

The relative abundance of *Dehalococcoides*-like organisms significantly increased at the interface of a hydrophobic liquid when compared to their abundance in initial liquid culture samples. This was true with the PCB-enriched sediment-free culture and the PCE-enriched digester sludge when both TCE (P <0.001 and P =0.02, respectively) and HD (P <0.001 for both cultures) were used as the hydrophobic liquid (Figure 3.1 and Figure 3.2, respectively). After only 20 min the ratio of the 16S rRNA genes of *Dehalococcoides*-like organisms to those of *Bacteria* increased by a factor of 4.0 and 8.1 using TCE and HD, respectively, as the NAPL in the PCB-enriched sediment-free culture, and by a factor of 6.3 and 6.2 using TCE and HD, respectively, as the NAPL in the digester sludge. Because of the short time period associated with these experiments, the interaction of the *Dehalococcoides*-like organisms with the hydrophobic liquid interface must have been a result of physical-chemical interactions rather than metabolic ones (*i.e.*, growth). HD, compared to TCE, increased the relative abundance of *Dehalococcoides*-like bacteria more effectively (P <0.005). No significant differences were observed in the relative abundance of *Dehalococcoides*-like organisms at the interface when the method was performed with larger initial culture volumes (1, 3, 6 ml of the sediment-free culture) (P=0.29). As expected, the total number of 16S rRNA genes present (*Dehalococcoides*-like and total *Bacteria*) did change in the interface sample with the initial mass used in the separation procedure; therefore, larger initial volumes could be used to obtain a larger overall quantity of 16S rRNA genes if desired. *Dehalogenimonas* and *Dehalobacter* were not detected in initial PCB-enriched sediment-free culture samples or in six randomly selected interface samples; they were present near the detection limit in the initial and

interface samples of the digester sludge. *Desulfitobacterium* was detected at levels near the detection limit in the initial and interface samples of the PCB-enriched sediment-free culture but not in samples taken from the digester sludge experiments. Because of the low levels or absence of these other putative organohalide respiring organisms from the two cultures tested, it is uncertain whether this method would result in their partial separation.

The relative abundance of *Dehalococcoides*-like organisms was also significantly (P =0.02) increased at the TCE-RAMM interface when uncontaminated sediment samples

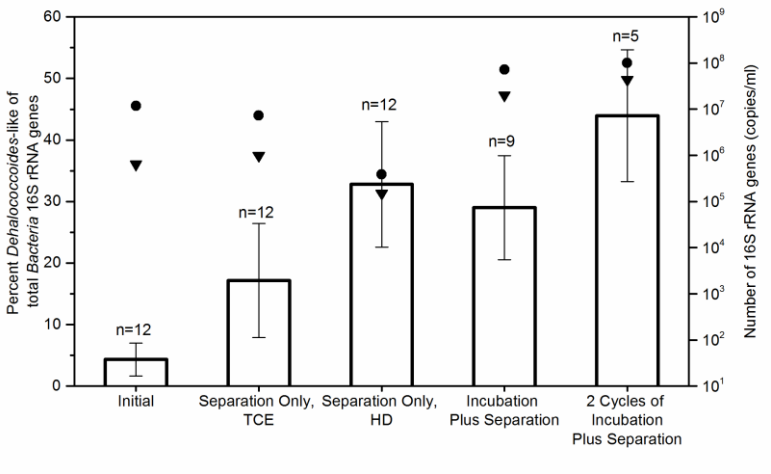

Figure 3.1 Percent Dehalococcodies-like gene for separation only method for PCB-enriched, sediment-free culture. The increase in the percent of Dehalococcoides-like 16S rRNA genes out of the total number of Bacteria 16S rRNA genes and the total number of Dehalococcoides-like 16S rRNA genes are shown for the separation only method applied to the PCB-enriched, sediment-free culture. Open bars represent the percent of Dehalococcoides-like organisms out of the total number of Bacteria l6S rRNA genes. Filled triangles (▼) show the quantity of Dehalococcoides-like 16S rRNA genes per ml sample and filled circles (●) show the quantity of total Bacteria 16S rRNA genes per ml sample. Error bars represent the 95% confidence intervals. The numbers above each bar represent the number of replicates of each experiment. The incubation plus separation is an average of the results for separation.

were subjected to the separation procedure, increasing from 8.8±2.4% to 45.7±16.2% (Figure 3.2). These results were supported by Illumina sequencing (Figure A.2), where an increase in the *Chloroflexi* was observed in interface samples. One OTU, OP8, also appeared to be partially separated at the interface (Figure A.2). The role of this phylum is unknown; nevertheless, OP8 has been observed in high concentrations in hydrocarbon-contaminated soil (40), suggesting that it might also have a niche that benefits from a

hydrophobic cell surface. *Dehalogenimonas* and *Desulfitobacterium* were present in the initial and interface samples, but at numbers below the quantification limit of $5x10^2$ copies/ml of sample. There was no detection of *Dehalobacter* in any of the sediment samples. Mass balances on *Bacteria* and *Dehalococcoides*-like organisms were calculated for several separation experiments. These data are shown in the Supporting Information (Table A.2). Overall, the number of 16S rRNA genes for *Dehalococcoides*-like bacteria and *Bacteria* in the interface samples plus that in the supernatant samples were within about an order of magnitude of those in initial samples. We considered these mass balances to be reasonable, given that we did not attempt to quantify DNA extraction efficiencies throughout our experiments, which may have been impacted by the TCE or HD present in some samples, and qPCR quantification is log-normal.

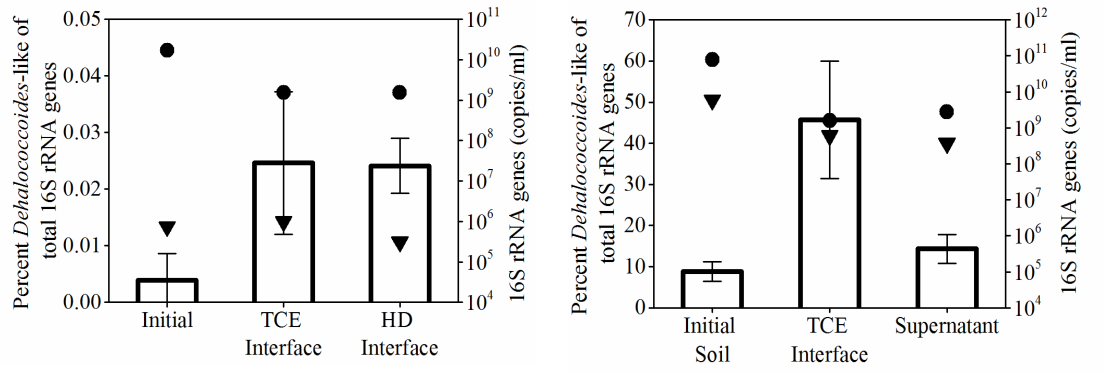

Figure 3.2 Increase in Dehaloccocoides-like for separation only method. The increase in the percent of Dehalococcoides-like 16S rRNA genes out of the total number of Bacteria 16S rRNA genes are shown for the separation-only method using (left) both TCE and HD with anaerobic digester sludge and (right) TCE with uncontaminated sediment. Filled triangles (▼) show the quantity of Dehalococcoides-like 16S rRNA genes per ml sample and filled circles (●) show the quantity of total Bacteria 16S rRNA genes per ml sample. Data for each of the separation methods with the PCB-enriched sediment-free culture are presented. Error bars represent the 95% confidence intervals. Note, the supernatant from the digester sludge experiment was not analyzed.

The organisms present in the interface samples from the PCE-enriched digester material were also assayed for dechlorination activity as a way to determine viability after the separation procedure was performed. Dechlorination was tracked by the formation of

the daughter product cis-DCE. Cis-DCE formation was delayed in both the resuspended HD and TCE interface samples (45 days) compared to that observed in the positive control (35 days). This is most likely a result of the smaller amount of biomass in the interface samples compared to that in the positive control and also the fact that there may have been some partitioning of both PCE and daughter products into the small amount of NAPL that remained in the interface samples. Cis-DCE was formed at maximum observed rates of 15.2, 7.8, and 1.5 µg cis-DCE/day in the positive control, HD interface samples, and TCE interface samples, respectively (Figure 3.3). The separation procedure did not appear to negatively impact cell activity and viability (Figure A.4). Indeed, the increase in the number of Dehalococcoides-like bacteria per mole of chloride released was higher in the TCE interface samples (4.6×1013 16S rRNA gene copies/mol chloride released or 1.4×10-7 µg cis-DCE produced/16S gene copy/day) compared to that in the positive control (8.9×10^{11} 16S rRNA gene copies/mole chloride released or 7.6×10^{-6} µg *cis*-DCE produced /16S gene copy/day) (P=0.004). No difference was seen in the increase of *Dehalococcoides*-like bacteria per mole of chloride released in the HD interface samples (8.2×10^{12} 16S rRNA gene copies/mole chloride released or 7.6×10^{-6} µg *cis*-DCE produced /16S gene copy/day) compared to the positive control (P=0.33).

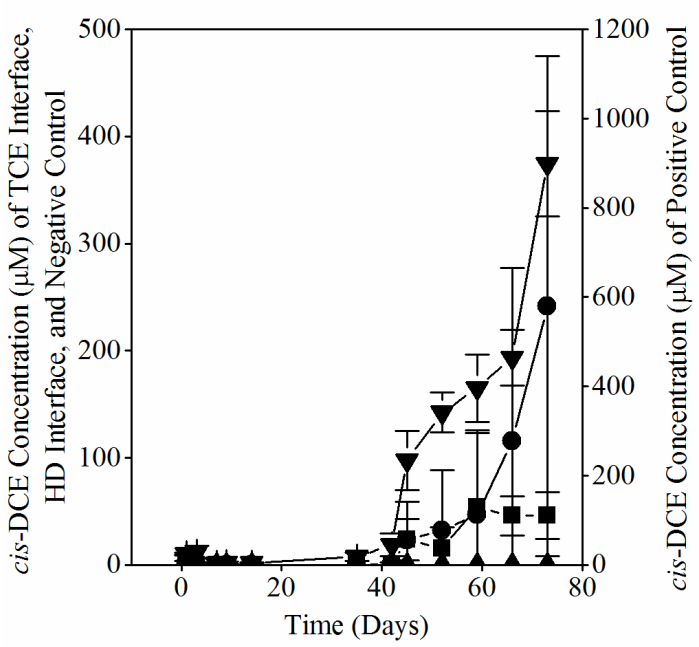

*Figure 3.3 Concentration of cis-DCE as a result of PCE dechlorination.*The concentration of cis-DCE is shown over time as a result of PCE dechlorination in the sterile negative control (▲), a positive control (▼), resuspended interface samples separated with TCE (■), and resuspended interface samples separated with HD (●).Error bars represent the 95% confidence intervals.

3.5.2 Incubation Plus Separation

When TCE was used as the hydrophobic phase and partial physical separation was combined with incubation, both the number of *Dehalococcoides*-like 16S rRNA genes and their relative abundance was affected (Figure 3.1). After a 3-hour incubation period, the total number of *Dehalococcoides*-like 16S rRNA genes increased at the interface from 9.9×10^5 to 1.3×10^7 copies/ml sample but resulted in a simultaneous decrease in the relative abundance of *Dehalococcoides*-like bacteria, dropping from 17.2% to 9.2%. For longer periods of incubation with TCE followed by the separation procedure, *Dehalococcoides*-like bacteria significantly increased in relative abundance at the interface. For example, after four days the relative abundance of *Dehalococcoides*-like bacteria increased to 34.5±10.31% (P <10^{-6} when compared to the initial culture and P

<0.001 when compared to separation only with TCE), as did the total number of *Dehalococcoides*-like 16S rRNA genes, from $9.9\times10^5 \pm 1.0\times10^5$ copes/ml sample after separation only, to $2.0\times10^7 \pm 1.5\times10^6$ copies/ml sample after 4-6 days of incubation plus separation (Figure 3.1).

This 20.4-fold increase in the number of *Dehalococcoides*-like 16S rRNA genes was observed over a four-day period, in contrast to only a 9.8-fold increase in total *Bacteria* 16S rRNA genes over the same period. This increase in *Dehalococcoides*-like 16S rRNA genes could be a result of additional partitioning and/or growth at the interface, suggesting that the *Dehalococcoides*-like bacteria were viable and were partially separated as a result of their physical cell properties and also likely were growing at the TCE-media interface. These results were also supported by Illumina sequencing (Figure A.3), where an increase in the *Chloroflexi* was observed after one week of incubation. The percentage of *Dehalococcoides*-like 16S rRNA genes decreased insignificantly in the supernatant, to 4.3%, suggesting that if growth was occurring, it did not occur in the bulk culture media or growth in the bulk media was coupled to rapid cell partitioning to the interface. After four days the total number of *Dehalococcoides*-like bacteria remained constant (Figure 3.4). This is unsurprising, as others have shown that the buildup of toxic dechlorination products during enrichment and extended exposure to saturation concentrations of TCE inhibit the growth of *Dehalococcoides mccartyi* strains (61, 219, 220).

No detection of *Dehalogenimonas* or *Dehalobacter* occurred during the incubation plus separation experiment. *Desulfitobacterium* were present but were below the quantification limit of $5x10^2$ copies/ml sample in all samples.

No increase in the relative abundance (Figure 3.4) or the total number of *Dehalococcoides*-like, *Dehalogenimonas, Desulfitobacterium*, or *Dehalobacter* 16S rRNA

genes were observed when the culture was incubated with HD or HD amended with TCE followed by separation (data not shown). The total number of *Bacteria* 16S rRNA genes did increase by approximately a factor of three in those vials.

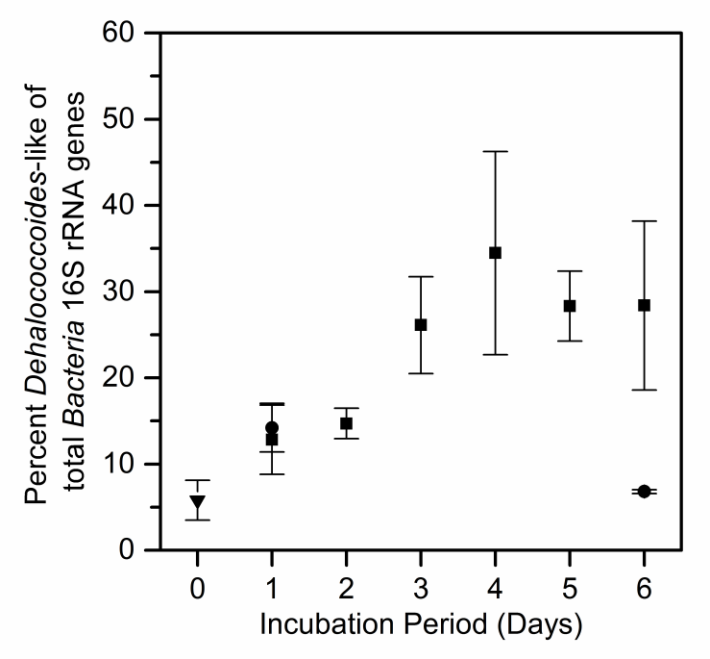

Figure 3.4 Change in Dehalococcodies-like genese for incubation plus separation experiements. The change in the percent Dehalococcoides-like 16S rRNA genes out of the total number of Bacteria 16S rRNA genes are shown for the incubation plus separation experiments with the PCB-enriched sediment-free culture. Incubation periods ranged from 1-6 days. Initial percent is shown as a filled triangle (▼), and experiments with HD and TCE are shown with filled circles (●) and filled squares (■), respectively. Error bars represent the 95% confidence intervals.

3.5.3 Cycles of Incubation and Separation

The intent of cycling incubation and separation steps was to further increase the percentage of, and possibly the total number of, putative organohalide respiring bacteria by providing a longer period of incubation at the interface while also removing inhibitors (*e.g.*, dechlorination products) that could build up over time in the vials (219). This was not observed with *Dehalococcoides*-like organisms, as they began to decrease in number after two cycles and any presumed enrichment of these organisms became very

inconsistent. Nevertheless, a consistent increase in total 16S rRNA genes was observed in these experiments, demonstrating that some other organism(s) were apparently enriched. Interestingly, although *Dehalococcoides*-like bacteria did not increase in number or relative abundance through cycles of incubation and separation, another putative organohalide respiring bacteria, *Desulfitobacterium* spp., did increase in number by over three orders of magnitude (Figure 3.5). It is therefore possible that this method could be used to gain insight into additional low-abundance organisms that may play a role in the biodegradation or reduction of hydrophobic organics. Neither *Dehalogenimonas* nor *Dehalobacter* were detected in any of the samples.

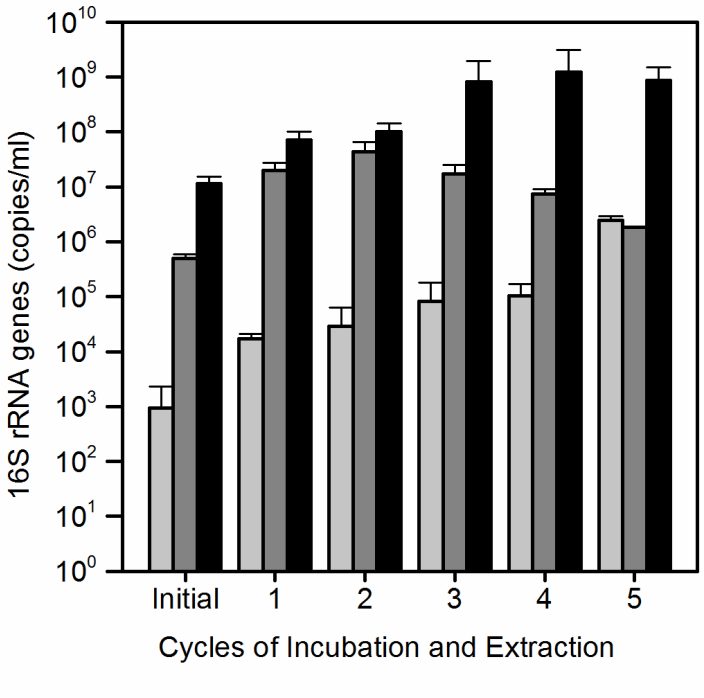

Figure 3.5 Number of genes at interface after enrichment and separation cycles. The number of 16S rRNA genes (copies/ml sample) of Desulfitobacterium (light grey), Dehalococcoides-like bacteria (medium grey), and total Bacteria (black) are shown from the interface samples after cycles of enrichment and separation with TCE. Error bars represent the 95% confidence intervals.

3.6 Discussion

This study provided evidence that *Dehalococcoides*-like bacteria and *Desulfitobacterium* spp., both of which include putative organohalide respiring bacteria, are partially separated at the interface between a NAPL and media and therefore, are likely to have a more hydrophobic cell surface than many other organisms. Indeed, the method developed herein to aid in the partial separation of *Dehalococcoides*-like bacteria was based on the assumption that these organisms might possess hydrophobic cell surface properties that would enable them to better attract and respire hydrophobic electron acceptors. A hydrophobic cell surface could provide an evolutionary advantage to *Dehalococcoides*-like, and other organohalide respiring bacteria, as has been suggested for a hydrophobic alkane degrader (11, 12, 44). Many chlorinated compounds are sparingly soluble in water, limiting their bioavailability (41). A more hydrophobic cell surface would provide a mechanism for organohalide respiring bacteria to attach to surfaces where contaminants are sorbed, perhaps enabling better contact between the cell and its hydrophobic electron acceptor.

These results also clearly showed that dechlorinating organisms remained viable after separation (Figure A.4), and that some *Dehalococcoides*-like bacteria and *Desulfitobacterium* spp. appeared to be capable of growth at the media-hydrophobic liquid interface if the hydrophobic phase was able to serve as an electron acceptor (*i.e.*, TCE). Growth of *Dehalococcoides mccartyi* at a NAPL phase has been suggested by others as well (203–205), further supporting these conclusions.

Two different hydrophobic liquids, TCE and HD, exhibited different separation capabilities, which was likely a result of HD's extreme hydrophobicity (log K_{ow}=8.2). More hydrophilic cells were potentially excluded from the interface based on their surface free

energy (15). This is supported by the lower total number of 16S rRNA genes (both *Dehalococcoides*-like and *Bacteria*) at the interface of HD and the PCB-enriched sediment-free culture compared to the number of 16S rRNA genes at the interface of TCE and the PCB-enriched sediment-free culture (Figure 3.1).

The research presented herein provides a simple and rapid method to partially separate *Dehalococcoides*-like organisms from a mixed microbial community, allowing for an improved study of *Dehalococcoides*-like bacteria, especially in environmental samples and in previously understudied niches such as uncontaminated lake sediments (34, 36). This is a group of organisms that are thought to be useful from a biotechnological perspective for the bioremediation of chlorinated contaminants and may be important for chloride and carbon cycling in the natural environment (34, 36), but can be difficult to study. Others have developed similar methods to separate cells based on surface properties to characterize a population (e.g., the Microbial Adhesion to Hydrocarbons (MATH) assay), but these are rarely used for cell enrichment and study (196, 199, 200). Indeed, the partial separation of bacteria from one another based on cell surface properties has been confined to a limited number of taxa (200) or to the selection of strains with varying properties from a single pure culture (195). This work, therefore, represents a new use of this approach and promises to improve our ability to rapidly identify and separate new organisms of interest. At its simplest, this rapid method for cell separation relies only on the interaction between the cell surface and the interface of a hydrophobic liquid, providing additional opportunities to optimize the method further by manipulating factors that change cell surface characteristics, including pH and ionic strength.

Despite exciting results, this method still has limitations. For example, the incubation and separation procedure is based on the hypothesis that the hydrophobic

liquid used can serve as an electron acceptor; without knowledge or availability of preferred electron acceptors, however, this might not be possible. Additionally, as the error bars on the figures indicate, the method can be variable, especially when multiple incubation and separation steps are used. Nevertheless, multiple individuals performed these experiments, providing validation that, although the method is variable, it is reproducible and effective. Finally, as with most methods designed to partially separate or enrich organisms of interest, this method is not able to isolate organisms of interest and is also not likely to separate all organohalide respiring bacteria. There are likely to be organohalide respiring bacteria that do not have a hydrophobic surface and bacteria incapable of organohalide respiration that do. The data presented, however, show that *Dehalococcoides*-like bacteria collected at a much higher fraction than overall *Bacteria* at the interface between aqueous culture media and a hydrophobic liquid, supporting the utility of this method for enhancing our knowledge of this important group of bacteria.

3.7 Acknowledgements

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Chapter 4

Presence, Diversity, and Enrichment of Reductive Dehalogenases and Dehalogenases in a Variety of Environments

4.1 Chapter Summary

Putative organohalide respiring bacteria have been found in many terrestrial soils and sediments and it is thought that these bacteria are capable of dechlorinating chlorinated natural organic matter (Cl-NOM). The research presented here analyzes the dechlorination processes in soil and freshwater sediment by analyzing metagenomes of bacteria enriched with Cl-NOM and metagenomes of lake sediment. The abundance of the genes encoding for enzymes capable of dehalogenation was further explored in lake sediments with varying amounts of urban impact to see how possible changes in the organochlorines present could affect the dehalogenation genes. All of the samples tested had the potential ability to dechlorinate because both reductive dehalogenase genes and non-respiratory dehalogenase genes were found in all of the samples. The reductive dehalogenase genes were shown to increase when uncontaminated lake sediment was enriched high concentrations of Cl-NOM; however, in the unenriched lake sediments that are naturally exposed to low concentrations of organochlorines, the non-respiratory dehalogenase genes were at a higher frequency. This supports that at low concentrations of organochlorines, non-respiratory dehalogenation may be more dominant than reductive

dehalogenation. Additionally, 5 of the lakes sediments with varying levels of urban impact were tested for their ability to dechlorinate trichloroethene (TCE) but only two the lakes were capable of TCE dechlorination. This differential dechlorination ability could not be explained by urban impact or gene concentration.

4.2 Introduction

Chlorinated organic chemicals, including polychlorinated biphenyls (PCBs), chlorinated ethenes, and chlorinated natural organic matter (Cl-NOM), are commonly found in soils and sediments (4, 110, 122, 221, 222). Although organochlorines can be present in high concentrations as a result of contamination events (4), many sites are impacted by low concentrations of contaminants, as a result of stormwater runoff and atmospheric deposition (223, 224). Additionally, Cl-NOM is thought to be present in most soils and sediments, made naturally by fungi, bacteria, and plants (114, 118, 225, 226). Cl-NOM is also thought to consist of several thousand different chemical structures (227), and can even include moieties typically thought of as contaminants, including TCE (228) and chlorinated phenols (108).

Bacterial dehalogenation has been well studied, with the majority of focus on contaminant dechlorination (6, 14, 27), but with some more recent attention also given to the bacterial dechlorination of Cl-NOM at uncontaminated sites (34, 75). Organohalide respiring bacteria use reductive dechlorination to obtain energy for growth by removing chlorine atoms from an organic backbone in a strictly anaerobic process (78). Interest in applying reductive dehalogenation for the bioremediation of chlorinated contaminants has led to the identification of reductive dehalogenase genes (rdhs) (229). Rdhs and organohalide respiring bacteria have also been found at uncontaminated sites, where they are thought to respire Cl-NOM (35, 38, 39). Other non-respiratory bacterial dehalogenation

processes also exist and use a variety of different dehalogenase (dh) enzymes to remove chlorine atoms from an organic backbone (18). One of the more common classes of dh enzymes are the haloalkane dehalogenases, which are capable of dechlorinating contaminants (31, 174) and may also be capable of dechlorinating Cl-NOM (40). The majority of these non-respiratory dehalogenation processes were thought to be aerobic, but recently dhs have also been found in anaerobic and microaerophilic environments (31, 230).

Understanding which of these dechlorination pathways, organohalide respiration vs. non-respiratory dechlorination, dominate under different environmental conditions could be beneficial for improving the bioremediation of chlorinated contaminants. Bioremediation strategies typically focus on stimulating organohalide respiration (92, 94); this approach has been successful with high concentration contaminant plumes; nevertheless, this process may not be as successful once contaminant concentrations become low (23). In such cases obligate organohalide respiring bacteria may no longer have enough electron acceptor to maintain an active dechlorinating population (27). Analysis of the natural chlorine cycle and how bacteria capable of dechlorination survive at uncontaminated sites where the organochlorine concentration is likely to be much lower can provide insights into which dehalogenation processes are active at low organochlorine concentration (226, 206). The goal of the work presented herein was to analyze the genes encoding rdh and dh enzymes in microbial communities that had been enriched with Cl-NOM. Additionally, sediment from lakes draining watersheds with varying levels of urbanization were analyzed for rdh and dh genes as well. These lake sediments could be receiving low concentrations of chlorinated contaminants from runoff (223, 224) and also receive runoff containing elevated chloride concentrations from road salt (124). Areas with

higher chloride concentrations have been shown to have higher concentrations of Cl-NOM (113), suggesting that these lakes could harbor bacteria with a variety of rdh and dh genes based on different concentrations of, and structures of, the organochlorines present.

4.3 Methods

4.3.1 Sample Collection and Processing

Pelican Lake sediment samples were collected at a depth of 0.2-0.5 m below the water-sediment interface. Samples were placed in sterile air-tight containers with no headspace and were transferred to an anaerobic chamber (Coy) within 12 hours of collection. The PCB-contaminated soil sample was supplied by a consulting company working on a remediation site. This sample contained low concentrations of metals (< 75 mg/kg) and moderate concentrations of total PCBs (1.43 – 104.9 mg/kg). This material was shipped to the laboratory on ice and also placed in an anaerobic chamber upon arrival.

The sediment of fourteen lakes in the Minneapolis-St. Paul, MN metropolitan area was also sampled to study the effect that the degree of urbanization had on the presence of rdh or dh genes and dechlorination capability (Table 4.1). These lakes were monitored by the Minnesota Pollution Control Agency (MPCA) for 11 to 61 years (231). The median chloride concentration in each lake in 2007 (the last year reported for all lakes) was used as an indicator of urban impact. Initial samples were taken in November 2013 at a depth of 0.2 m below the water-sediment interface. Samples were placed in an anaerobic chamber within 12 hours of collection. Five of the lakes were selected for additional sampling on April 7, May 30, July 24, September 11, and November 20, all 2016. Final samples of these five lakes were taken on July 22, 2017 and used for metagenomic sequencing. Sampling locations (Table 4.1) were the same for each sampling event and samples were always handled identically.

Within 48 hours of collection, the water and organic matter content of the sediment, and the sediment pore water concentrations of chloride, bromide, nitrate, sulfate, and phosphate, were determined as described below. DNA was extracted from each sediment sample using the MP Bio soil extraction kit, according to the manufacturer's protocol, and stored at -20ºC until analysis.

Table 4.1. Lakes sampled in November 2013. The median chloride concentration, chloride impairment, and watershed road density were published by the Minnesota Pollution Control Agency. Lakes are considered impaired for chloride if they have chloride concentrations above 230 mg/L during low flow conditions. The high risk lakes are lakes with higher chloride concentration that have been increasing (231).

Lake	Sampling Coordinates	Median 2007 chloride concentration (mg/L)	Chloride Impairments	watershed road density %
Big Marine	45.206892, -92.872495	11	no	<18
Tanners	44.953605, -92.978937	161.7	yes	29
White Bear	45.092946, -92.995624	35.3	no	<18
Gervais	45.014649, -93.075463	150	no, high risk	29
Carver	44.904675, -92.979677	190	yes	25
Bennett	45.019063, -93.141042	160	no, high risk	23
Square	45.154908, -92.795003	7	no	<18
Long	45.076739, -93.200628	151.3	yes	33
Turtle	45.091829, -93.131323	40	no	<18
Wakefield	44.994585, -93.034181	146	no, high risk	26
Johanna	45.047598, -93.173953	152.5	no, high risk	36
Centerville	45.160146, -93.081317	26	no, high risk	<18
Como	44.982706, -93.140447	192.5	yes	32
Josephine	45.034476, -93.149392	65	no	21

4.3.2 Enrichment

The PCB-contaminated soil and Pelican Lake sediment microcosms were constructed and enriched over 150 days in triplicate. Microcosms were constructed in 140-ml serum bottles containing 5 g of soil or sediment, 100 ml of reduced anaerobic mineral media (RAMM) (207), and 25 µl of either Cl-NOM or NOM (biological negative control). The Cl-NOM was synthesized from extracted NOM from Pelican Lake using chloroperoxidase enzymes, as described by Krzmarzick et.al (34). NOM-amended treatments contained the extracted

NOM subject to the same processing as the Cl-NOM, except that the chloroperoxidase enzyme was not added. Chemical negative controls were also prepared in triplicate and were identical to the Cl-NOM-amended microcosms except that the soil or sediment had been autoclaved 2 times for 45 min before it was added to the serum bottles. Dechlorination was measured over time via chloride evolution. T-tests were used to determine if the chloride evolution was significantly different from the controls. Samples were taken for DNA analysis, extracted, and analyzed as described below.

Five of the urban lake samples (Bennett, Tanners, Square, Carver, and Turtle) were also used to construct microcosms. Microcosms were again constructed in 140-ml serum bottles, but contained 10 g sediment, 70 ml of RAMM, and 100 µM TCE. Autoclaved chemical negative controls were also constructed and maintained in the same manner. All microcosms were constructed in triplicate. TCE and *cis*-DCE were measured in the microcosm headspace over time as described below. Samples were taken for DNA analysis and again extracted and analyzed as described below. A comparison of the amount of *cis*-DCE was calculated using ANOVA to determine significance.

4.3.3 DNA Analysis

Metagenomic Sequencing

Metagenomic sequencing was completed on five of the urban lake sediments and on the Pelican Lake sediment enrichments and PCB-contaminated soil enrichments that had been incubated with Cl-NOM and NOM for 150 days. For the enrichments, the triplicate microcosms were each sampled and mixed in equal volumes for pooled metagenomic analysis. Extracted DNA was submitted to the University of Minnesota Genomics Center (UMGC) for sequencing on an Illumina HiSeq with paired end sequencing at 125 bp long. Nextera XT was used for library creation. The sequences were tagged then the enrichment

cultures and lake sediments were pooled separately at equal concentrations so each sample set could be sequenced on a separate HiSeq lane (i.e., enrichments were sequenced on one lane and the lake samples were sequenced on a second lane).

The sequencing data was analyzed at the Minnesota Supercomputing Institute (MSI). The Illumina adapters and primers were removed with Scythe. The reads were quality filtered and trimmed with Sickle, using the default values for Sanger sequencing. FastQC was used to verify the read quality. The samples were then interleaved to join the forward and reverse reads. Sequences without both forward and reverse reads were discarded. IDBA was used for assembly, with a minimum k value of 52, a maximum k value of 92, and a step of 8.

Putative rdh and dh genes were identified using DIAMOND in both the quality-filtered unassembled reads and in the assembled contigs (232). A database of rdh and dh enzymes was compiled from the curated Uniprot database, and only contained enzyme sequences from bacteria that had been experimentally shown to be capable of dechlorination. This is a similar database to that used in previous research (40), with the addition of enzymes verified since the cited work was published (2016). This database included rdhs, haloalkane dehalogenases, S-2-haloacid dehalogenases, haloacetate dehalogenases, and dechlorination-specific mono- and dioxygenases. It did not include enzymes that are known to nonspecifically dechlorinate, such as methane monooxygenase genes. DIAMOND was used to align the known rdh and dh enzymes to the unassembled reads using a cutoff of 60% protein identity and an e-value of 10^{-9} (233). The abundance of each of the genes was calculated by taking the number of hits and dividing by the total number of quality-filtered reads (40). Only one metagenome was analyzed per enrichment condition, so it was not possible to perform statistics on these

results. A less-stringent cutoff of 40% protein identity was used search the assembled reads for putative rdhs and dhs, as the identified sequences were all manually checked against the NCBI database.

qPCR Primer Development and Analysis

The putative rdh, haloalkane dehalogenase, and S-2-haloacid dehalogenase genes identified from the assembled contigs were used to create qPCR primers. The IDT PrimerQuest Tool was used to identify potential qPCR primers from the sequences. The identified primers were further analyzed for the potential to form primer dimers and secondary structures that would interfere with primer binding to the DNA. Multiple primer sets were selected for each gene type and tested on pooled sample DNA. The pooled DNA used for testing consisted of equal volume aliquots of the DNA from the five July 2016 lake samples and the final samples taken from each of the enrichment cultures. This was done to allow for efficient testing of amplification with the primer sets. The samples were run using the qPCR method described below, but with a gradient of annealing temperatures ranging from 53ºC to 62ºC to determine optimal annealing temperatures. The primer sets that resulted in amplification were further analyzed for specificity by sequencing the amplicons generated with those primer sets on an Illumina Miseq at UMGC (300 bp paired end sequencing). The sequences were processed on MSI. The reads were demultiplexed based on the primer sequences and then trimmed to 50 base pairs less than the expected amplicon length using trimmomatic. The reads were further trimmed if needed, based on a Q-score of 30. The paired ends were interleaved and reads without matching paired reads were discarded. Using QIIME, the reads were clustered at 100% similarity and clusters with less than 3 reads were disregarded. The sequences were compared to the NCBI database to determine whether the correct sequence had

been amplified by the primers. Those primers that resulted in amplification of only the targeted sequences were used in further sample analysis and are shown in Table 4.2. Table B.1 shows the specificity of the primers based on the Illumina sequencing.

Primers for the quantification of the 16S rRNA genes of Dehalococcoides mccartyi, Dehalococcoidia, Desulfitobacterium, Geobacter, Anaeromyxobacter, Desulfomonile, Dehalobium, Sulfurospirillum, Desulfovibrio, Dehalogenimonas, and Dehalobacter were also used in sample analysis, in addition to determining the total 16S rRNA gene concentration (209, 234, 212, 235–240). Because these primers were obtained from the literature, they were not further tested for specificity.

The qPCR mixture was the same for all of the primer sets and contained: 1X SYBR green MasterMix (Bio-Rad Laboratories), 100 nM of each primer, 1 mg/L of BSA, and 1 µl of undiluted template. The general qPCR cycle was an initial 95ºC denaturation for 2 minutes followed by 40 cycles of 95ºC denaturation for 15 s and 30 s anneal/extension at the specific annealing temperature of each primer set (Table 4.2). A melt curve analysis was completed at the end of each run for quality control/assurance and consistently a single peak that matched the standards. The number of gene copies in each sample was determined with a standard curve of dilutions ranging from 10^8 to 10^0. These standards were purchased as gblocks from IDT, based on the sequence assembled from the metagenomes or on the known 16S rRNA gene sequences, available in the NCBI database. The general 16S rRNA gene standards for quantifying total *Bacteria* were prepared by ligating the 16S rRNA gene from *Escherichia coli* into pGEM-T Easy vectors (Promega) according to the manufacturer instructions. This was transformed into *E. coli* JM109. Plasmids were purified using a MiniPrep Kit (Qiagen).

Table 4.2 qPCR primers developed from metagenomes. The design target is the gene and location of the original gene the primers were designed from.

Primer Name	Sequence	amplicon length (bp)	annealing temp (ºC)	Design Target
TannersRdhF	ACTATCGATCCGGAGAAGGT	104	58.0	Rdh in Tanners Lake
TannersRdhR	TCCTCCTCACTCCTCATATAGC			
BennettRdhF	CGAGGTCAACAGGCTTATC	87	58.0	Rdh in Bennett Lake
BennettRdhR	GGCACGGACTTCTCATTAC			
PCBRdhF	CCTGAACAGCTATGGGAATAC	129	56.0	Rdh in PCB Contaminated Soil Cl-NOM Enrichment
PCBRdhR	CAGCCGGTAATCAATACTCC			
PelicanRdhF	GCTCGCCACCTTCATTACT	114	59.0	Rdh in Pelican Lake Cl-NOM Enrichment
PelicanRdhR	GCCGTTCCGTCCCATTT			
TannersHaDhgF	GAGAACCCTCATGGTCCTATCT	157	56.0	Haloalkane Dehalogenase in Tanners Lake
TannersHaDhgR	CAGTTCTGCATCCAGTCCAC			
PCBHaDhg1F	CCATCAAATCGGGAGCTAAA	129	59.0	Haloalkane Dehalogenase in PCB Contaminated Soil Cl-NOM Enrichment
PCBHaDhg1R	CGTATGTGGATACAGGAAAGG			
PCBHaDhg2F	GGAACGCTTGATCTTGGAA	110	55.0	Haloalkane Dehalogenase in PCB Contaminated Soil Cl-NOM Enrichment
PCBHaDhg2R	CAAGGTAAGGGCGATGATATG			
CarverHaDhgF	GTAGATGAGGGACCCAAGAA	114	56.0	Haloalkane Dehalogenase in Carver Lake
CarverHaDhgR	CACTCGATAACCTGCAACTG			
PCB 2-haloacidDhgF	GTTTCGCATCCGGGTAAA	104	58.0	2-Haloacid Dehalogenase in PCB Contaminated Soil Cl-NOM Enrichment
PCB 2-haloacidDhgR	GCTGACTTTCACGCTCAA			
Pelican 2-haloacidDhgF	CGATCCATGCCACATTCA	122	56.0	2-Haloacid Dehalogenase in Pelican Lake Cl-NOM Enrichment
Pelican 2-haloacidDhgR	GCGAACCTACGAACTGATT			

4.3.4 Analytical Methods

The water content of the collected sediments was measured by weighing the samples before and after they were dried for 4 hours at 105ºC. The organic matter loss on ignition was measured by gravimetrically comparing the dry samples before and after burning at 550ºC for 4 hours.

Ion chromatography (IC) was used to measure bromide, chloride, nitrate, phosphate, and sulfate in the pore water of the urban lake sediments within 24 hours of sample collection. IC was also used to quantify chloride release in the Cl-NOM enrichment cultures. The same method was used for both measurements. Samples were centrifuged and the supernatant was injected into a Metrohm 930 Compact IC Flex with a Metrosep A sup 5 anion separation column. The flow rate of the eluent of carbonate buffer (3.2 mM Na_2CO_3 and 1.0 mM $NaHCO_3$) was 0.7 ml/min. The detection limits for bromide, nitrate, phosphate, and sulfate were 0.005 mg/L while the detection limit for chloride was 0.01 mg/L.

TCE and *cis*-DCE was measured in the headspace of the serum bottles. Samples (10 µl) were taken via a gas-tight locking syringe (Hamilton) and samples were injected onto a gas chromatograph (HP 6890 Series) coupled to a micro electron capture detector (GC-µECD). TCE and *cis*-DCE were separated on an Rtx-1701 (Restek) column with a nitrogen flow rate of 2.5 ml/min. The oven was maintained at a constant temperature of 35ºC. The detection limits for TCE and *cis*-DCE were 0.2 µM and 0.4 µM, respectively.

4.4 Results

4.4.1 Dechlorination and frequency of rdh genes

Rdhs were present in all the samples sequenced, regardless of the degree of prior exposure to organochlorines (e.g., the PCB-contaminated soil versus the uncontaminated Pelican Lake sediment) or degree of urban impact. In fact, putative rdh genes were found in all of the enrichments, regardless of previous exposure to Cl-NOM or PCBs or whether they were enriched on NOM or Cl-NOM (Table 4.3). Similarly, the frequency of detection of putative rdh genes in the metagenomes of the five urban lakes was similar, regardless

of the degree of urbanization in the watershed (Table 4.4). This shows that all of the terrestrial environments sampled had the potential to dechlorinate.

Table 4.3 Frequency of genes in metagenomes. The numbers are copies per 10 million reads

	Pelican ClNOM	Pelican NOM	PCB ClNOM	PCB NOM
Reductive Dehalogenase	84	2	16	18
Haloalkane Dehalogenase	20	22	49	85
2-Haloacid Dehalogenase	1	1	17	10
Haloacetate Dehalogenase	2	2	1	3
Di- and Monooxygenase Dehalogenase	8	4	20	8
Other Dehalogenase	5	0.7	6	4

Table 4.4 Frequency of genes in lake sediment metagenomes. The numbers are copies per 10 million reads

	Square	Bennett	Turtle	Tanners	Carver
Reductive Dehalogenase	23	17	11	30	25
Haloalkane Dehalogenase	83	53	79	64	59
2-Haloacid Dehalogenase	12	8	20	4	7
Haloacetate Dehalogenase	23	12	23	9	13
Di- and monooxygenase Dehalogenase	11	13	17	9	12
Other Dehalogenase	8	10	5	6	9
nirK	283	47	773	244	276
nirS	13	9	41	3	7
amoA	23	6	10	7	6
amoB	26	3	15	0	8
Median Chloride	35.3	160	150	146	151.3
Watershed Road Density (percent)	>18	23	<18	29	25

Interestingly, despite their inherent, and similar, potential, the dechlorination capability of the different lakes and enrichments did vary (Figure 4.1, Figure 4.2). Indeed, the uncontaminated sediment from Pelican Lake had the ability to dechlorinate Cl-NOM, but the PCB-contaminated soil did not (Figure 4.1), with the Pelican Lake microcosms also releasing significantly more chloride in the Cl-NOM-amended versus NOM-amended microcosms (P= 0.001). This was not the case with the PCB contaminated soil (P=0.13) With respect to TCE dechlorination, Tanners Lake, the most impacted of the five, had the

greatest ability to dechlorinate TCE, while Carver and Bennett, the next-most impacted lakes, were incapable of TCE dechlorination over the 75-day incubation period (Figure 4.2). Enrichments of Turtle Lake sediment, a moderately impacted lake, was the only other lake of the five that was capable of TCE dechlorination.

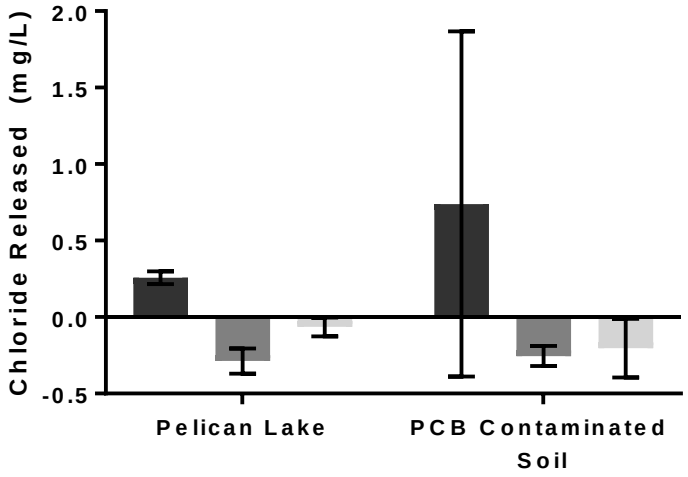

Figure 4.1. Chloride released during enrichment with Cl-NOM. Chloride released signifies Cl-NOM dechlorination. Dark grey bars are the Cl-NOM enrichments, medium grey are the NOM enrichments, and light grey are the sterile negative controls. Error bars are standard deviations of triplicate enrichments.

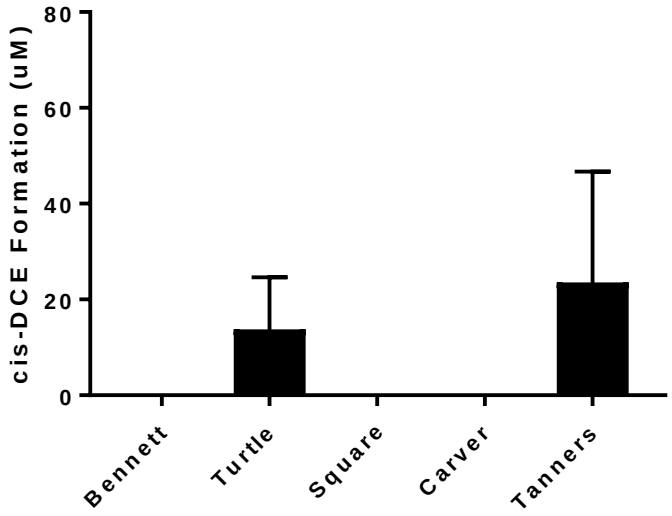

Figure 4.2. cis-DCE formation in sediment enriched with TCE. Error bars represented standard deviations of triplicate enrichments.

When analyzed quantitatively by qPCR or semi-quantitatively using the metagenomes, the results suggest that multiple rdh enzymes were present and some were differentially enriched. In the Pelican Lake microcosms, the metagenomes taken after 150 days of enrichment showed a higher frequency of rdh genes in the Cl-NOM enrichments compared to the NOM-amended controls (Table 4.1). In the metagenomes of the PCB-contaminated soil microcosms, the frequency of rdh genes was similar between the Cl-NOM and NOM enrichments (Table 4.4). This mirrored the chloride release patterns and suggested that high concentrations of Cl-NOM could enrich for rdh genes in some sediments. In contrast, the qPCR results showed that a putative rdh gene originally identified in the Pelican Lake metagenomes did increase over the 150-day enrichment, but there was no differential enrichment between the Cl-NOM- and NOM-amended treatments (P= 0.15) (Figure 4.1). This putative rdh was not present in the Cl-NOM- and NOM-amended PCB-contaminated soil after 150 days. This suggests that

multiple rdh genes were likely present and differentially enriched by Cl-NOM, with the metagenomic analysis picking up multiple rdh genes, including ones that were not specifically quantified by the qPCR method developed. Moreover, these results also suggest that the rdh gene that was quantified via the qPCR method was not the primary gene responsible for Cl-NOM dechlorination in the Pelican Lake microcosms.

With respect to the urban lakes, the metagenomes again provided a different picture than the more specific qPCR methods, but both again indicated the presence of multiple rdh enzymes. The metagenomic analysis of the five lakes showed that the total number of rdh genes across the lakes was fairly constant both based on the concentrations (Table 4.2) and based on the ratio of the total 16S rRNA genes (data not shown). The rdh quantification by qPCR in all 14 lakes, however, showed that there was variation in specific genes, with some rdh genes found in multiple lake sediments and enrichment cultures (e.g., Tanners rdh, Pelican rdh) and others only found in a single lake (Bennett rdh) (Figure 4.3). Indeed, the rdh gene originally found in the Tanners metagenome was present above the limit of quantification in 8 of the 14 lakes, an rdh found in the Pelican Lake metagenome was also detected in 9 of the 14 lake sediments, and, perhaps surprisingly, an rdh from the PCB-contaminated soil enrichments was found in 5 of the 14 lake sediments. This shows that some rdh genes were widespread, while others were much more lake-specific. In addition, variations in specific rdh genes in the 14 lakes did not correlate to the degree of urbanization in the watershed, nor were these variations predictive of the capacity to dechlorinate TCE in later experiments. The concentration of the rdh genes did not change significantly in the five lakes that were monitored over the course of the growing season (Table B.3), remaining at a relatively constant abundance, other than in November, when the total number of 16S rRNA genes

also decreased. The changes in the ratio of the genes compared to total 16S rRNA gene concentration was also calculated but the trends were similar.

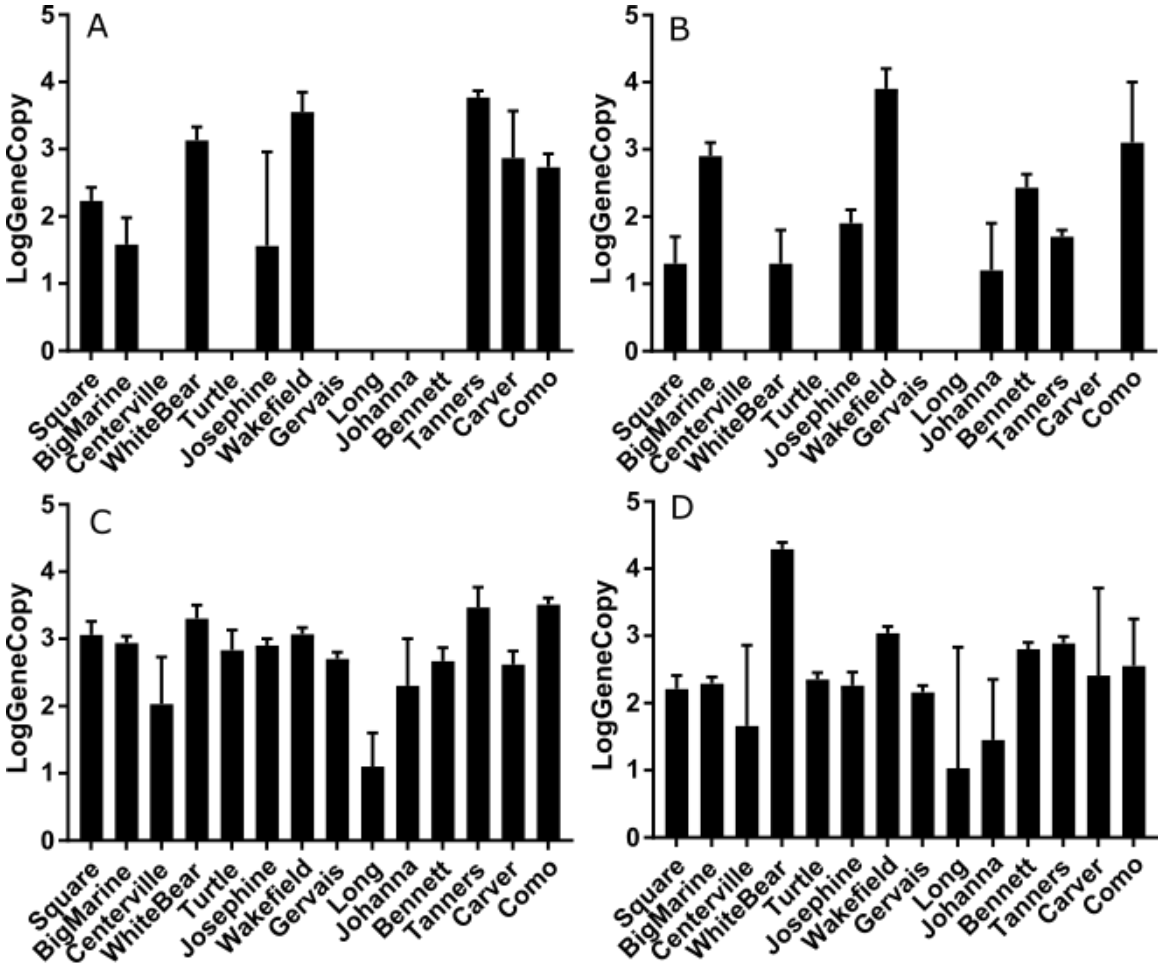

Figure 4.3. qPCR results of selected primer sets from lakes with varying urban impact. The lakes are ordered in increasing order of median salt concentration as a measure of urban impact. All measuresments are in log gene copy per gram sediment. A. Tanners rdh B. PelicanClNOM rdh C. Carver haloalkane dh D. Tanners haloalakane dh.

4.4.2 Dechlorination and frequency of dh genes

According to both the metagenomes and the qPCR data, dhs were also present in all of the enrichment culture samples and were typically more abundant than rdhs. In the metagenomes, the most common of these were the haloalkane dehalogenases (Table

4.1,Table 4.2), which hydrolytically dechlorinate haloalkanes and the 2-haloacid dehalogenases, which convert short-chain haloacids to the corresponding hydroxyacid (18). The frequency of the different types of dh genes in the metagenomes of the Cl-NOM enrichments was similar between the Cl-NOM- and NOM-amended enrichments containing Pelican Lake sediment and the PCB-contaminated soil. This again suggests that these systems have the potential to dechlorinate, although their actual Cl-NOM dechlorination ability differed. The qPCR data showed that specific dhs were enriched as Cl-NOM was dechlorinated in the Pelican Lake sediment, with two different 2-haloacid dehalogenases increasing in number significantly more in the enrichments in which Cl-NOM was dechlorinated, compared to those amended with NOM (P = 0.03, P= 0.04); one haloalkane dehalogenase also increased in number in the Pelican Lake enrichments in which Cl-NOM was dechlorinated, but this increase was not significant when compared to that observed in the NOM-amended treatments (P= 0.09) (Figure 4.4). All of the genes monitored by qPCR decreased in the Cl-NOM- and NOM-amended enrichments containing the PCB contaminated soil (data not shown). The metagenomic data did not show a general increase in dh genes during Cl-NOM dechlorination, nor did it show any differential trends in the different enrichments, again indicating that the potential for dechlorination was generally present in both the previously contaminated soil and uncontaminated sediment and only a subset of dh genes was actually responsible for Cl-NOM dechlorination in these systems.

As observed with the enrichments, both the metagenomes and the qPCR data showed that dh genes were present in all of the lakes sampled and again, were typically more abundant than rdhs. The presence of the different dh genes was similar among the five lake sediment metagenomes, with haloalkane dehalogenases again commonly detected.

The dh genes were also detected by qPCR in the 14 lakes sampled, with two of the haloalkane dehalogenases that were originally sequenced in the lake metagenomes present in all 14 lakes. One of the haloalkane dehalogenases originally sequenced in the Cl-NOM enrichments of the PCB-contaminated soil was also found in five out of the 14 lakes, but was generally at lower levels than the other haloalkane dehalogenases (Figure 4.3). The 2-haloacid dehalogenases were at lower concentrations in the lake sediments, but the qPCR primers used to quantify this dh gene were based on genes originally sequenced in the Cl-NOM enrichments. The number of haloalkane dehalogenases and 2-haloacid dehalogenases in the lake sediments did not correlate with the amount of urbanization in the watershed or with the average chloride concentrations in the lakes with the strongest correlation belonging to the PCB 2-haloacid dehalogenase (Spearmans ρ = 0.32, P-value = 0.27). In addition, the numbers of the genes present did not tend to vary over the growing season, except for the same decrease observed in November that was also observed in the total 16S rRNA gene levels and in the rdh gene levels (Table B.3).

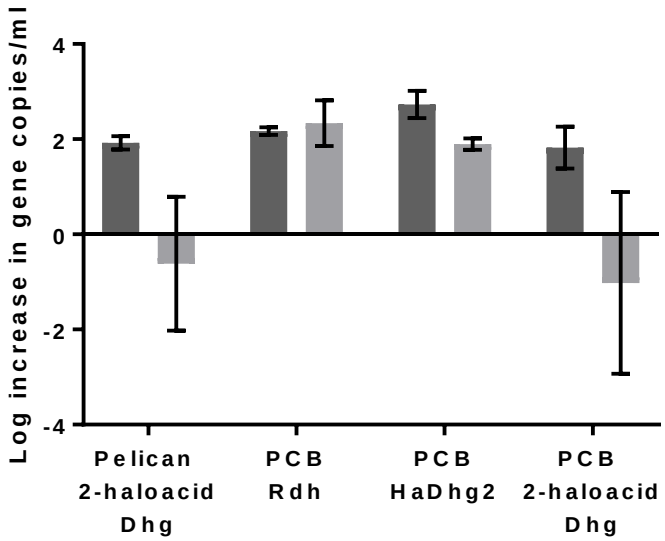

Figure 4.4. Rdh and Dh genes detected in the Cl-NOM Pelican Lake enrichment cultures. The dark grey is the Cl-NOM and the light grey is the NOM negative controls. The two 2-haloacid dehalogenase are significant at P-value < 0.05 while the haloakane dehalogenase is significant at P-value < 0.1.

4.4.3 Phylogeny of rdh and dh genes

Although *Dehalococcoides*, *Geobacter*, *Desulfomonile*, *Anaeromyxobacter*, *Desulfovibrio*, and *Dehalogenimonas* spp. were all present in numbers generally greater than 10^3 gene copies/g wet sediment (Specific Gene Concentrations in Lake Sediments

Table B.2), phylogenetically, the rdhs detected in both the enrichments and lake sediments were most similar to rdhs found in the *Firmicutes* and *Proteobacteria* phyla (Figure 4.5). One rdh found in Tanners Lake was similar to those found in *Dehalococcoides* spp.; nevertheless, the majority of the rdhs sequenced in this study grouped with those rdhs belonging to *Desulfitobacter, Dehalobacter,* and *Sulfurosprillum*. This is interesting because qPCR results showed higher numbers of 16S rRNA genes from *Dehalococcoides* and *Dehalogenimonas* spp. compared to these other organisms, suggesting that the rdhs detected may reside in other *Firmicutes* and *Proteobacteria* that

were not quantified by qPCR. Previous work has found that the number of potential organohalide respiring bacteria in both the *Firmicutes* and *Proteobacteria* phyla may be more extensive than the organohalide respiring bacteria that have been isolated to date (75). These bacteria are also known to have other metabolic capabilities besides reductive dehalogenation (17, 71, 73, 99), which could allow them to survive, and continue dechlorinating, at low organochlorine concentrations. Some of the rdhs identified in this study did not cluster with previously identified rdhs. This is unsurprising, as limited work has been performed to understand the diversity of rdh genes in uncontaminated sediments and it is therefore likely that novel rdhs could be present in these environments. The phylogeny of the rdh genes identified again did not seem to be affected by urbanization or previous organohalide exposure. For example, rdhs from Square Lake (least impacted) and Tanners Lake (most impacted) clustered near each other on two occasions.

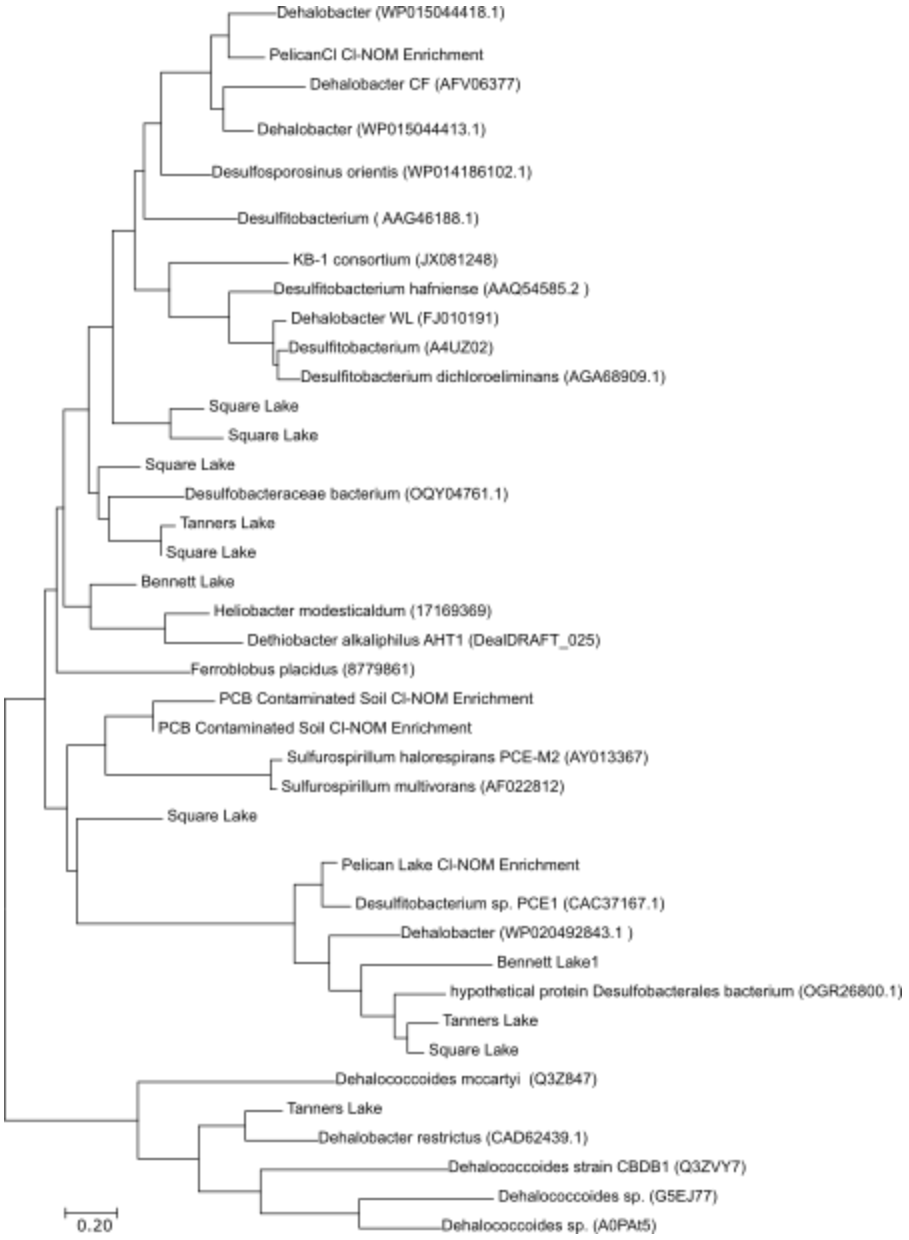

Figure 4.5 Phylogeny of the putative rdh genes sequenced in the urban lakes and the Cl-NOM enrichments. The NCBI, JGI, or UniProt ID numbers are in parenthesis. This is not a comprehensive list of all of the rdh genes and includes disproportionally more of the rdhs found in the Firmicutes phylum. Haloalkane dehalogenases were used as an outgroup. The tree was constructed using the maximum likelihood algorithm in Mega5. Sequences were aligned using Clustal.

The haloalkane dehalogenases in the lake sediments grouped separately from those in the Cl-NOM enrichments (Figure 4.6). Haloalkane dehalogenases have been

found to separate into distinct groups (241), and from the phylogenetic analysis, the haloalkane dehalogenase in the lake samples are in a different group from those in the Cl-NOM enrichments. The PCB-contaminated soil and Pelican Lake sediment, however, do contain similar genes (Figure), which could be a result of the 150-day enrichment on NOM (which would contain some Cl-NOM) or Cl-NOM prior to the metagenomic analysis. Within the lake samples, the haloalkane genes in Tanners Lake and Square Lake seem the most similar. Fewer genes were identified in Carver and Turtle Lakes, but these two lakes are also more similar to each other. No haloalkane dehalogenase genes were identified in Bennett Lake. Because dh genes are non-respiratory and are broadly distributed phylogenetically (241, 242), it is impossible to speculate on the specific types of organisms that harbor these genes and their inherent metabolic capabilities.

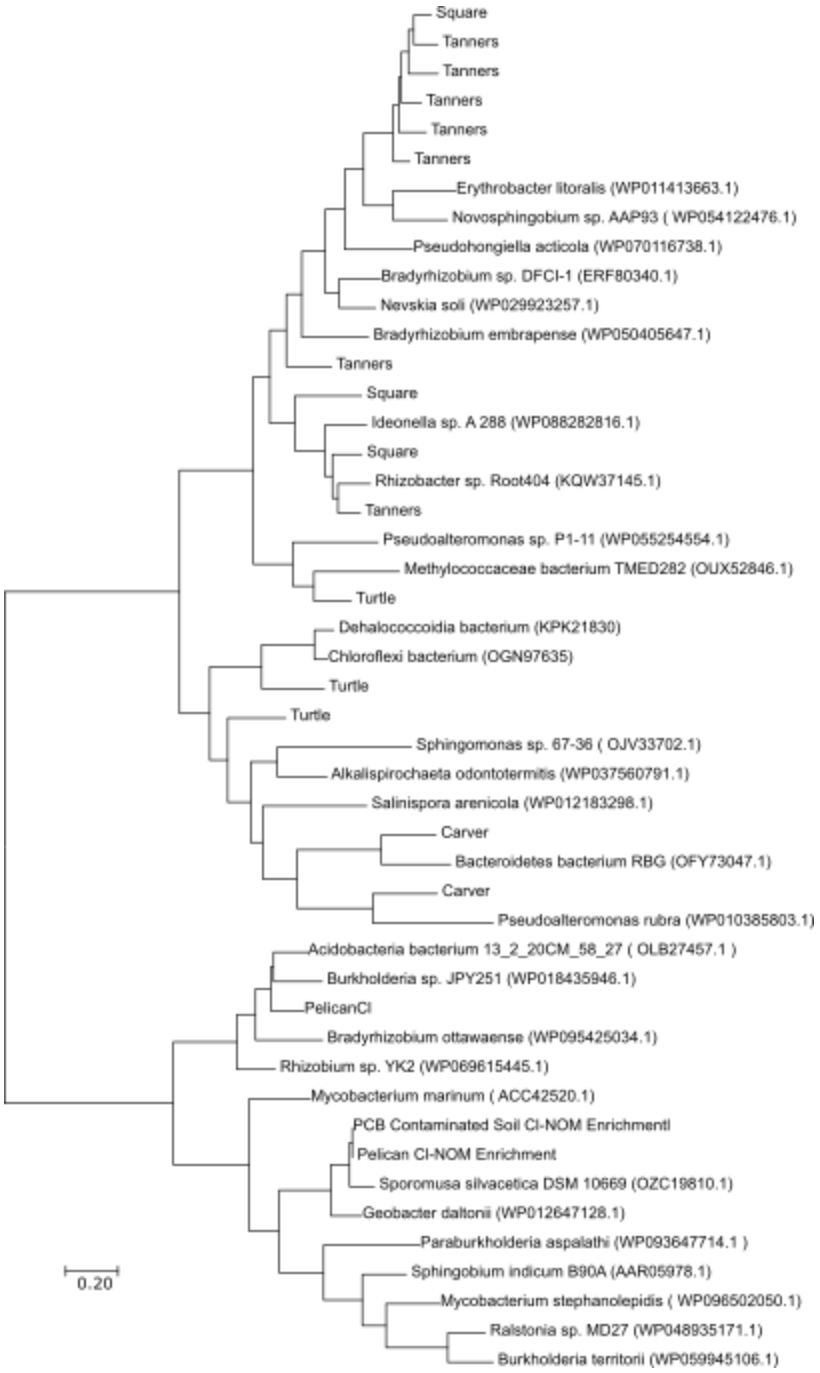

Figure 4.6 Maximum likelihood tree of haloalkane dehalogenase genes identified in the metagenomes of the urban impacted lakes and Cl-NOM enrichments. The putative haloalkane dehalogenase genes found were compared to haloalkane dehalogenase genes found in the NCBI database. Reductive dehalogenases were used as an outgroup. The tree was constructed using the maximum likelihood algorithm in Mega5. Sequences were aligned using Clustal.

4.5 Discussion

The presence of rdh and dh genes in multiple urban lakes and contaminated soil and uncontaminated sediment enriched on NOM and Cl-NOM shows that the capability for dehalogenation is widespread in the environment. Similar rdh and dh genes were found in multiple lakes and enrichments while some genes were more specific to a single location. Each single location also contained a variety of genes encoding for enzymes thought to be capable of dehalogenation. Previously published work found that the rdh genes of known dechlorinating enzymes were widely present at contaminated sites (95, 152, 243). Rdh genes have also been identified in uncontaminated marine environments where concentrations of Cl-NOM are higher (38, 39), and in select terrestrial environments (40). Known organohalide respiring bacteria have also been previously found in uncontaminated marine and terrestrial environments (35, 36, 39); nevertheless, the presence of these bacteria does not confirm the ability to dechlorinate (127). This work furthers our knowledge regarding the dechlorination potential in uncontaminated terrestrial environments, showing that this potential is widespread.

While the presence or abundance of a particular gene was not necessarily predictive of the ability to dechlorinate, both rdh and dh genes increased in detection frequency during Cl-NOM dechlorination, suggesting enrichment of these genes during dechlorination. Sediments from Tanners Lake and Turtle Lake were capable of dechlorinating TCE and had a variety of rdh and dh genes present, but a single gene could not be identified as responsible for TCE dechlorination. This shows that rdh and dh genes are likely to be involved in Cl-NOM dechlorination and may also be important for the dechlorination of the contaminant TCE. The latter is particularly interesting, as dh enzymes and genes have not been a subject of focused study in contaminated anaerobic

environments. Dh enzymes have been found to be capable of dechlorinating contaminants (163, 174, 175). In addition, in a contaminant plume with signs of active dechlorination, haloalkane dehalogenase genes were found at higher concentrations than rdh genes (31). These enzymes have primarily been studied in aerobic environments, but their presence at higher frequencies and similar individual concentrations to rdh genes in our samples and recent literature evidence that they are active in anaerobic environments suggests that dh enzymes might be as important as rdh enzymes in anaerobic environments (31, 230). Our evidence of dh gene involvement in anaerobic dechlorination processes is particularly interesting because dh enzymes and genes have not been a subject of focused study with respect to their role in anaerobic Cl-NOM cycling or role in anaerobic contaminant dechlorination.

As mentioned above, studying the natural chlorine cycle can provide insights that could be helpful for bioremediation efforts, especially the bioremediation of chlorinated contaminants at low concentrations. Organohalide respiring bacteria such as *Dehalococcoides* spp. are often found at contaminated sites where the concentration of chlorinated electron acceptors are high and are also thought to be among the most useful organisms for contaminant remediation (45, 51, 53). When the concentration of contaminant decreases at these sites, however, dechlorination can “stall”, as there may be insufficient chlorinated electron acceptor to support an active organohalide respiring population (23, 27). The results presented herein suggest that both rdh and dh enzymes from non-obligate dechlorinators are widespread and can be stimulated. Perhaps then, a focus on the stimulation of non-obligate organohalide respiring bacteria in the *Firmicutes* and *Proteobacteria* phyla and of bacteria containing non-respiratory dh genes through the supply of nutrients but limited quantities of carbon may allow for dechlorination to continue

at lower concentrations. It is possible that a more expansive view of bioremediation, including efforts at stimulating both organohalide respirers and non-respiratory dechlorinators, could be more effective in the clean-up of contaminants, particularly those present at lower concentrations.

Chapter 5

Differential Priming of Contaminant Dechlorination through Amendment of different Cl-NOM Fractions

5.1 Chapter Summary

Chlorinated natural organic matter (Cl-NOM) is thought to provide a substrate of bacteria capable of dehalogenation in uncontaminated environments. The work presented here studies what type of Cl-NOM is preferentially dechlorinated and how enrichment with different types of Cl-NOM affects the ability of bacteria to dechlorinated contaminants. The Cl-NOM was fractionated based on three hydrophobicities into three groups. Separate enrichments of PCB contaminated soil with the fractions showed that bacteria preferentially dechlorinated the least hydrophobic fraction followed by the moderate hydrophobic fraction. No dechlorination was observed in the most hydrophobic fraction. Although the least hydrophobic fraction showed the largest dechlorination, the moderate hydrophobic fraction was better primed to dechlorinate trichloroethene and tetrachlorobenzene. Community analysis of the enrichment cultures showed that although known organohalide respiring bacteria of may have played a role in the dechlorination, many other bacteria were also enriched with the Cl-NOM. Indeed, other dechlorination processes besides organohalide respiration were also shown to be important as two haloalkane dehalogenases increased over the enrichment period.

5.2 Introduction

Biological dechlorination can be effective for the remediation of chlorinated contaminants; nevertheless, there are still problems during implementation, including the stalling of dechlorination before remediation is complete (5, 244, 245). This could be a result of lowered concentrations of contaminants being unable to sustain the activity and growth of organohalide respiring bacteria, thought to be critical actors in the treatment of chlorinated contaminants (23, 27, 246). Priming the respiration of chlorinated contaminants with alternative electron acceptors, such as non-toxic chlorinated or brominated compounds, can be an effective method to stimulate dechlorination *in situ* (27). Unfortunately, the chemicals that have been thus far found to be effective primers are also toxic (27). Finding primers that are not toxic should help prevent organohalide respiration, and therefore bioremediation, from stalling prior to reaching clean-up goals.

Organohalide respiring bacteria, including *Dehalococcoides mccartyi*, *Geobacter* spp.*, Desulfitobacterium* spp.*, Dehalobacter* spp.*,* and *Dehalogenimonas* spp., have historically been a focus of bioremediation research (e.g. 7–13). These organisms make use of reductive dehalogenase (rdh) enzymes to generate energy during dechlorination and can dechlorinate multiple contaminants, including trichloroethene (TCE) (17, 83), polychlorinated biphenyls (PCBs) (49, 85), trichloroethane (105), chlorinated benzenes (8, 107), and chlorinated phenols (6). Contaminants can also be dechlorinated via the activity of non-respiratory dehalogenase (dh) enzymes (152, 163, 174). Bacteria make use of dh enzymes to remove the chlorine atom from chlorinated organics, freeing the organic base molecule for use as a carbon source for growth (reviewed in 24). These dh enzymes include haloalkane dehalogenases, which have been found to dechlorinate 24 chlorinated chemicals for subsequent use as a source of carbon for growth (18). Research on

bioremediation, however, has not focused on the activity of dh enzymes, the organisms that might contribute to bioremediation via the activity of dh enzymes, or the environmental conditions that favor dechlorination via dh enzymes. In addition, it is unknown whether primers can have a stimulatory effect on populations that dechlorinate via non-respiratory processes as well as those that make use of organohalide respiration.

Uncontaminated environments rich in chlorinated natural organic matter (Cl-NOM) could provide a source of non-toxic primers, as well as a niche for microbial populations containing both rdh and dh enzymes. Indeed, bacteria capable of degrading anthropogenic chemicals may also be able to degrade, and grow on, Cl-NOM. Several thousand chlorinated chemicals are produced naturally and might offer an opportunity to stimulate contaminant dechlorinators without the addition of toxic primers (32). Understanding the presence and enrichment patterns of rdh vs. dh genes on Cl-NOM could also enable the engineering of remediation systems to enrich bacteria that utilize both respiratory and non-respiratory processes during dechlorination depending on the contaminant type, contaminant concentration, and the bacteria present at the site.

In this research we explored whether particular fractions of Cl-NOM are preferentially dechlorinated, whether Cl-NOM could prime dechlorination of the contaminants TCE and tetrachlorobenzene (TeCB), and whether dh or rdh genes were enriched during either of these processes. The specific enrichment of various genera was also explored during both the dechlorination of Cl-NOM and dechlorination of the contaminants TCE and TeCB. This research highlights the potential for Cl-NOM to serve as a primer for contaminant dechlorination, while further illustrating the variety of bacteria involved in dechlorination and the likely involvement of both respiratory and non-respiratory processes in dechlorination.

5.3 Materials and Methods

5.3.1 Preparation of Cl-NOM

The dechlorination of different fractions of Cl-NOM was studied through the addition of both extracted NOM, which was expected to contain some natural Cl-NOM, and additional laboratory-generated Cl-NOM. Briefly, organic-rich lake sediment was dried and sequentially extracted into three fractions with (1) water ("least hydrophobic"), (2) methanol ("moderately hydrophobic"), and (3) hexane:acetone (50:50) ("most hydrophobic") using an accelerated solvent extractor (Thermo Scientific). Sequential extraction helped to limit cross over of specific chemical structures between the fractions. These fractions contained NOM from the sediment and any Cl-NOM naturally present in that sediment, and are referred to herein as "NOM". Additional Cl-NOM (herein referred to as "Cl-NOM") was generated from each individual NOM-rich fraction via the addition of hypochlorous acid (modified from 26, 27). The extraction and chlorination methods are described in detail in the Appendix C. All fractions, including the three NOM fractions and the three amended Cl-NOM fractions were cleaned via extraction through C18 columns into methanol.

5.3.2 Enrichment of soil with Different Hydrophobicities of NOM and Cl-NOM

Soil contaminated with PCBs was collected from upstate New York by a consulting firm and was stored in an anaerobic chamber (Coy) after arrival until its use. Enrichment cultures (140-ml) were set up in the anaerobic chamber and contained 5 g of soil, 100 ml of reduced anaerobic mineral media (RAMM) (207), and 25 µl of different concentrated NOM or Cl-NOM fractions. The amount of Cl-NOM could not be normalized in each fraction because of the variety of chemical structures that could be present. The headspace of the serum bottles contained 5% hydrogen and 95% nitrogen. Treatments

consisted of bottles to which the least, moderately, or most hydrophobic NOM or Cl-NOM fractions were added, for a total of 6 different treatments, each in triplicate. Sterile negative controls were autoclaved three times for 40 minutes before the addition of a mixture of the three different fractions of additional Cl-NOM.

Once started via the addition of the NOM or Cl-NOM fractions, enrichment cultures were sampled every 2-4 weeks. Sterile pipettes were used to sample the cultures, removing 1 ml and 5 ml of well-mixed slurry for DNA analysis and chloride analysis, respectively. The release of chloride was assumed to result from the dechlorination of either the amended Cl-NOM or the Cl-NOM naturally present in the NOM extracts. The sterile controls were also sampled to monitor any abiotic chloride release. The amount of chloride released in the different treatments was compared using Kruskal-Wallis test to determine if the chloride release was different between all of the treatments. Individual samples were compared using the FDR corrected *P*-value in GraphPad Prism.

5.3.3 Enrichment with Contaminants

After enrichment with the different NOM or Cl-NOM fractions for 115 days, the triplicate serum bottles for each treatment were first mixed together, then subdivided into 6 new serum bottles with 45 ml of the initial enrichment each. The total volume of half the new serum bottles was brought up to 70 ml and the total volume of the other half of the new serum bottles was brought up to 100 ml. TCE was added to each of the new 70-ml-containing bottles at 100 µM and 1,2,3,5-tetrachlorobenzene (TeCB) was added to each of the new 100-ml-containing bottles at 20 µM. Because TeCB is much less soluble in water, a second addition of 20 µM TeCB was added after 20 days. The loss of the parent compounds (TCE and TeCB) and the formation of possible daughter products (1,1-dichloroethene (1,1-DCE), *cis*-DCE, *trans*-DCE, 1,2,3-TCB, 1,3,5-TCB, 1,3-DCB, and 1,2-

DCB) were analyzed over time. The experiment lasted 65 days and samples were taken for chemical and DNA analysis. Abiotic controls were set up in the same manner. The first order degradation rate coefficients for TCE and TeCB were calculated, as were the first order formation rate coefficients for *cis*-DCE and 1,2,3-TCB. The rates were compared using Kruskal-Wallis test to determine if the rates were different. If this was significant, individual samples were compared using the FDR corrected *P*-value in GraphPad Prism.

5.3.4 DNA Analysis Methods

Samples taken for DNA extraction were centrifuged for 15 min at 8000 g. The supernatant was discarded and the pellets were extracted with the FastDNA Extraction Kit (MP Biomedicals) according to the manufacturer's protocol. The DNA was analyzed by both quantitative polymerase chain reaction (qPCR) and Illumina sequencing of the 16S rRNA gene sequence. qPCR was used to quantify three dh, one rdh gene, and the total number of 16S rRNA genes as described in Appendix C. For bacterial community analysis, Illumina paired end sequencing (2x300) was completed on the V4-V6 region of the 16S rRNA gene. Amplification and sequencing were completed at the University of Minnesota Genomics Center. Details are also provided in the Supporting Information. The community data was analyzed using multiple statistical methods, described in detail in Appendix C; in all cases a $P<0.05$ was considered significant.

5.3.5 Analytical Methods

Chloride concentration was measured with ion chromatography. Centrifuged samples were injected into a Metrohm 930 Compact IC Flex with an eluent of carbonate buffer (3.2 mM Na_2CO_3 and 1.0 mM $NaHCO_3$). Concentrations were determined via an external calibration curve. The detection limit was 0.01 mg/L.

TeCB and 1,2,3-TCB were extracted from 1 ml of the sample slurry with hexane and measured via a gas chromatograph (HP 5890) coupled to an electron capture detector (GC-ECD). Briefly, 2 ml of the culture was measured into a glass vial with a Teflon lined top. 1 ml of hexane was added to the sample and shaken for 2 min. The hexane layer was removed. This was repeated and the two hexane layers were combined and dried with sodium sulfate. Hexane extract (5 µl) was injected onto an Agilent HP-5 column via autosampler (HP 7673). The initial oven temperature was maintained at 110°C for 1 min then increased 10°C/min until 200°C where it was held for an additional 5 min. All possible degradation products of TeCB were analyzed (1,2,3-TCB, 1,3,5-TCB, 1,3-dichlorobenzen (1,3-DCB), and 1,2-DCB), but only 1,2,3-TCB was ever detected. The detection limit of each of the TCB congeners were 50 nM and the DCB congeners 100 nM.

TCE was measured via headspace injection (10 µl) onto an Agilent HP-5 column on a GC-ECD. Standards were prepared in the same way as the cultures, with different known quantities of TCE added to 70 ml pure water (milli-Q, Millipore) in sealed 140-ml serum bottles. The GC method was a constant oven temperature at 35°C. All possible isomers of DCE were also measured by this method (1,1-DCE, *trans*-DCE, and *cis*-DCE). Only *cis*-DCE was ever detected. The detection limits of the DCE congeners and TCE were 1 µM and less than 100 nM, respectively.

5.4 Results and Discussion

5.4.1 Cl-NOM dechlorination and use as a primer

Bacteria preferentially dechlorinated the fraction of Cl-NOM that was the least hydrophobic, as shown by the larger release of chloride in that fraction compared to the others (P=0.022) (Figure 5.1). The three enrichments did release significantly different amounts of chloride (Kruskal-Wallis P = 0.0036). This preferential dechlorination could be

a result of several factors: the compounds in the less hydrophobic Cl-NOM may be more bioavailable and/or the less hydrophobic compounds may be degraded by a greater variety of microbial populations. The moderately hydrophobic Cl-NOM fraction appeared to be dechlorinated at a slower rate (Figure 5.1), with less total chloride released, while the most hydrophobic Cl-NOM fraction was not dechlorinated. In all cases similar trends with respect to dechlorination and NOM hydrophobicity were observed in the treatments amended with the three different NOM fractions, but with lower total quantities of chloride released. This was expected, as the NOM extracts likely contained Cl-NOM as well, only in lower concentrations.

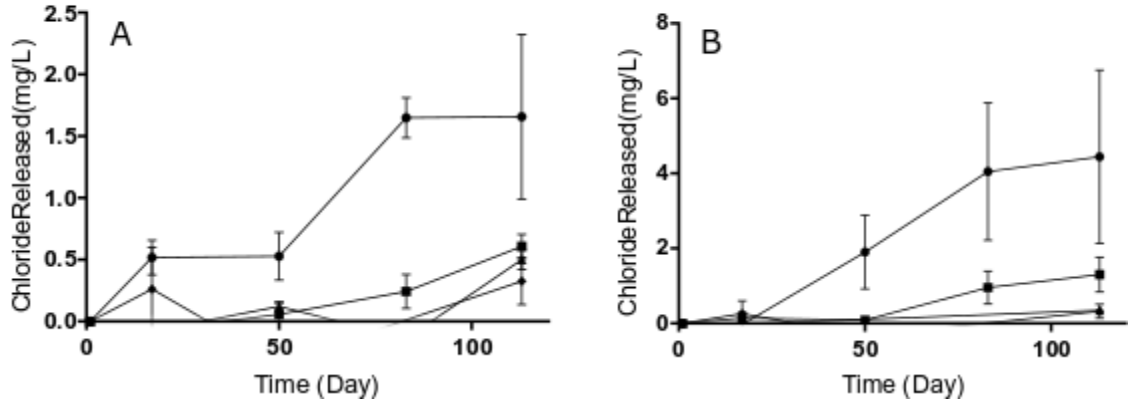

Figure 5.1 Panels A and B show the release of chloride upon Cl-NOM dechlorination with time in the enrichments amended with (A) NOM and (B) HOCl-generated Cl-NOM. Circles (●) show the least hydrophobic fraction, squares (■) show the moderately hydrophobic fraction, triangles (▲) show the most hydrophobic fraction, and diamonds (◆) show the sterile negative control. Note: the y-axes of panels A and B are on different scales to better show the chloride release with the extracted NOM, which was also expected to contain lower concentrations of naturally occurring Cl-NOM.

Not only was the Cl-NOM dechlorinated, but perhaps more importantly, it was also able to act as a primer, stimulating the dechlorination of subsequent amendments of both TCE and TeCB (Appendix C.3). Parent compound degradation rate coefficients of TCE and daughter product formation rate coefficients (cis-DCE and 1,2,3-TCB) are presented

in Table 5.2 and Table 5.3 for all of the treatments. TeCB degradation coefficients were not calculated because additional TeCB was added during the experiments. Previous enrichment with amended Cl-NOM fractions that were the least and moderately hydrophobic resulted in a larger rate of cis-DCE formation (Least: P-value = 0.065, Moderate: P-value=0.007) and more rapid onset of cis-DCE formation. Similarly, previous enrichment on the moderately hydrophobic fraction of Cl-NOM may have also stimulated the degradation of TeCB, with 1,2,3-TCB formation only being detected in the moderate hydrophobic enrichment with both Cl-NOM and NOM and the least hydrophobic Cl-NOM. The rate of formation was faster in the moderate hydrophobic Cl-NOM compared to the least hydrophobic Cl-NOM (P=0.0219). There was no significant difference between the moderate hydrophobic Cl-NOM and NOM (P=0.58) most likely caused by the large variance in the rates of formation. Previous enrichment on the moderately hydrophobic fraction of NOM also appeared to be capable of priming TeCB dechlorination, perhaps as a result of enrichment on the lower concentrations of natural Cl-NOM, which was also dechlorinated (Figure 5.1). Indeed, degradation was observed after 21 days in TeCB-amended treatments enriched with moderately hydrophobic Cl-NOM and NOM, but not until Day 36 in treatments previously enriched on the least hydrophobic Cl-NOM fraction. No 1,2,3-TCB or cis-DCE formation was observed during the 65-day experiment in the sterile treatment or the treatment previously enriched on the least hydrophobic NOM fraction. No dechlorination was observed in the treatments previously enriched on the most hydrophobic Cl-NOM or NOM fractions either, which was consistent with the observation of no Cl-NOM dechlorination in the original enrichments amended with the most hydrophobic Cl-NOM or NOM fractions. These results suggest that Cl-NOM can act as a primer for dechlorination, with some Cl-NOM fractions (particularly the moderately

hydrophobic fractions) priming more effectively than others. Although encouraging, the use of Cl-NOM to prime the bioremediation of weathered and more recalcitrant contaminants, such as PCBs, is unknown and should be investigated.

Table 5.1 First order degradation coefficient for the TCE dechlorination experiments in day^{-1}.

	Average	Standard Deviation
Least Hydrophobic Cl-NOM	0.120	0.059
Moderately Hydrophobic Cl-NOM	0.240	0.092
Most Hydrophobic Cl-NOM	0.006	0.020
Least Hydrophobic NOM	0.042	0.040
Moderately Hydrophobic NOM	0.018	0.002
Most Hydrophobic NOM	0.001	0.006
Negative	0.007	0.008

Table 5.2. Formation rate of cis-DCE in uM/day.

	Average	Standard Deviation
Least Hydrophobic Cl-NOM	1.91	0.41
Moderately Hydrophobic Cl-NOM	4.67	0.30
Most Hydrophobic Cl-NOM	0.00	0.00
Least Hydrophobic NOM	2.28	0.21
Moderately Hydrophobic NOM	2.49	0.09
Most Hydrophobic NOM	0.00	0.00
Negative	0.00	0.00

Table 5.3 Rate of formation of 1,2,3-TCB in nM/day

	Average	Standard Deviation
Least Hydrophobic Cl-NOM	0.59	0.20
Moderately Hydrophobic Cl-NOM	10.31	5.78
Most Hydrophobic Cl-NOM	0.00	0.00
Least Hydrophobic NOM	0.00	0.00
Moderately Hydrophobic NOM	3.49	3.23
Most Hydrophobic NOM	0.00	0.00
Negative	0.00	0.00

5.4.2 Microbial Community Structure and the Presence of Multiple Putative Dechlorinators

Certain suspected organohalide respiring bacteria were detected with qPCR in both the initial Cl-NOM enrichments and in subsequent TCE- or TeCB-dechlorinating enrichments. The genera *Geobacter, Dehalobacter,* and *Anaermyxobacter,* all known organohalide respiring bacteria, increased in concentration in all of the NOM- and Cl-NOM-amended treatments over time (Figure 5.2). The growth patterns of *Geobacter* spp. were the most different when comparing the NOM-amended and Cl-NOM-amended treatments (*P*-value = 0.03), with the treatments amended with the moderately hydrophobic fraction of Cl-NOM showing the most growth overall as well as more growth than the moderately hydrophobic NOM-amended treatments (*P*-value 0.024). *Geobacter* did increase in the TCE enrichments with the moderate hydrophobic Cl-NOM (ρ=0.95, *P*=0.0001) but not in any of the others. No significant increase of *Geobacter* was observed in the TeCB enrichment. *Anaeromyxobacter* spp. also showed patterns of differential growth between the NOM- versus Cl-NOM-amended treatments (*P*-value = 0.029), with more growth observed in the treatments amended with the least hydrophobic Cl-NOM (0.011) and, surprisingly, the most hydrophobic fractions of Cl-NOM at the 10% significance level (*P*= 0.010). Differential growth was not observed between the moderate hydrophobic Cl-NOM and NOM (*P*-value = 0.41) although growth did occur in both. Anaeromyxobacter did also grow in the TCE enrichments with the least hydrophobic Cl-NOM (ρ=0.94, $P < 0.0001$) and moderate hydrophobic Cl-NOM (ρ=0.95, *P*=0.0001). No growth was observed in the most hydrophobic Cl-NOM or any of the NOM enrichments. Additionally, no growth was observed in the TeCB enrichments (*P*=0.61). While *Dehalobacter* grew, there were no statistical differences in the quantity of *Dehalobacter* that grew over the experimental

period in any of the treatments (*P*=0.89). *Dehalobacter* did, however, grow in the TCE enrichments with the least hydrophobic Cl-NOM (ρ=0.95, *P*<0.0001), moderate hydrophobic Cl-NOM (ρ=0.94, *P*=0.0002), moderate hydrophobic NOM (ρ=0.95, *P*=0.0001). Growth was less significant in the most hydrophobic Cl-NOM (ρ=0.79, *P*=0.085). No growth was observed in the TeCB enrichments. It is possible that all three of these genera were stimulated by the Cl-NOM and had a role in the dechlorination, as the NOM added would have also contained Cl-NOM naturally present in the soil organic fraction (206); nevertheless, if Cl-NOM was able to serve as an electron acceptor during organohalide respiration, one would have expected these populations, including *Dehalobacter*, to grow substantially more in those treatments receiving additional HOCl-generated Cl-NOM. Although this was observed with some of the Cl-NOM fractions and *Geobacter* and *Anaeromyxobacter*, the results were more subtle than expected if these organohalide respiring organisms were playing a primary role in Cl-NOM dechlorination. Additionally, the growth patterns observed in the Cl-NOM could not explain the dechlorination and growth patterns observed in the TCE and TeCB enrichment cultures. For example, *Geobacter* did grow more in the moderate hydrophobic Cl-NOM enrichment cultures which also had the fastest TCE dechlorination; however, no significant growth was also observed in the other enrichment cultures that also showed TCE dechlorination. The commonly studied organohalide respiring bacteria *Dehalococcoides mccartyi* was not detected in any of the treatments by Illumina sequencing, neither in the initial Cl-NOM enrichments nor in the subsequent contaminant-amended enrichments. *Desulfitobacterium*, another organohalide respiring bacteria, was detected but did not grow over the enrichment period in any of the Cl-NOM or NOM enrichments (*P*=0.56).

Significant growth was observed in the TCE enrichment with the least hydrophobic Cl-NOM (ρ=0.86, *P*=0.005).

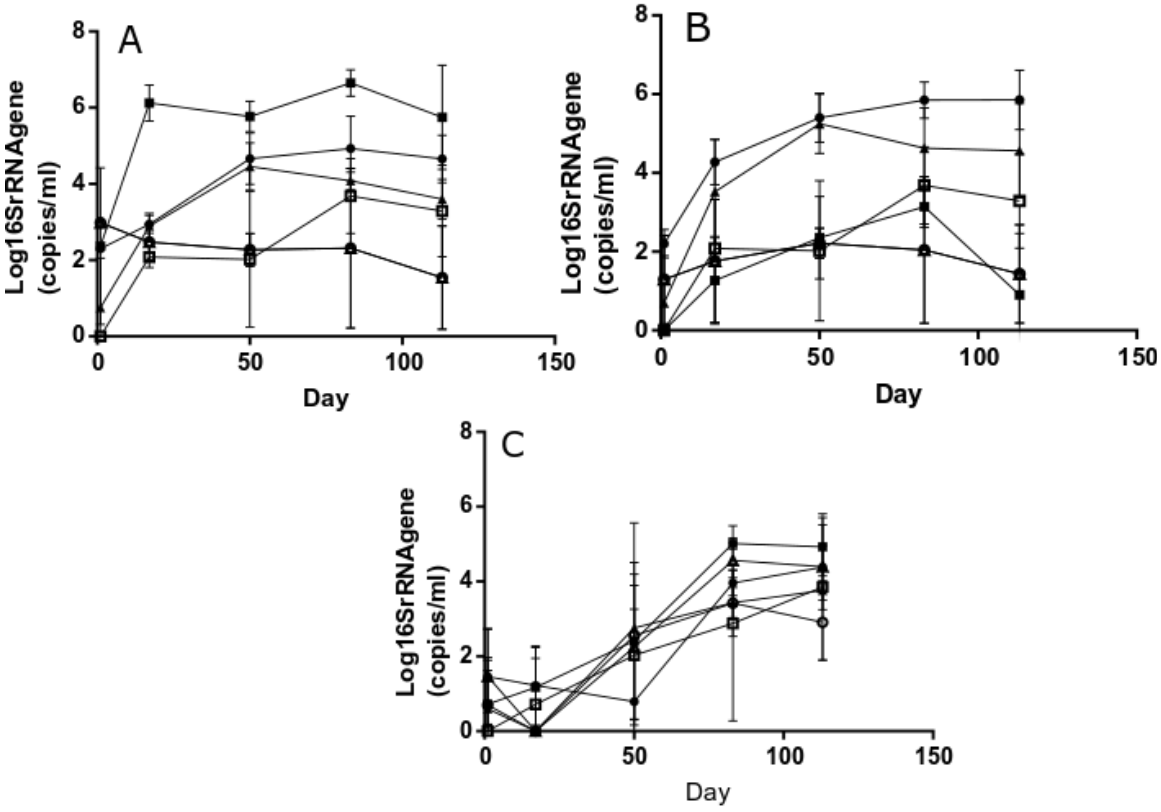

Figure 5.2 Concentration of (A) Geobacter, (B) Anaeromyxobacter, and (C) Dehalobacter in Cl-NOM/NOM enrichment cultures. Error bars represent standard deviation of triplicate enrichments. Filled symbols represent the Cl-NOM enrichments while open symbols represent the NOM enrichments. Circles (●) represent the least hydrophobic fraction, squares (■) represent the moderate hydrophobic fraction, and triangles (▲) represent the most hydrophobic fraction.

Interestingly, although the growth of known organohalide respiring bacteria could not explain all of the differences observed in the Cl-NOM- versus NOM-amended treatments, multiple genera potentially capable of non-respiratory dehalogenation also grew in the Cl-NOM-amended treatments. The Spearman's rank correlation analysis (see Appendix C.2.3, Table C.2, **Error! Reference source not found.**, and **Error! Reference**

source not found.) revealed that multiple genera grew more in treatments to which additional Cl-NOM was added Figure 5.3, as well as in treatments in which the dechlorination of TCE and TeCB appeared to have been primed with Cl-NOM addition (Figure 5.4 and Figure 5.5) The treatments amended with the least hydrophobic Cl-NOM fractions contained more genera that preferentially grew compared to the treatments amended with the moderately or most hydrophobic Cl-NOM fractions (Figure 5.3, Table C.2). Even though there was no measurable chloride release in the treatments amended with the most hydrophobic Cl-NOM fraction, some genera did grow in these treatments when compared statistically to the corresponding NOM-amended treatments. It is possible that these bacteria did participate in some dechlorination, but not enough to detect via chloride measurement. From annotated genomes available on IMG, several of the genera that grew preferentially in the Cl-NOM-amended treatments or in the primed/TCE- or TeCB-dechlorinating treatments contain non-respiratory dehalogenase genes, including haloacid dehalogenases and haloalkane dehalogenases. None of the growth observed with the genera could explain the dechlorination results of the Cl-NOM and the priming of the TCE and TeCB. This inconsistency may partially be a result of these bacteria, including the majority of the organohalide respiring bacteria, are capable of multiple metabolic schemes, allowing this bacteria to utilize other electron acceptors when an appropriate organochlorine was not present. These results are consistent with the previously discussed results of target organohalide respiring organisms and suggest that multiple populations are involved in the dechlorination of Cl-NOM, they can be primed for TCE and/or TeCB dechlorination, and non-respiratory dehalogenases may play and important, and previously unappreciated, role in chlorine cycling and contaminant degradation.

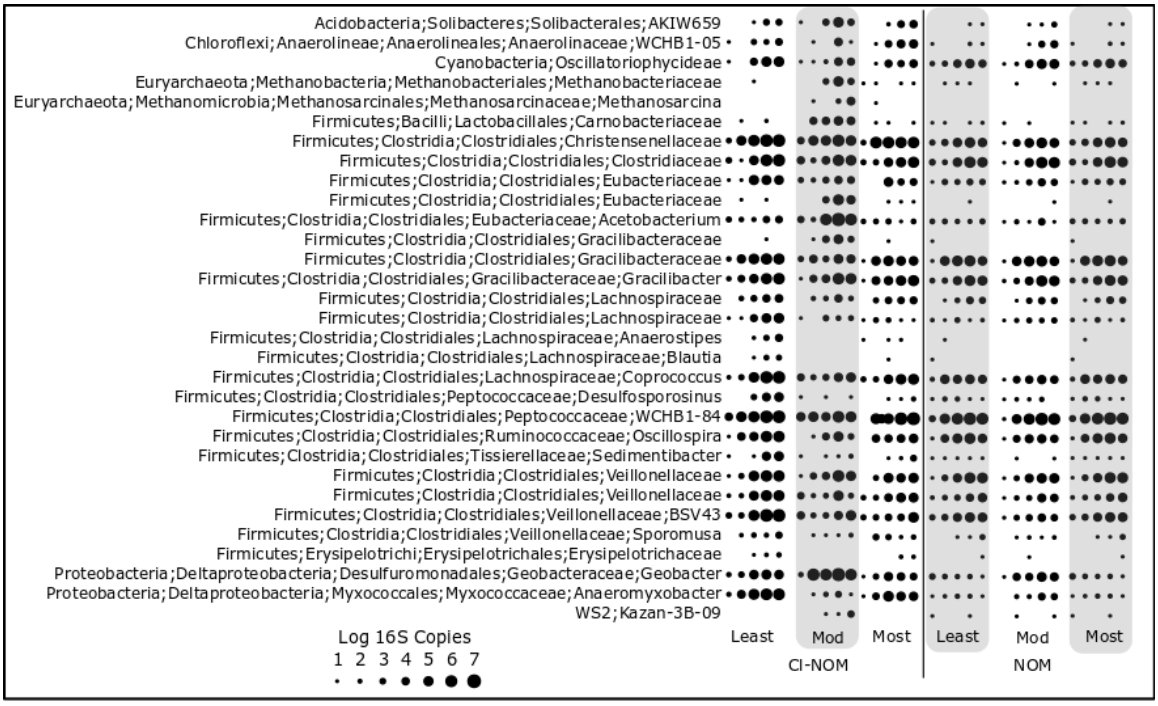

Figure 5.3 The genera that significantly grew over the enrichment period with the different hydrophobicities of Cl-NOM to a greater extend in the enrichment cultures with additional Cl-NOM compared to the NOM. The size of the dot indicates the log concentration of the 16S rRNA gene per ml of enrichment culture. Each dot corresponds to a day where a measurement was taken (1, 17, 50, 83, and 113)

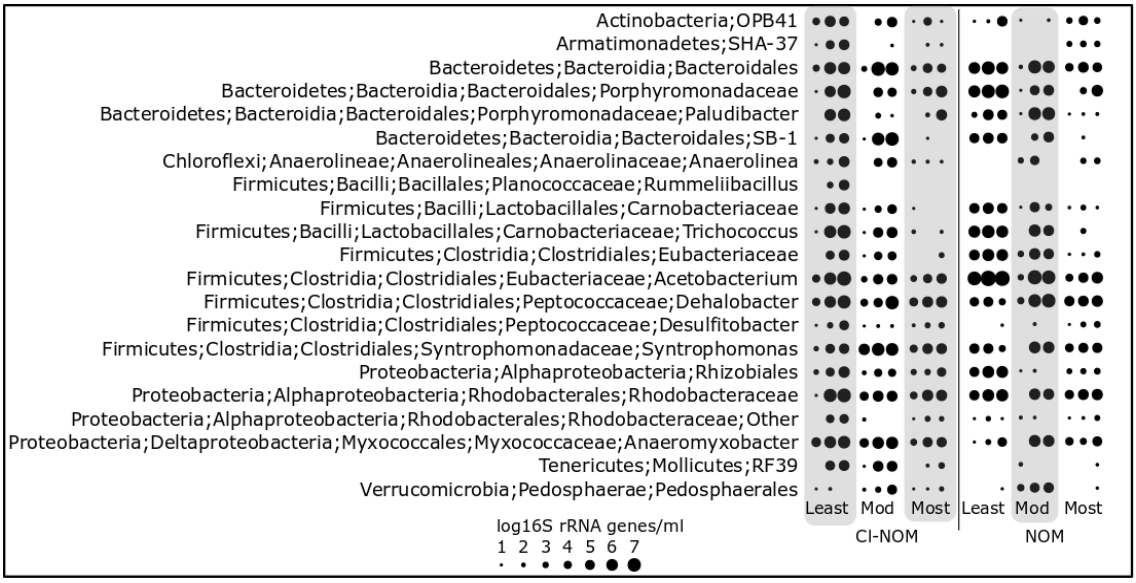

Figure 5.4 The genera that significantly grew in the TCE enrichments when more TCE was dechlorinated. The size of the dot indicates the log 16S rRNA genes per ml of enrichment culture. The complete phylogeny of each genera could not be determined. Each dot represents the concentration on the day sampled (1, 20, 50).

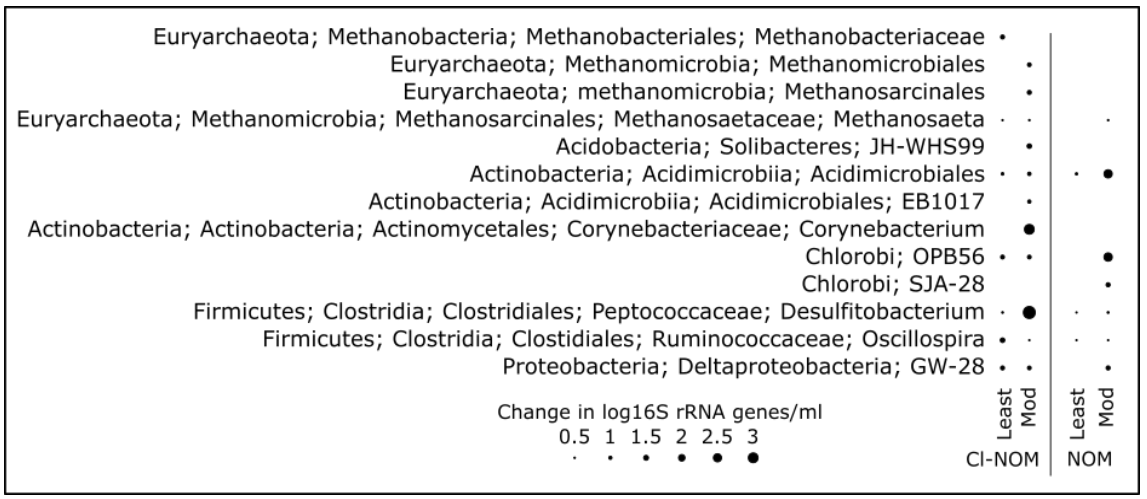

Figure 5.5 The increase in genera that significantly grew more (Kurskall-Wallis test, P<0.05) in the enrichments with TeCB dechlorination over the 50 day enrichment period. None of these genera showed growth in the most hydrophobic fraction.

5.4.3 Dehalogenase genes stimulated

Because the microbial community results suggested the enrichment of organisms likely to contain non-respiratory dehalogenases in addition to possible organohalide respiring bacteria, qPCR was used to determine whether there was evidence of dh or rdh genes increasing over the enrichment period. Four primer sets were previously developed based on metagenomic analysis of the PCB-contaminated soil used in these experiments (Developed in Chapter 4). Of these, the two haloalkane dehalogenase genes showed increased over the period of Cl-NOM dechlorination. The results varied between replicate treatment, but the moderately hydrophobic Cl-NOM-amended treatments had both the largest average gene number increases and the smallest variability between replicates for these two haloalkane dehalogenases (Figure 5.6). When the Cl-NOM enrichments were compared to the NOM enrichments, one of the haloalkane dehalogenase genes (PCB HAdhg 1) significantly increased at the 95% confidence level (P=0.047) while the other (PCB HAdhg2) was only significant at the 90% confidence level (P=0.085). Neither the least hydrophobic or most hydrophobic fractions were statistically different between the Cl-NOM and NOM enrichments. The same haloalkane dehalogenase genes were present in the TCE- and TeCB-amended treatments but did not increase over time. The increase in both of these haloalkane dehalogenase genes suggests that they might be active in dechlorinating moderately hydrophobic Cl-NOM compounds. A 2-haloacid dehalogenase gene (PCB 2-haloacidDhg primer set) was detected in all of the least and moderate hydrophobicity Cl-NOM and NOM enrichments but did not increase with time or chloride evolution. An rdh gene (PCBrdh primer set) was detected at the end of the enrichment with the most hydrophobic Cl-NOM and NOM fractions with average rdh quantities on Day 113 of $3.1x10^3 \pm 1.4x10^3$ and $3.8x10^3 \pm 3.7x10^3$ gene copies/ml slurry, respectively. The

rdh genes was also detected in the TCE and TeCB enrichments but were not correlated with degradation. While we observed the enrichment of two dh genes, it is likely that other, unidentified genes were also active in this process.

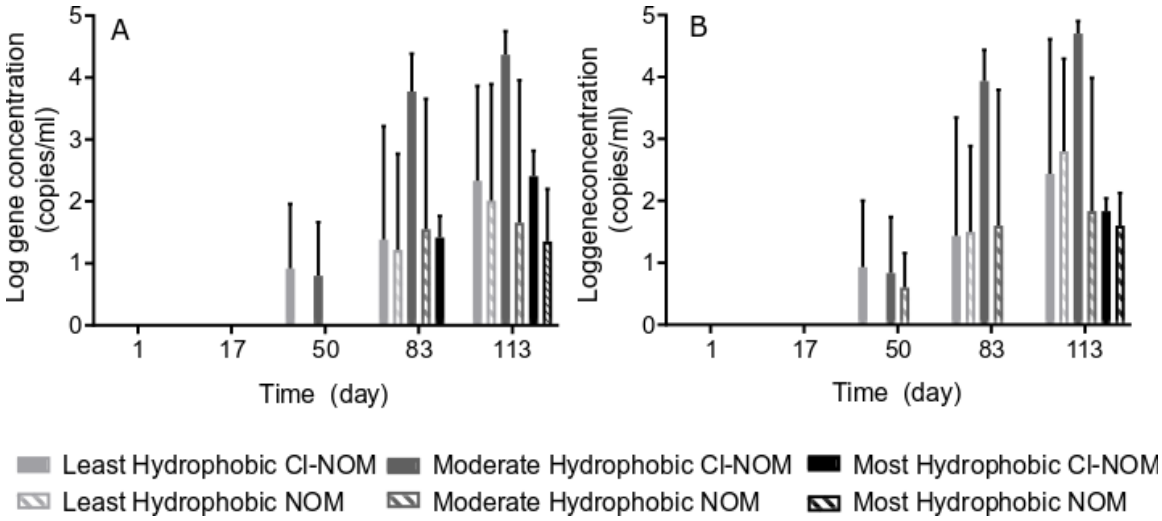

Figure 5.6 Increase in haloalkane dehalogenases of primer sets (A) PCB HAdhg1 and (B) PCB HAdhg2. Error bars are standard deviation of triplicate enrichments.

5.4.4 Environmental Implications

Previous results have shown that bacteria phylogenetically similar to *Dehalococcoides* are enriched with Cl-NOM but the results presented here show that other bacteria may also be capable of dechlorinating Cl-NOM (34). This includes bacteria besides organohalide respiring bacteria that have non-respiratory dh enzymes (40). Both types of bacteria capable of dehalogenation could play a role in the natural chlorine cycle. Improved understanding in the types of Cl-NOM that are preferentially dechlorinated by different bacteria and dehalogenating processes can give insight into the specificity of those bacteria for different chlorinated contaminants to improve bioremediation efforts. Dh enzymes have been found capable of dechlorinating contaminants but are often not the focus of bioremediation because they are typically thought to be active only in aerobic

systems but this work and others have shown them to be enriched in anaerobic environments as well (163, 230). The enrichment of dh genes and ability for different types of Cl-NOM to prime for dechlorination of contaminants shows that a better understanding of the natural chlorine cycle can improve bioremediation efforts.

Chapter 6

Dehalogenation Potential in Soudan Mine Metagenomes

6.1 Chapter Summary

Bacterial metagenomes from the Soudan Mine were analyzed for the potential dehalogenation ability of bacteria in this environment. The Soudan Mine is an abandoned iron mine with groundwater that has very low organic carbon, high chloride, and high iron concentrations, as well as no known chlorinated contaminants. The dehalogenation potential in uncontaminated environments has not been well studied and analysis of bacteria in low carbon environments can provide insight into whether dehalogenation could occur in low nutrient environments. In the metagenomes, putative reductive dehalogenase and non-respiratory dehalogenase genes were found with non-respiratory dehalogenase genes present at higher frequencies. These genes could allow the bacteria to utilize chlorinated natural organic matter as a carbon source, which would be beneficial in a carbon-limited environment. The ability of bacteria to dehalogenate, in addition to their use of other metabolic schemes, could give them metabolic advantages in a nutrient-limiting environment.

6.2 Introduction

Bacteria with the potential to dehalogenate chlorinated chemicals have been found in many environments. There are two broad groups of enzymes that catalyze this ability: reductive dehalogenase enzymes (rdhs) or non-respiratory dehalogenase enzymes (dhs)

(18). Rdhs allow bacteria to use chlorinated chemicals as electron acceptors during respiration. Dhs cleave off chlorine atoms to either allow the chemical to be utilized as a carbon source or to detoxify an inhibitory chlorinated chemical (249). Bacteria have been found to be capable of dechlorinating chlorinated natural organic matter (Cl-NOM) (34); therefore, dh and rdh enzymes are thought to play a role in the natural chlorine cycle. Indeed, while bacteria capable of dehalogenation have predominantly been studied for their ability to dechlorinate contaminants, genes encoding rdh enzymes have also been found in uncontaminated soil and sediment, as well as in deep sea vents (38–40). Genes encoding dhs have also been found in uncontaminated soils and sediments, but are much less commonly investigated (40). The findings of both rdh and dh genes in varied environments shows that dehalogenation is not an isolated process; nevertheless, it is not clear how widespread this process is, or which environments promote different modes of dechlorination.

In low-nutrient, low-carbon, anaerobic environments the metabolic strategy of lithotrophy can dominate. For example, bacterial cycling of sulfur, iron, manganese, and other metals for energy generation is common in low carbon environments. Autotrophic bacteria are also common in such environments, where bacteria will fix inorganic carbon sources to serve their anabolic needs (250). Dehalogenation has not been well studied in these environments, although iron can react with natural organic matter and chloride to form Cl-NOM under certain unique geochemical conditions (10). Indeed, organohalide respiring bacteria are lithotrophs, requiring hydrogen as electron donor, and as such, they could potentially survive in such an environment. In addition, an ability to utilize a non-respiratory dh enzyme to dechlorinate Cl-NOM in a carbon-limited environment could give bacteria a metabolic advantage. Nevertheless, organohalide respiring bacteria also

typically utilize acetate as a carbon source for growth (251) and bacteria that perform non-respiratory dechlorination via dh enzymes tend to be heterotrophs (18). Both non-respiratory and respiratory dechlorination processes have therefore been predominantly studied in soils and sediments where organic carbon is more plentiful and predominantly biotic reaction form Cl-NOM (34, 40, 75).

Metagenomes from the Soudan Mine in northern Minnesota were analyzed for genes that suggest an ability to dehalogenate, provide insight into the range of environments where dehalogenation could occur. The Soudan Mine is an old iron mine that has since been converted to a state park and is open to the public. Metagenomes from the water in three deep boreholes were analyzed as a part of a larger study to characterize the microbial and chemical processes in this environment. These boreholes are in the lowest level of the mine and the water is suspected to have come from deep within the Earth (correspondence with Brandy Toner, University of Minnesota). This reduced system (-56mV to -320mV) has high concentrations of iron (87 ppm – 130 ppm) and chloride (48750 ppm – 75600 ppm) but low carbon concentrations (below detection limits) (Unpublished data provided by Brandy Toner). Analysis of the dechlorination capabilities in this unique environment can provide insight into the ecological role dehalogenation plays and the types of environments it can be expected to occur in.

6.3 Materials and Methods

6.3.1 Sample Collection and Processing

All sample collection and processing was completed by Daniel Bond and Jon Badalamenti. In summary, the samples were collected by pumping and filtering the water. The DNA on the filters was extracted for further analysis. The DNA was sequenced by the University of Minnesota Genomics Center (UMGC) using Illumina HiSeq. The samples were quality

filtered and trimmed before assembly into contigs. All computational analysis of the metagenomes was completed on the Minnesota Supercomputing Institute (MSI). This work was completed by Jon Badalamenti as a part of a larger analysis of the microbial community.

6.3.2 Rdh and Dh Gene Search

The quality filtered DNA sequences were aligned to a database of protein sequences known to be capable of dehalogenation. This database of rdh and dh enzymes was compiled from the curated Uniprot database and is a similar database to that used in previous research (40), with the addition of enzymes verified since the cited work was published (2016). This database included rdhs, haloalkane dehalogenases, S-2-haloacid dehalogenases, haloacetate dehalogenases, and dechlorination-specific mono- and dioxygenases. It did not include enzymes that are known to nonspecifically dechlorinate, such as methane monooxygenase genes. DIAMOND was used to align the known rdh and dh enzymes to the unassembled and assembled reads using a cutoff of 60% protein identity and an e-value of 10^{-9} (233). The abundance of each of the genes was calculated by taking the number of hits and dividing by the number of quality filtered reads.

6.3.3 Identifying Longer Gene Fragments

DIAMOND was also use to align the database of enzymes known to be capable of dehalogenation to the assembled contigs. The matches were filtered to those having an e-value less than 10^{-6} but no minimum protein identity percent. This less stringent search was used because the results could be individually analyzed using the NCBI Blast database to confirm whether or not the identified protein sequence was likely to be an rdh or dh gene. The identified rdh and haloalkane dehalogenase genes were translated and phylogenetically compared to known proteins. The two classes of enzymes were

separately aligned to published sequences using Clustal with Mega5. A maximum likelihood phylogenetic tree with 1000 bootstraps was used to determine which proteins were most similar to those identified in the genome.

6.3.4 Binning of Contigs

MetaBAT was used to separate the contigs into bins (252). A maximum length of 1500 bp was required for a contig to be binned and the minimum class size for a bin to be recognized was 100000 bp. Besides these two changes, the default values in metaBAT were used. These changes were made to accommodate smaller contig sizes. CheckM was used to determine the phylogeny of the bins (253). The bins were searched for the contigs previously determined to contain rdh and dh genes to determine which phylogenies found in the metagenomes could be capable of dehalogenation.

6.4 Results and Discussion

6.4.1 Phylogeny of Detected Rdh and Dh Genes

Rdh genes were found that were phylogenetically similar to those in the *Chloroflexi* and *Firmicutes* phyla (Figure 6.1). Four out of the six rdh genes identified in the assemblies grouped closely together near rdhA proteins from *Desulfitobacter* and another was similar to other rdh genes found in the *Firmicutes* phylum. Another rdhA was more closely related to rdhA found in *Dehalogenimonas* and *Dehalococcoides. Desulfitobacterium* is known to have diverse metabolic capabilities and can use a variety of electron acceptors and donors (105) while *Dehalococcoides* are thought to be obligate organohalide respiring bacteria (14). The finding of rdh genes similar to non-obligate organohalide respiring bacteria shows that bacteria in this nutrient-limited environment may have evolved to have more diverse metabolic capabilities. Although one of the rdh genes was similar to those found in *Dehalococcoides* spp., one cannot speculate whether this gene is from an obligate

organohalide respiring bacterium, as bacteria phylogenetically similar to *Dehalococcoides* spp. that do not appear to respire organochlorines have been identified in deep sea marine sediments (127).

Several types of dh genes were also identified in the Soudan Mine metagenomes, including those putatively encoding for a dioxygenase capable of dechlorination, haloalkane dehalogenases, and haloacetate dehalogenases. The most common individual gene found was one that encoded for a 2-halobenzoate 1,2-dioxygenase originally identified in *Burkholderia cepacia* but which has now been found to be present in multiple bacteria (167, 249). This enzyme has predominantly been studied with respect to its ability to dechlorinate monohalobenzoates to catechol, but it also has dechlorination activity with other chlorinated chemicals of similar molecular structure (254). Genes similar to pentachlorophenol 4-monooxygenase and 2,4-dichlorophenol 6-monooxygenase enzymes were also identified in the Soudan Mine metagenomes. Haloalkane dehalogenases were also common. These enzymes have the ability to dechlorinate various chlorinated alkanes (163, 168). Haloalkane dehalogenases have been found in several bacteria, but all of the genes identified in the Soudan Mine metagenomes were phylogenetically similar to those found in *Marinobacter* (Figure 6.2) (255, 256). DDH 951 also contained two additional haloalkane dehalogenases which were more similar to another haloalkane dehalogenase found in a *Marinobacter* species (257, 258). *Marinobacter* has been isolated from biofilms on interfaces between alkanes and water and are extremely halotolerant; haloalkane dehalogenases have been characterized from this genera (259).

To better determine which bacterial genomes contained rdh and dh enzymes, the assembled reads were grouped, or binned, into 23, 21, and 16 bins for boreholes DDH

932, 944, and 951, respectively. Very few of the contigs that were binned contained genes encoding enzymes capable of dehalogenation, and of the ones that were, only one of the bins could be identified past the kingdom level. This bin was identified as that of the genus *Marinobacter*, and contained 2-halobenzoate 1,2-dioxygenase, 2,4-dichlorophenol 6-monooxygenase, and a haloalkane dehalogenase. The low binning rate of the other rdh and dh genes could be a result of their presence in low-abundance organisms, or the fact that this unusual environment may contain relatively unstudied bacteria.

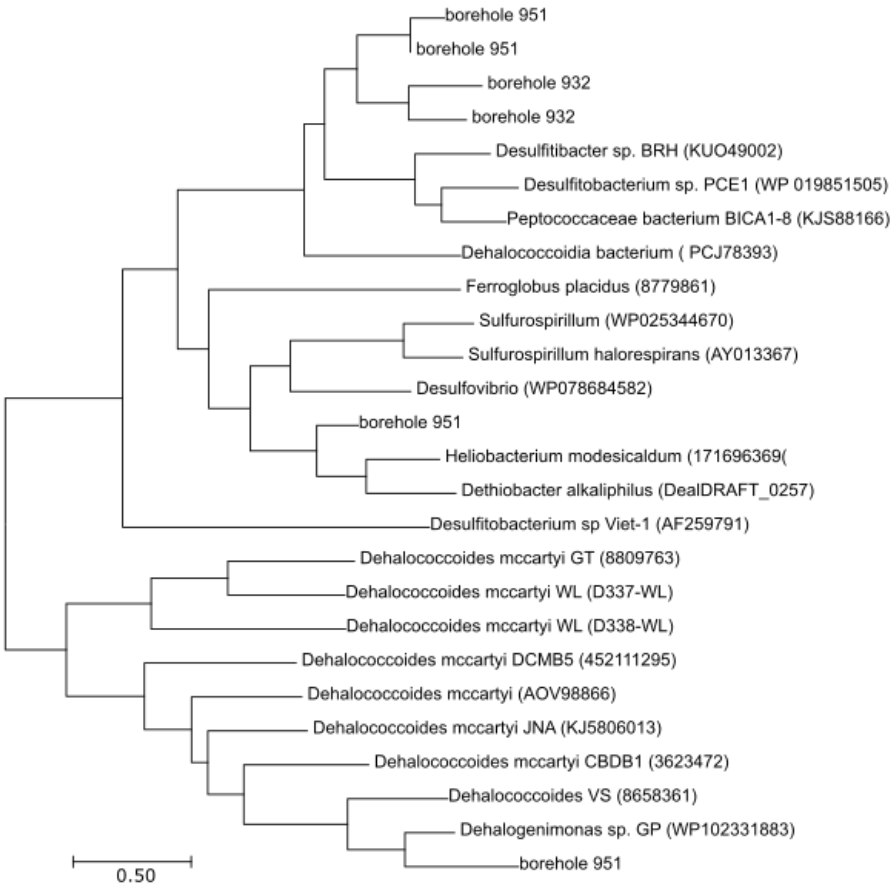

Figure 6.1 Reductive dehalogenase phylogeny.

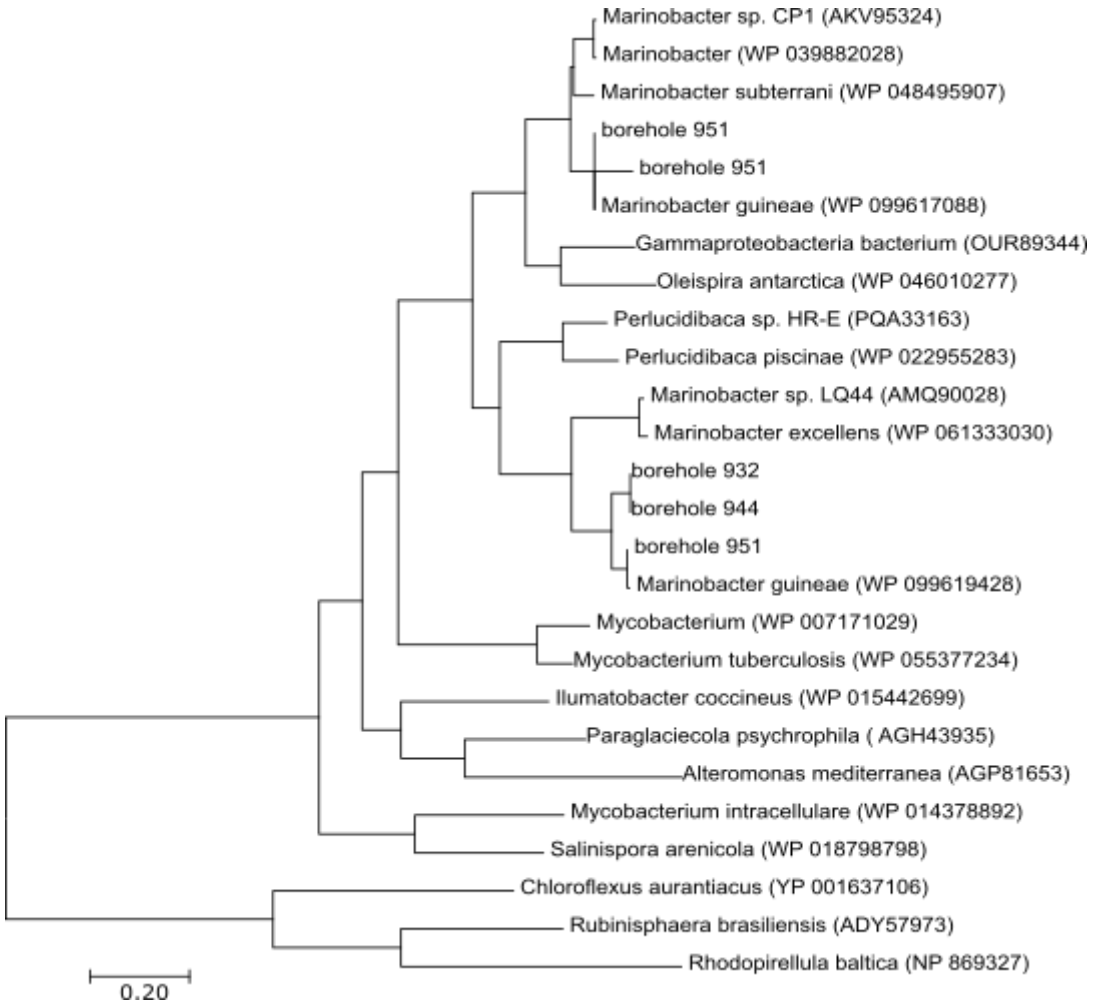

Figure 6.2 Haloalkane dehalogenase tree by maximum likelihood method. The names are the genomes with the annotated haloalkane dehalogenase. All of the genomes have been published. The numbers in parenthesis are the NCBI ascension numbers.

6.4.2 Abundance of Rdh and Dh genes

The abundance of both rdh and dh genes in the unassembled reads in the three boreholes is presented in Table 6.1. Non-respiratory dh genes were much more common in the boreholes than rdh genes (Table 6.1), with a particularly large number of mono- and di-oxygenase genes specific to dechlorination detected. Although dehalogenation for either energy production or generation of a carbon source is not expected to be dominant metabolisms in the Soudan Mine groundwater, bacteria having a large metabolic diversity

that includes dehalogenation could have a metabolic advantage in this environment. Iron cycling is thought to be the primary metabolism in the Soudan Mine groundwater, as iron reducing and oxidizing bacteria have been previously isolated and characterized (260, 261). These results suggest again that although dechlorination is not likely to be a dominant respiratory pathway in this type of anaerobic, nutrient-limited environment, it could provide certain organisms with a competitive advantage over heterotrophs that are unable to utilize chlorinated organics as a carbon source and strict autotrophs who must fix carbon. Based on the stoichiometry of the Calvin Cycle, a total of 18 molecules of ATP are used to fix 6 CO_2 molecules (250). If one considers an organism growing on H_2 and Fe^{3+}, generating approximately 2.5 mol ATP/mol H_2, the ability to utilize an organic carbon source rather than fix CO_2 can greatly improve their ability to compete and grow.

These results further support that dehalogenation is a common process, including one supported in low nutrient systems. Although it may not be a dominant metabolism in uncontaminated environments, it can provide bacteria with a metabolic advantage.

Table 6.1 The number of hits per 10 million quality filtered reads with 60% protein identity and a maximum e-value of 10-9. The forward and revers reads were averaged.

	DDH 932	DDH 944	DDH 951
Reductive Dehalogenase	3.2	1.0	1.9
Haloalkane Dehalogenase	108.3	52.5	70.0
2-Haloacid Dehalogenase	1.6	8.0	0.5
Haloacetate Dehalogenase	12.6	17.7	0.2
Dechlorination specific mono- and di-oxygenase	237.0	103.7	10.0
Other Dehalogenases	0.2	0.5	0.7

Chapter 7

Conclusion

7.1 Conclusions

This dissertation expands our understanding of dechlorination in uncontaminated environments and connects the dechlorination of chlorinated natural organic matter (Cl-NOM) to what has been previously found about the dechlorination of contaminants. The following are the specific conclusions drawn from this research.

- Putative organohalide respiring bacteria can be selected for at the interface between aqueous culture media and a hydrophobic liquid. Both trichloroethene (TCE) and hexadecane were tested as hydrophobic liquids. The fraction of *Dehalococcoides*-like bacteria increased 20.4 times at the interface compared to the culture media in just 20 minutes with the bacteria retaining the ability to dechlorinate after extraction. This method provides a rapid way to select for putative organohalide respiring bacteria for further study.
- Organohalide respiring bacteria may not be the only ones playing a role in the natural chlorine cycle and the dehalogenation of Cl-NOM. This was shown via several lines of evidence. First, non-respiratory dehalogenase (dh) genes increased during the enrichment of uncontaminated lake sediment with Cl-NOM concomitant to Cl-NOM dechlorination. Second, dh genes were much more common than reductive dehalogenase (rdh) genes in the metagenomes of several lake sediments and PCB-contaminated soil. Finally, other bacteria besides known

organohalide respiring bacteria were enriched by the different hydrophobic fractions of Cl-NOM.

- Genes indicating the potential to dechlorinate are common, though not necessarily correlated to the dechlorination of a pollutant. Indeed, sediments of all 14 lakes sampled in this research and in enrichment cultures contained multiple putative rdh and dh genes, suggesting dehalogenation capability. When experiments were subsequently performed on 5 of the lake sediments, however, neither the quantity nor types of rdh or dh genes previously identified in the sediments could predict the ability of that sediment microbial community to dechlorinate TCE. Some of the putative rdh genes and dh genes were more common and detected in multiple lakes, while others were only detected once.
- Rdh genes can be enriched during dechlorination of Cl-NOM. In the metagenome of uncontaminated lake sediment enriched with Cl-NOM, the frequency of rdh genes increased compared to the same sediment enriched with unchlorinated NOM. This indicates that rdh genes play a role in the natural chlorine cycle in addition to the bioremediation of chlorinated contaminants.
- Bacteria preferentially dechlorinate different types of Cl-NOM and initial enrichment on these different Cl-NOM fractions can “prime” contaminant dechlorination. When the Cl-NOM was fractionated into three groups based on hydrophobicity, the least hydrophobic fraction was dechlorinated to the greatest extent, followed by the moderately hydrophobic Cl-NOM fraction. No dechlorination was observed in the most hydrophobic Cl-NOM fraction. Furthermore, the bacteria enriched on the moderately hydrophobic Cl-NOM were able to dechlorinate TCE and tetrachlorobenzene more quickly than the

communities enriched on other Cl-NOM fractions, or enriched on NOM. This shows that bacteria enriched with specific types of Cl-NOM, whether the Cl-NOM is added during bioremediation or is already present as a result of natural halogenation reactions, can be predisposed to dechlorinate specific contaminants. Cl-NOM could be an option for a non-toxic primer to help prevent stalling of bioremediation when contaminant concentrations decrease to levels too low to support organohalide respiring bacteria.

- Metagenomes from the low carbon environment of the Soudan Mine suggest that this environment also has the potential to dechlorinate chlorinated organics. Dh genes were present at higher frequencies compared to rdh genes in Soudan Mine metagenomes. Dh enzymes could provide the bacteria in carbon limited environments such as this one with a metabolic advantage by allowing the bacteria to use Cl-NOM as a carbon source after dechlorination.

7.2 Recommendations

Studying the dechlorination of Cl-NOM can improve bioremediation techniques for chlorinated contaminants. Bacteria capable of dechlorination are naturally exposed to low concentrations of a variety of chlorinated chemicals in uncontaminated environments. The types of Cl-NOM that the bacteria are exposed to, either naturally or through amendment, could impact the types of contaminants the bacteria are capable of dechlorinating. A better understanding of this relationship could help prevent stalling of dechlorination during bioremediation and help lower contaminant concentrations to safe levels more quickly. Further work should be completed to determine what types of molecular structures promote the dechlorination of specific contaminants. Efforts should be made to analyze

the entire microbial community capable of dechlorination, as different dechlorination processes may be primed by different types of Cl-NOM.

Additionally, studying the natural chlorine cycle can also indicate which bacteria and microbial processes are important under different conditions. Often studies focus on *Dehalococcoides* spp. because these organisms have been well characterized and are often found at sites with higher contaminant concentrations; nevertheless, other bacteria and dehalogenation processes may also play important roles in contaminant dechlorination. Instead of looking for a single process or for the stimulation of a particular genus to successfully remediate all sites over the course of the entire remediation period, efforts should be made to understand whether, and when, other bacteria and dehalogenation processes, such as non-respiratory processes, may be more effective in achieving bioremediation goals. This is especially true at low contaminant concentrations when obligate organohalide respiring bacteria may not have enough electron acceptor to maintain an active population. Work should be performed that focuses on how different ratios of carbon to chlorinated organic compounds impacts the initial microbial community present, their dechlorination potential, their ability to be stimulated, and their actual dechlorination abilities. This could help practitioners better design remediation systems capable of utilizing this natural potential better.

Chapter 8

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Appendix A

Additional Information for Chapter 3

A.1 Separation Only

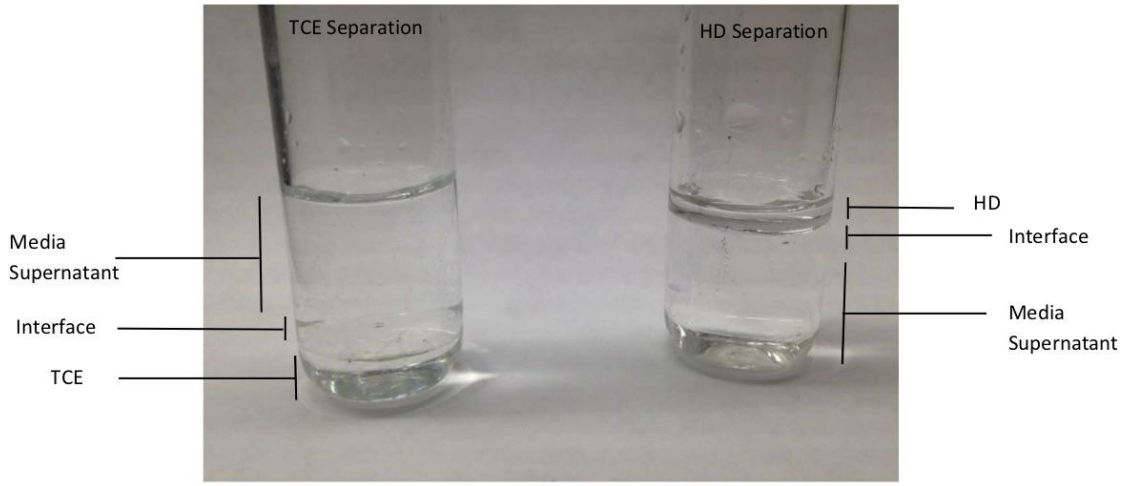

Figure A.1 A photograph of the experimental set-up for the separation only experiments. The image shows the interface between the NAPL and media phases, the light nature of HD, and the dense nature of TCE.

Figure A.1 shows an image of the system used for the separation only experiments, clearly showing the interface between the NAPL and media phases, the light nature of HD, and the dense nature of TCE.

A.2 Illumina-Based Community Analysis

Illumina sequencing was used to determine which bacteria were being separated at the hydrophobic interface with the various methods described. Insufficient DNA was present in samples from the sediment-free culture separation only experiments (<10^6 copies/µl) for Illumina analysis. Illumina analysis of samples from the separation only experiments with Pelican Lake sediment showed increases in the fraction of *Chloroflexi* OTUs at the interface compared to the initial sediment sample and the supernatant sample (Figure

A.2). In general, *Proteobacteria* showed the largest decrease in the percentage of OTUs in the interface samples. Only two OTUs within the *Chloroflexi* phylum with greater than 0.05% of the population were lost during the extraction process, both belonged to the *Anaerolinea* class. Besides *Chloroflexi*, candidate phylum OP8 also consistently increased during extraction from 1.4% in the initial sediment sample to 8.6% in the interface sample (Figure A.2). Table A.1 displays the percentage of the total Illumina sequences of the orders within the *Chloroflexi* phylum with greater than 0.1% of the total reads. All of the class *Dehalococcoidia*, which includes *Dehalococcoides mccartyi*, either increased or remained the same abundance when comparing the initial and interface samples. Additionally, classes *Ellin6529* and *S085* also increased in the interface samples, while half of the orders within the class *Anaerolineae* showed increases in the fraction of Illumina reads in the interface samples. Those classified as undetermined could not be placed into any of the orders as they may be novel 16S rRNA genes that have yet to be classified. An increase in the fraction of 16S rRNA genes may indicate that these organisms more likely to be putative organohalide respiring bacteria; more work is required to determine the ecological niche of these bacteria, however. It is important to note that phylogeny alone cannot be used to predict which bacteria have dechlorinating capabilities, as bacteria similar to *D. mccartyi* have been found to contain no reductive dehalogenase genes (1), while bacteria more distantly related have been enriched by chlorinated chemicals (2).

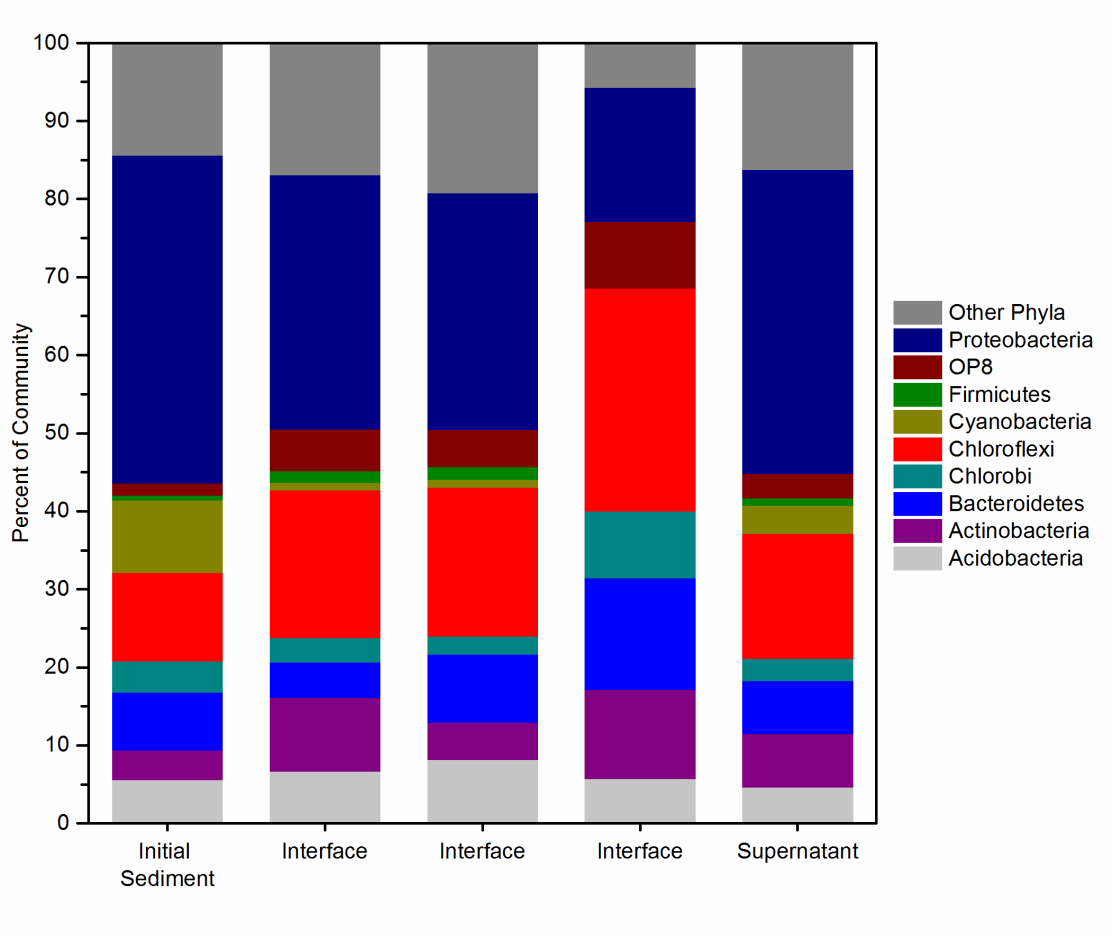

Figure A.2 Illumina sequencing results of the separation only experiments performed with Pelican Lake sediment and TCE as the hydrophobic solvent. Data at the phylum level, specifically, the percent of each phylum within the overall community, are shown. Three interface sample replicates were sequenced to show the variability in the method. One of the supernatant samples was also sequenced to determine which bacteria were excluded from the interface. The Chloroflexi phylum is shown in red.

Table A.1 The percentage of the total 16S rRNA sequences that is represented by each order within the Chloroflexi phylum, based on Illumina DNA sequencing of the separation only experiments performed with Pelican Lake sediment and TCE as the hydrophobic solvent. The OTUs that are undetermined could not be classified beyond the class level.

	Sediment Interface sample		Other Locations Family Found	Reference
Class Anaerolineae				
Undetermined	0.91%	2.14%		
A31	0.35%	0.09%	Soil	(3, 4)
Anaerolineales	0.12%	0.37%	Okinawa Trough (deep sea)	(5)
GCA004	0.57%	0.93%	Marine sediment	(6, 7)
O4D2Z37	0.02%	0.19%	Guerrero Negro hypersaline microbial mat	(8)
OPB11	0.14%	0.09%	Hot Spring in Tibet, Yellowstone hot spring	(9, 10)
S0208	0.35%	0.19%	Subsurface gold mine, Lake Superior Sediment	(11, 12)
SB-34	0.11%	0.28%	Lake Superior sediment	(12)
SBR1031	0.73%	0.56%	Activated sludge, agricultural soil	(13, 14)
SHA-20	0.88%	1.40%	Lake Superior sediment	(12)
SJA-15	1.04%	1.02%	Trichlorobenzene enrichment	(15)
envOPS12	0.69%	0.93%	Saanich Inlet (marine), membrane bioreactor	(16, 17)
pLW-97	1.03%	0.28%	Lake Washington	(18)
Class Dehalococcoidia				
Undetermined	0.23%	0.47%		
Dehalococcoidales	0.51%	1.58%	Several soils, aquifers, and freshwater and marine sediment	
FS117-23B-02	0.13%	0.47%	Marine sediment	(19, 20)
GIF9	0.43%	0.47%	Marine sediment	(21–23)
Class Ellin6529				
Undetermined	1.22%	3.35%	Agricultural soil, marine sediment	(24, 25)
Class S085				
Undetermined	0.10%	0.74%	Marine sediment, agricultural soil	(21, 26)

Samples from the four-day incubation plus separation experiments were also analyzed by Illumina sequencing and showed a large increase in the fraction of Illumina reads belonging to the *Chloroflexi* phylum (Figure A.3). The only order containing more than 0.1% of the reads was *Anaerolineales*. This order increased from 8% in the initial culture to 30.3% after four days of incubation. That same increase did not occur in the

supernatant samples, suggesting that growth was occurring at the hydrophobic interface and not in the bulk media. This supports the speculation of others that this order contains organohalide respiring bacteria capable of degrading PCBs and other chlorinated organics (2, 3, 4).

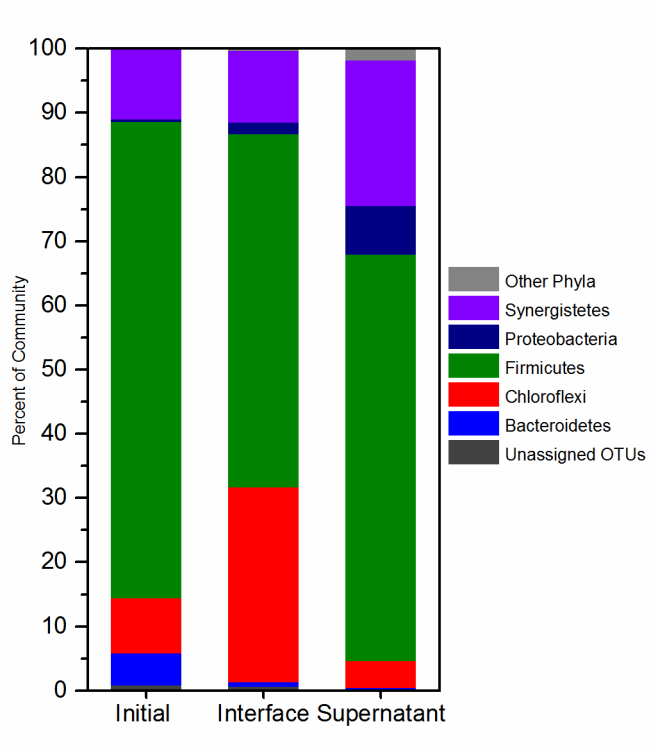

Figure A.3 Illumina sequencing results for the four-day incubation plus separation experiments. The percent of each phylum within the overall community is shown. The initial sample is the microbial community prior to enrichment or separation. The supernatant in the vial after four days was also sequenced to verify that separation and growth occurred only at the interface and not within the bulk liquid. The *Chloroflexi* phylum is shown in red.

A.3 Mass Balance of 16S rRNA Genes

A mass balance was completed on the 16S rRNA genes for both the *Dehalococcoides*-like (Table A.2) and the total 16S rRNA genes (Table A.3) for the separation only experiments in which the supernatant was also sampled. The total number of 16S rRNA genes in the interface and supernatant (taking volume of sample into account) were added and compared to that in the initial sample. The sum of the interface and supernatant were

consistently less than the total number of 16S rRNA genes in the initial sample for reasons described within the manuscript.

Table A.2 Mass balance of 16S rRNA gene copies for Dehalococcoides*-like genes.*

Sample	Concentration of 16S rRNA genes (gene copies/µl DNA extract)	16S rRNA gene copies in DNA extract (gene copies)	Sample volume (ml) extracted (into 50 µl)	16S rRNA gene copies in original sample (gene copies)	16S rRNA gene copies in original sample (average ± standard deviation)	[Interface + Supernatant] (average ± standard deviation)
Initial A	2.02×10^{3}	1.01×10^{5}	3.0	3.03×10^{5}	3.03×10^{5}	
Interface A1	4.71×10^{2}	2.35×10^{4}	0.2	4.71×10^{3}	$3.06\times10^{3} \pm 2.33\times10^{3}$	$4.95\times10^{4} \pm 2.58\times10^{3}$
Interface A2	1.42×10^{2}	7.08×10^{3}	0.2	1.42×10^{3}		
Supernatant A1	3.37×10^{2}	1.69×10^{4}	2.8	4.72×10^{4}	$4.64\times10^{4} \pm 1.10\times10^{3}$	
Supernatant A2	3.26×10^{2}	1.63×10^{4}	2.8	4.57×10^{4}		
Initial B	3.70×10^{3}	1.85×10^{5}	3.0	5.54×10^{5}	5.54×10^{5}	
Interface B1	3.41×10^{3}	1.71×10^{5}	0.2	3.41×10^{4}	$2.39\times10^{4} \pm 1.44\times10^{4}$	$8.54\times10^{4} \pm 2.76\times10^{4}$
Interface B2	1.38×10^{3}	6.88×10^{4}	0.2	1.38×10^{4}		
Supernatant B1	3.20×10^{2}	1.60×10^{4}	2.8	4.48×10^{4}	$6.14\times10^{4} \pm 2.36\times10^{4}$	
Supernatant B2	5.58×10^{2}	2.79×10^{4}	2.8	7.81×10^{4}		
Initial sediment	4.96×10^{7}	2.48×10^{9}	3.0	7.45×10^{9}	7.45×10^{9}	
Interface sediment 1	1.24×10^{6}	6.18×10^{8}	0.2	1.24×10^{7}	$2.43\times10^{7} \pm 1.18\times10^{7}$	$1.07\times10^{9} \pm 1.31\times10^{8}$
Interface sediment 2	2.45×10^{6}	1.23×10^{8}	0.2	2.45×10^{7}		
Interface sediment 3	3.59×10^{6}	1.79×10^{8}	0.2	3.59×10^{7}		
Supernatant sediment 1	6.40×10^{6}	3.20×10^{8}	2.8	8.97×10^{8}	$1.05\times10^{9} \pm 1.31\times10^{8}$	
Supernatant sediment 2	7.90×10^{6}	3.95×10^{8}	2.8	1.11×10^{9}		
Supernatant sediment 3	8.12×10^{6}	4.06×10^{8}	0.2	1.14×10^{9}		

Table A.3 Mass balance of 16S rRNA gene copies for total Bacteria 16S rRNA genes.

Sample	Concentration of 16S rRNA genes (gene copies/µl DNA extract)	16S rRNA gene copies in DNA extract (gene copies)	Sample volume (ml) extracted (into 50 µl)	16S rRNA gene copies in original sample (gene copies)	16S rRNA gene copies in original sample (average ± standard deviation)	[Interface + Supernatant] (average ± standard deviation)
Initial A	9.44×10^{4}	4.72×10^{6}	3.0	1.42×10^{7}	1.42×10^{7}	
Interface A1	1.51×10^{3}	7.55×10^{4}	0.2	1.51×10^{4}	1.26×10^{4} ± 3.52×10^{3}	2.40×10^{6} ± 1.45×10^{6}
Interface A2	1.01×10^{3}	5.06×10^{4}	0.2	1.01×10^{4}		
Supernatant A1	9.74×10^{3}	4.87×10^{5}	2.8	1.36×10^{6}	2.39×10^{6} ± 1.45×10^{6}	
Supernatant A2	2.43×10^{4}	1.22×10^{6}	2.8	3.41×10^{6}		
Initial B	2.53×10^{5}	1.26×10^{7}	3.0	3.79×10^{7}	3.79×10^{7}	
Interface B1	1.56×10^{4}	7.78×10^{5}	0.2	1.56×10^{5}	8.96×10^{4} ± 9.35×10^{4}	2.79×10^{6}± 1.32×10^{6}
Interface B2	2.35×10^{3}	1.17×10^{5}	0.2	2.35×10^{4}		
Supernatant B1	1.26×10^{4}	6.32×10^{5}	2.8	1.77×10^{6}	2.70×10^{6} ± 1.32×10^{6}	
Supernatant B2	2.60×10^{4}	1.30×10^{6}	2.8	3.64×10^{6}		
Initial sediment	3.79×10^{8}	1.89×10^{10}	3.0	5.68×10^{10}	5.68×10^{10}	
Interface sediment 1	2.18×10^{6}	1.09×10^{8}	0.2	2.18×10^{7}	6.37×10^{7} ± 5.15×10^{7}	7.82×10^{9} ± 2.90×10^{9}
Interface sediment 2	4.83×10^{6}	2.41×10^{8}	0.2	4.83×10^{7}		
Interface sediment 3	1.21×10^{7}	6.06×10^{8}	0.2	1.21×10^{8}		
Supernatant sediment 1	3.90×10^{7}	1.95×10^{9}	2.8	5.47×10^{9}	7.76×10^{9} ± 2.90×10^{9}	
Supernatant sediment 2	4.85×10^{7}	2.42×10^{9}	2.8	6.79×10^{9}		
Supernatant sediment 3	7.87×10^{7}	3.94×10^{9}	0.2	1.10×10^{10}		

A.4 Growth of Dehalococcoides-like Bacteria during PCE Enrichment after Separation at the Interface of a Hydrophobic liquid

Dehalococcoides-like bacteria were quantified at Day 0 and Day 73 of the PCE enrichment enrichment experiment performed after the separation only experiment on the digester culture for the positive control samples, HD interface samples, and TCE interface samples using the procedures described in the methods section. All three of the conditions resulted

in small increases in growth; nevertheless, the increase in the 16S rRNA genes for the HD interface samples was not as large (P=0.06) compared to that within the positive control samples (P=0.03) and the TCE interface samples (P=0.02).

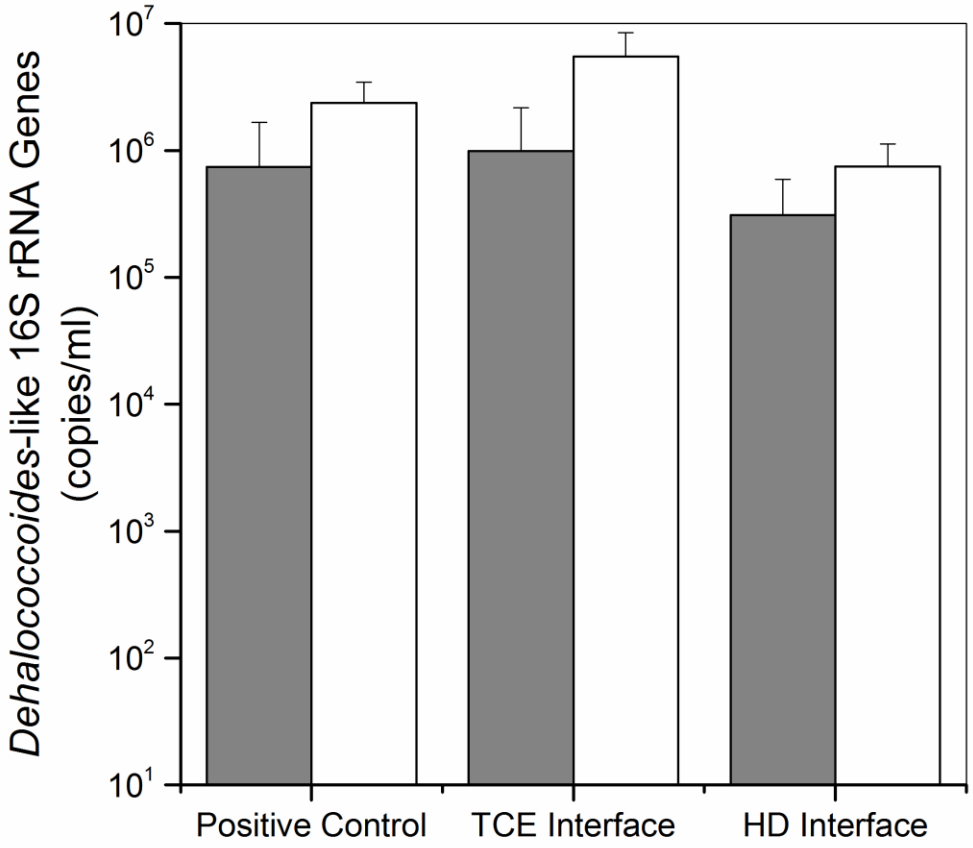

Figure A.4 Increase in Dehalococcoides-like bacteria 16S rRNA genes over the PCE incubation and degradation experiment of 73 days (see also Figure 3.5). The white bars show the initial 16S rRNA gene copy numbers/mL sample and the grey bars show the final 16S rRNA gene copy numbers/mL sample. Error bars are the 95% confidence intervals.

A.5 Additional References for Appendix A

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Appendix B

Additional Information for Chapter 4

B.1 qPCR Primer Efficiency

The target efficiency for each primer set was determined by dividing the total number of Illumina sequencing reads by the number of reads that were the correct target gene. More PCR primers were tested but only those with correct target amplification are included. The most common non target gene amplification were presumed to be primer dimers because no match was made in the NCBI database. The Tanners Rdh and PCBHaDhg2 primer sets also did also amplify 2-haloacid dehalogenase genes. A target efficiency of greater than 90% was deemed acceptable because this is within the error of qPCR.

Table B.1 Efficiency of primer sets based on amplicon sequencing.

Primer Set Name	Percent Target Efficiency
TannersRdh	90.2
BennettRdh	96
PCBRdh	95.6
PelicanRdh	97.3
TannersHaDhg	90.2
PCBHaDhg1	>99.9
PCBHaDhg2	93.8
CarverHaDhg	91.9
PCB 2-haloacidDhg	>99.9
Pelican 2-haloacidDhg	96.9

The following are the sequences of the standards of the gBlocks gene fragments that were purchased through IDT.

B.2 qPCR Standards

>TannersRdh
CACTGCGCGAGTACCGGCGAACCCATCGATTTGCCACACACGCACGCTATCGGGATACTGGTCGACCAAGA
CTATCCCACGTCCCACGCCTCGAACGGGCACGACTGGATCAGCAACTCCATGAGTTTCATGGCCTATTCGA
GCTCAGGG
TTCATCGCCTGCATACTAGCTGACTACATACGCCGGTTGGGCTACTCCGCGCGTGCCCACCACGCCCGCAA
CTATCAAGTCATGGTGCCCCCGATCCTGCTTTGGGCGGGACTTGGCGAGATGTGCCGTATCGGCGATACGG
TGCTGCACCCCTATCTTGGACCTCGGTTCAAAGCCGCGGTAGTGACG

>BennettRdh
GTTATTTGATACAGATGCAGGCTCGGTAGACCTCTTCCCTGAGGAGATCGCTTTTCCAGGGCATTCTTGGG
CGCACGCTCCGCAAGATTCGCAAAAGTCCTGAATCCCGAGGTCAACAGGCTTATCTGGCTTGAGAGGGAGA
TCGGTAAACACTTTCGACAACCTTACCCTTGGGCCAAACTGCTCTGTAATGAGAAGTCCGTGCCTCCCCAA
TTCCCCCAGGCCCGCATCGATCGCTAATGGAATACTCAGGGCCGTGTCATTTCCCATGGGAATTGCCTTAT
AACCCAGATTCCTGATAAACTCTGCCATCGTTACCGCAGTGATGGCCATTTTCGAATAACCCAAACCCGTT
GCGGCCGCCGATAATGCCCTGGGAGATTCTTTCATCAGTCCATAATCCATCTCAATGGCCATGAC

>PCBRdh
ATCATTCCCATCCTGCTCAATTCTCCAAGACCTGCCTGAACAGCTATGGGAATACTTAATGCAGTGTCATT
GCCGCAAGGTATAGCCTGATATCCCAGCTGTCTCACAAACGTGGCTACCTTGTGGGCTGTTGCAGCCATGT
TGGAGTATTGATTACCGGCTGCAGCGCTTGCGATTAATGACGGTGCAGTCCGGAAAGCATCATGATTCATT
TCTAAAGCCAAGGCAATTACACTTTTGGGTTCAAA

>PelicanRdh
GGGGCCGATCGGGTGGGCATCACGCGCCTGAACCCGCTCTGGATCTACACGCACTGGGGGATGCAGAACGT
CCACTACTCCGGCGCGGCCCAGGCGGGCGACCCGATCGACATcccccccGAGTACCAGACGGTGATCGTCA
TGATCCACCGCATGGATTACGACGTGATCCTGAGGTCGCCGGCGGTCGAGCATGAAACCGACATCGGGTAC
TCCAAGGCCGCCTGGAGCGCGGCATCGCTCGCCACCTTCATTACTGAACTTGGGTACAAGGCCATCCCCGC
GTGCAACGAGCTCGGCATCAGCATCGCCATGGCGGTCGATGCCGGCCTTGGCGAAATGGGACGGAACGGCC
AGCTG

>TannersHaDhg
TTCAACGGCCTGCCGGGCTTCGCTTTCGCGCCGAACTACCTCGAGTCGCTGCACGGCTACGAATGGCTGCG
CATGCATTACCTCGACGAGCGCCCCATGGGGACCGCCAGCGGCCGTACCGTGTTGTGCCTGCACGGCCAGC
CAACCTGGTGTTACCTGTATCGCAAGATGATCCCGGTGTTCCTGGCTGCCGGGCACCGCGTCGTCGCGCCG
GACTTCTTCGGTTTCGGCCGTTCCGACAAACCCGTCGACGACGCGGTCTACACGTTCGGTTTCCATCGCGG
GATGCTGATGCGCTTCATCGAGACGCTCGACCTCCACCGGGTGACG

>PCBHaDhg1

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ACATATCCCAACTGAGTGGTTGGACAATGGCTTCCATATAGACAATAGCCTTGACCCGCTCTGGATGGCGG
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TGTATCAAACCAGGCATCTAGGTAACGTGCGTGATCCACGAAACGATAAGTACCATTAGCAATTTTCCCGG
AATCTCCCATTCCCATCAAATCGGGAGCTAAACAACGGCCTACGCCTTCCAAATGGGGAATCACATTCCGC
CACAGATAGGAGGAAGTGGGATTCCCATGCAAAAAGACAATCGGCTCCCCCTTTCCTGTATCCACATACGC
CAT

>PCBHaDhg2

TGTCACCCTCGTGGTTCACGATTGGGGATCGGCGCTGGGTTTTCATTGGGCAGACCGCCATCCAGAGCGGG
TCAAGGCTATTGTCTATATGGAAGCCATTGTCCAACCACTCAGTTGGGATATGTGGCCCGAACATGCTCGG
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AACCAGGTGAGGTACGACGTCCCATGCTGATGTGGCCGCGCGAACTCCCGTTTGAGGGTGAACCGGGTGAT
GTGCATGATATCGTGGCTCACTATGCGGCTTGGTTGGCGACCAGTACCATTCCCAAGCTTTTCATTAACGC
CGATCCTGGTTCTATTTTGGTCGGGACACAACGCGAGTTTTGCCGTACCTGGCCGTGTCAGCAAGAAATCA
TCG

>CarverHaDhg

TTCGTACTCCTGAGAATCGATTCAAGAATCTACCTCAATATCCCTTTGAACCCCATTACACCCAGATAGAA
GGACTAAGAATGCACTATGTAGATGAGGGACCCAAGAACGGTGAAGTAGTTTTAATGCTTCATGGTCAGCC
AACTTGGTCCTATTTATACCGTAAAATGATTCCACCGTTAGCAGTTGCAGGTTATCGAGTGATTGCAGCTG
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CGGCTTAAAAAGTTCATTGGTGCTCTGGAACTTAGAGATGTTACCTTGTTCTGCCAAGACTGGGGTGGTTT
GATGGGACTGCGAATCGTCGGGGACCAGCCTGATATTTTTGCCAGAGTAGTGGCAGCCAACACAATGCTTC
CC

>PCB 2-haloacidDhg

AGCACCTCATGCGGCTTTACGCCGAGATTCGATTCAATCAGTTCGTAGGTTCGCGGCGACGGCTTGAAGGT
CTTCGTTGAGTCGATGCTGATGGTCGCGTCGAGAATGCTGTCGAGGCCGGTATTGCGCACCAGCGCATTCA
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GCCTGGTCCGTGACGGCAGCAACTGACTGCACATCGTAGAGTGTGCCGTAAGCGTCGAATACGACGGCCTT
GAT

>Pelican 2-haloacidDhg
ATGATCTTGGGCAGGTCGGCAAGCGCGGAGATTCGAAAGTCGGGTTCCATCCCGAACTCGTCCATTTGCAT
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GACACGAACAGCACCTCATGCGGCTTTACGCCGAGATTCGATTCAATCAGTTCGTAGGTTCGCGGCGACGG
CTTGAAGGTCTTCGTTGAGTCGATGCTGATGGTCGCGTC

B.3 Specific Gene Concentrations in Lake Sediments

Table B.2 Concentration of known organohalide respiring bacteria in the lake sediments (copies/g). Samples that were detected but below the limit of quantification are denoted as blq.

Copies/g wet weight	Geobacter	Desulfomonile	Dehalobium	Anaeromyxobacter	Sulfurospirillum	Desulfovibrio
Big Marine	1.3E+07	3.3E+03	8.8E+01	3.7E+05	2.3E+01	2.5E+03
Tanners	4.5E+07	4.1E+03	1.9E+02	2.2E+05	3.4E+02	5.9E+03
White Bear	2.7E+07	7.9E+03	1.2E+02	1.2E+05	9.1E+02	9.0E+03
Gervais	2.9E+06	8.3E+02	3.6E+01	7.8E+04	1.7E+01	1.6E+03
Carver	5.2E+06	1.4E+03	3.3E+02	5.8E+04	1.0E+01	2.0E+03
Bennett	4.8E+06	2.2E+03	1.5E+02	8.5E+04	9.1E+01	1.9E+03
Square	6.5E+06	2.7E+03	6.0E+01	2.8E+05	1.0E+00	8.8E+03
Long	6.8E+06	3.8E+03	2.7E+02	3.5E+05	2.4E+01	6.9E+03
Turtle	4.9E+06	8.9E+03	1.6E+02	2.1E+05	blq	3.7E+03
Wakefield	1.1E+08	3.7E+03	2.4E+02	2.4E+05	5.0E+02	4.2E+03
Johanna	1.7E+07	1.3E+02	1.1E+01	4.2E+05	3.3E+02	5.0E+03
Centerville	3.3E+06	3.0E+02	1.6E+02	8.8E+04	2.1E+08	1.3E+03
Como	3.4E+08	2.1E+04	2.4E+02	1.1E+06	2.2E+03	7.3E+03
Josephine	2.2E+07	1.5E+03	8.7E+01	6.5E+05	9.7E+01	2.8E+03

Copies/g wet weight	Dehalogenimonas	Dehalococcoidia	D. mccartyi	Dehalobacter	Desulfitobacterium	Total 16S
Big Marine	6.5E+04	8.0E+06	3.6E+03	5.9E+02	6.3E+01	4.65E+07
Tanners	2.7E+05	1.8E+07	1.1E+04	1.9E+03	6.5E+01	1.45E+07
White Bear	4.0E+04	1.7E+07	7.1E+03	8.8E+02	3.9E+02	3.93E+06
Gervais	3.2E+04	4.3E+06	5.7E+03	4.6E+02	2.6E+01	6.79E+07
Carver	9.7E+04	8.0E+07	9.2E+03	2.2E+02	3.1E+01	3.07E+07
Bennett	5.0E+04	1.7E+07	1.0E+04	3.5E+03	3.7E+01	2.15E+07
Square	1.8E+05	4.2E+06	7.5E+03	2.6E+02	3.3E+02	7.64E+07
Long	1.5E+05	1.9E+07	1.1E+04	2.0E+03	7.4E+01	7.58E+07
Turtle	1.3E+05	1.3E+07	2.9E+03	6.3E+02	5.3E+01	4.58E+07
Wakefield	1.4E+05	1.4E+07	4.0E+04	1.4E+03	1.1E+02	3.62E+07
Johanna	1.9E+04	5.8E+05	7.7E+03	1.4E+03	1.0E+02	1.09E+07
Centerville	4.9E+04	1.1E+06	1.8E+03	blq	2.3E+01	3.72E+07
Como	3.1E+05	3.5E+07	2.2E+04	2.3E+03	2.0E+02	8.92E+06
Josephine	9.7E+04	7.3E+06	2.5E+03	5.1E+02	5.0E+01	3.70E+07

Table B.3 Variation in rdh and dh genes over a growing season. Log gene concentrations of genes are listed for each of the sampling dates (copies/ml) Genes that were detected but below the limit of quantification are denoted as bql.

Lake	Genes (log copies/g sediment)	4/7/2016	5/30/2016	7/24/2016	9/11/2016	11/20/2016	3/25/2016	Average
Tanners	GeoNOM haloalkane dehalogenase	Bql	Bql	1.02	1.0	Bql	Bql	0.55
	GeoNOM Rdh2	Bql	Bql	Bql	Bql	0	Bql	Bql
	Tanners Rdh1	2.58	2.56	2.36	2.51	Bql	2.58	2.21
	Carver haloalkane dehalogenase	3.30	3.02	3.00	3.23	1.10	3.43	2.85
	Pelican Rdh 1	Bql	Bql	Bql	Bql	0	Bql	Bql
	Bennett Rdh	Bql	Bql	Bql	Bql	Bql	Bql	Bql
	Tanners haloalkane dehalogenase	2.83	2.68	2.65	2.85	1.36	3.01	2.56
	GeoCl S-2-haloacid	2.20	1.96	1.86	2.23	Bql	2.09	1.79
	GeoCl haloalkane dehalogenase	Bql	Bql	Bql	Bql	0	Bql	Bql
	Total 16S rRNA Gene	8.76	7.43	8.03	7.94	5.56	8.10	7.55
Bennett	GeoNOM haloalkane dehalogenase	1.10	Bql	Bql	Bql	Bql	Bql	Bql
	GeoNOM Rdh2	0	0	Bql	Bql	0	0	0
	Tanners Rdh1	0	0	0	0	0	0.00	0
	Carver haloalkane dehalogenase	3.01	3.30	2.81	2.02	1.65	3.25	2.67
	Pelican Rdh 1	2.64	3.33	2.79	1.37	1.71	2.73	2.43
	Bennett Rdh	1.07	2.70	2.41	2.82	1.97	3.07	2.34
	Tanners haloalkane dehalogenase	3.91	3.06	2.72	2.29	1.67	3.13	2.80
	GeoCl S-2-haloacid	1.59	1.75	1.32	1.21	1.05	1.08	1.34
	GeoCl haloalkane dehalogenase	0	0.30	0.52	-0.24	0.00	-0.99	-0.21
	Total 16S rRNA Gene	7.49	8.09	8.26	7.7	7.24	7.79	7.76
Carver	GeoNOM haloalkane dehalogenase	Bql	Bql	Bql	Bql	0	Bql	Bql
	GeoNOM Rdh2	0	0	Bql	0	0	0	Bql
	Tanners Rdh1	Bql	1.13	1.42	1.53	0	Bql	
	Carver haloalkane dehalogenase	3.21	3.06	3.74	3.47	2.40	3.41	3.22
	Pelican Rdh 1	Bql	Bql	1.15	Bql	0	Bql	0.50
	Bennett Rdh	0	Bql	0	4.72	Bql	Bql	0.97
	Tanners haloalkane dehalogenase	2.46	2.25	3.40	3.64	1.88	2.34	2.66
	GeoCl S-2-haloacid	Bql	1.31	1.49	1.57	Bql	1.17	1.18
	GeoCl haloalkane dehalogenase	0	0	0	0	0	0	0
	Total 16S rRNA Gene	7.27	7.38	8.18	7.66	6.41	7.33	7.38
Turtle	GeoNOM haloalkane dehalogenase	Bql	Bql	Bql	Bql	0	Bql	0.30

Lake	Genes (log copies/g sediment)	4/7/2016	5/30/2016	7/24/2016	9/11/2016	11/20/2016	3/25/2016	Average
	GeoNOM Rdh2	0	0	0	0	0	0	-0.68
	Tanners Rdh1	0	1.12	0	0	0	0	0.19
	Carver haloalkane dehalogenase	2.25	2.19	2.77	2.33	1.53	2.37	2.24
	Pelican Rdh 1	Bql	Bql	Bql	0	0	Bql	0.13
	Bennett Rdh	0	Bql	0	Bql	0	Bql	-0.24
	Tanners haloalkane dehalogenase	1.14	Bql	1.62	1.39	Bql	1.26	1.17
	GeoCl S-2-haloacid	Bql	Bql	Bql	Bql	Bql	Bql	Bql
	GeoCl haloalkane dehalogenase	0	0	0	0	0	0	0
	Total 16S rRNA Gene	7.35	7.41	7.64	7.16	6.57	7.38	7.22
Square	GeoNOM haloalkane dehalogenase	0	Bql	Bql	Bql	0.00	Bql	Bql
	GeoNOM Rdh2	0	0	0	0	0	Bql	Bql
	Tanners Rdh1	Bql	Bql	0	Bql	0.00	0	Bql
	Carver haloalkane dehalogenase	2.15	1.53	1.75	2.38	Bql	2.50	1.79
	Pelican Rdh 1	0	0.00	0.00	0	0.00	Bql	Bql
	Bennett Rdh	0	Bql	Bql	0	Bql	Bql	Bql
	Tanners haloalkane dehalogenase	2.28	2.34	2.49	2.60	Bql	3.20	2.31
	GeoCl S-2-haloacid	Bql	Bql	0.00	1.20	0	0.88	0.25
	GeoCl haloalkane dehalogenase	0.00	0.00	Bql	0.00	0.00	0.00	Bql
	Total 16S rRNA Gene	8.14	7.18	6.87	7.17	5.73	7.66	7.02

Table B.4 Log gene concentration (copies/g) of reductive dehalogenase genes targeted by qPCR in each of the lake sediments

	PCBRdh	PelRdh	Bennett Rdh	TannersRdh	Average Chloride	Percent Road Density
Big Marine	bql	2.86	0.00	1.58	11	<18
Tanners	1.08	1.75	bql	3.77	161.7	29
White Bear	1.30	1.27	bql	3.13	35.3	<18
Gervais	bql	bql	bql	bql	150	29
Carver	bql	bql	0.00	1.43	190	25
Bennett	0.00	2.43	2.34	0.00	160	23
Square	bql	1.28	bql	2.23	7	<18
Long	bql	bql	0.00	bql	151	33
Turtle	bql	bql	bql	0.00	40	<18
Wakefield	bql	3.87	bql	3.55	146	26
Johanna	1.50	1.23	0.00	bql	153	36
Centerville	bql	bql	0.00	0.00	26	<18
Como	1.01	3.10	bql	2.73	193	32
Josephine	1.00	1.92	bql	1.56	65	21

Table B.5 Log gene concentrations (copies/g) of the dehalogenase genes targeted by qPCR.

	PCB2 HAdhg	PCB 2haloacid	GeoClHA	CarverHA	TannersHA	PelS2haloacid	Average Chloride	Percent Road Density
Big Marine	bql	1.01	0.00	2.94	2.29	0.00	11	<18
Tanners	1.00	2.05	bql	3.47	2.89	bql	161.7	29
White Bear	1.04	bql	bql	3.30	4.29	1.04	35.3	<18
Gervais	bql	bql	0.00	1.80	1.44	bql	150	29
Carver	bql	bql	bql	2.19	2.41	bql	190	25
Bennett	0.00	1.34	0.00	2.67	2.80	0.00	160	23
Square	bql	bql	0.00	3.06	2.21	bql	7	<18
Long	bql	bql	bql	1.10	1.03	bql	151	33
Turtle	bql	bql	0.00	1.89	1.57	bql	40	<18
Wakefield	1.20	1.79	bql	3.07	3.04	1.03	146	26
Johanna	1.83	bql	bql	2.30	1.45	1.27	153	36
Centerville	1.00	bql	0.00	2.03	1.66	bql	26	<18
Como	bql	1.98	bql	3.51	2.55	bql	193	32
Josephine	bql	1.19	bql	2.90	2.26	0.00	65	21

Appendix C

Additional Information for Chapter 5

C.1 Preparation of Cl-NOM

Organic rich sediment was collected from Pelican Lake, MN and dried at 105ºC for 4 hrs. The organic matter was extracted sequentially from the sediment (100 g total) with an accelerated solvent extractor (ASE) (Thermo Scientific) using first water, then methanol, and finally hexane:acetone (50:50). Each extract (water, methanol, or hexane:acetone) was collected in separate bottles. The ASE method for each solvent had an oven temperature of 100ºC. The 50 ml ASE vials were filled and then had a static time of 10 min. Half of the solvent was then replaced in 2 subsequent extraction steps as is typical of ASE extractions. All of the extracts were rotovapped and blown down to dryness with nitrogen, after which they were resuspended in a phosphate buffer (100 mL, 0.1M, pH 4). The extract obtained with each solvent was then split evenly between two flasks, one which was chlorinated with hypochlorous acid (to generate the “amended Cl-NOM”), while the other was simply retained (“NOM”). For the chlorination step, hypochlorous acid was added to a final concentration of 1 µM every 20 minutes for a total of 10 additions to each fraction. As described in the manuscript, all three fractions of both the amended Cl-NOM and the NOM were cleaned via solid phase extraction with C18 columns into methanol.

C.2 Microbial Community Analysis and Quantification

C.2.1 Quantitative PCR

The qPCR primers used for the rdh and dh gene detection were developed in Chapter 3. Total 16S rRNA gene quantity was determined using a general primer set that targets the V3 region (262). The qPCR mixture for all of the primer sets contained 1X SYBR green

MasterMix (Bio-Rad Laboratories), 100 nM of each primer, 1 mg/L of BSA, and 1 µl of undiluted template. The general qPCR cycle was an initial 95ºC denaturation for 2 minutes followed by 40 cycles of 95ºC denaturation for 15 s and 30 s anneal/extension at the specific annealing temperature for each primer set Table C.1. A melting curve analysis was completed at the end of each run for quality control/assurance. The number of gene copies in each sample was determined with a standard curve of standard dilutions ranging from 10^8 to 10^0. These standards were purchased as gblocks from IDT. Standards for total 16S rRNA gene analysis were prepared as described in Chapter 3.

Table C.1 Primer sequences.

Primer Name	Sequence	amplicon length (bp)	annealing temp (ºC)
PCBRdhF	CCTGAACAGCTATGGGAATAC	129	56.0
PCBRdhR	CAGCCGGTAATCAATACTCC		
PCBHaDhg1F	CCATCAAATCGGGAGCTAAA	129	59.0
PCBHaDhg1R	CGTATGTGGATACAGGAAAGG		
PCBHaDhg2F	GGAACGCTTGATCTTGGAA	110	55.0
PCBHaDhg2R	CAAGGTAAGGGCGATGATATG		
PCB 2-haloacidDhgF	GTTTCGCATCCGGGTAAA	104	58.0
PCB 2-haloacidDhg	GCTGACTTTCACGCTCAA		

C.2.2 Community Analysis

As stated in the manuscript, Illumina paired end sequencing (2x300) was completed on the V4-V6 region of the 16S rRNA gene and used for bacterial community analysis. Amplification and sequencing were completed at UMGC. The V4-V6 region was amplified with primers 515F-GTGCCAGCMGCCGCGGTAA and 806R-GGACTACHVGGGTWTCTAAT. These were selected from those available at UMGC because they captured the highest percentage of the *Chloroflexi* bacteria in the Ribosomal Database Project database, which were anticipated to be important in Cl-NOM cycling (34). The data was analyzed via the Minnesota Supercomputing Institute (MSI). Quality filtering was performed with a pipeline available on MSI that uses Trimmommatic and FastQC. The number of reads in each sample were rarified to the least number of reads in one of the samples. The processed reads were then analyzed using QIIME using the denovo clustering pipeline. The reads were also analyzed using a pipeline with the MOTHUR clustering algorithms, but this did not significantly change the results (data not shown). Once the relative fraction of a given population was calculated, this was converted to a numerical value (number of 16S rRNA genes) based on the total number of 16S rRNA genes present in the sample as obtained via qPCR. This conversion from relative

abundance to quantities has been verified in the literature as a way to account for changes in biomass (263, 264).

C.2.3 Statistical analysis

To determine whether particular bacterial populations may have benefitted from the amendment of Cl-NOM, Spearman's rank correlation was used. The analysis was performed separately at each phylogenetic class, though family and genus levels were focused on. First, any family or genera in less than 5 of the samples (triplicate samples of the 5 time points of the 6 treatments) and with fewer than 1000 16S rRNA gene copies/ml in at least one sample were filtered out of the dataset. The Spearmen's rank correlation was then performed for each of the filtered genus and family to determine which populations increased over time in the treatments amended with Cl-NOM. The calculated *P*-values were corrected with FDR. The families or genera with negative Spearman's rho and *P*-values greater than 0.05 were deleted. Further filtering was conducted by determine which of the remaining genera or families increased more in treatments amended with fractions of Cl-NOM when compared to the treatments amended with the same fraction of NOM. The same method was used to determine which genera significantly grew with the dechlorination of TCE. The TeCB enrichments were analyzed differently because only two time points (Day 0 and Day 50) were taken. Instead the Kruskal Wallis test in QIIME was used to determine which genera grew significantly more in the samples with 1,2,3-TCB formation.

C.3 Contaminant Dechlorination Results

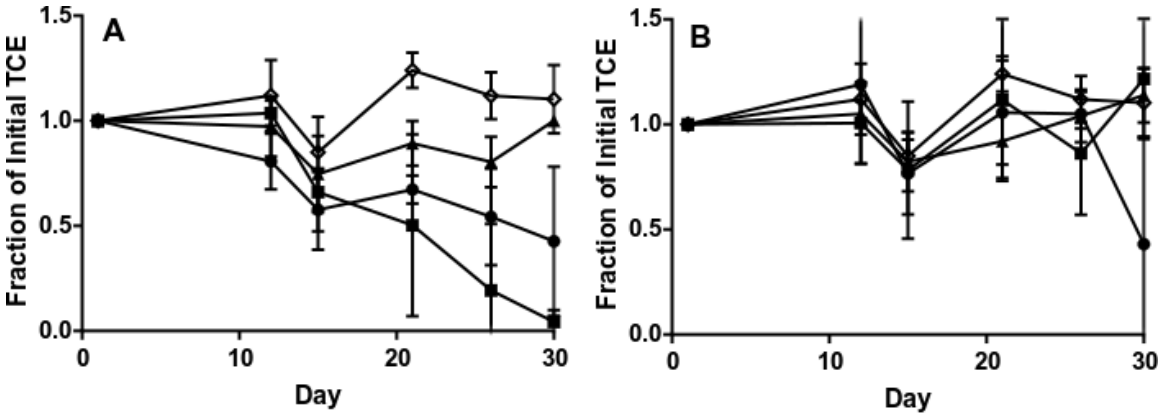

Figure 24. Fraction of initial TCE of (A) Cl-NOM enrichments and (B) NOM enrichments of the sterile negative control (◇), least hydrophobic fraction (●), moderate hydrophobic fraction (■), and most hydrophobic fraction (▲).

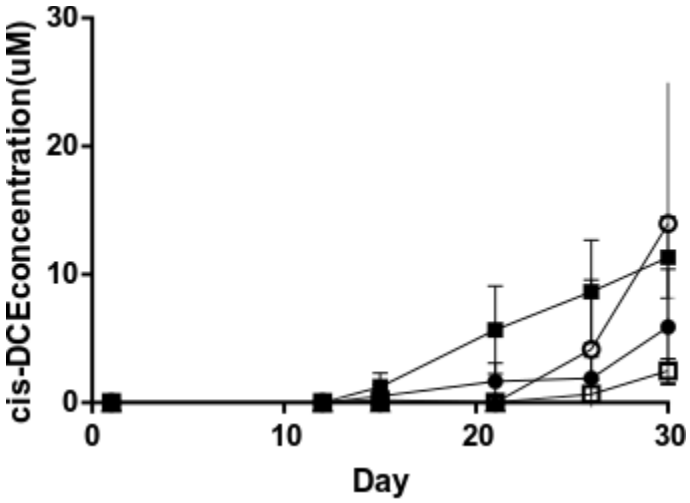

Figure 25. Cis-DCE formation of Cl-NOM enrichments (closed symbols) and NOM enrichments (open symbols) of the least hydrophobic fraction (circles) and moderate hydrophobic fraction (square). No cis-DCE was detected in the most hydrophobic fraction or sterile negative control.

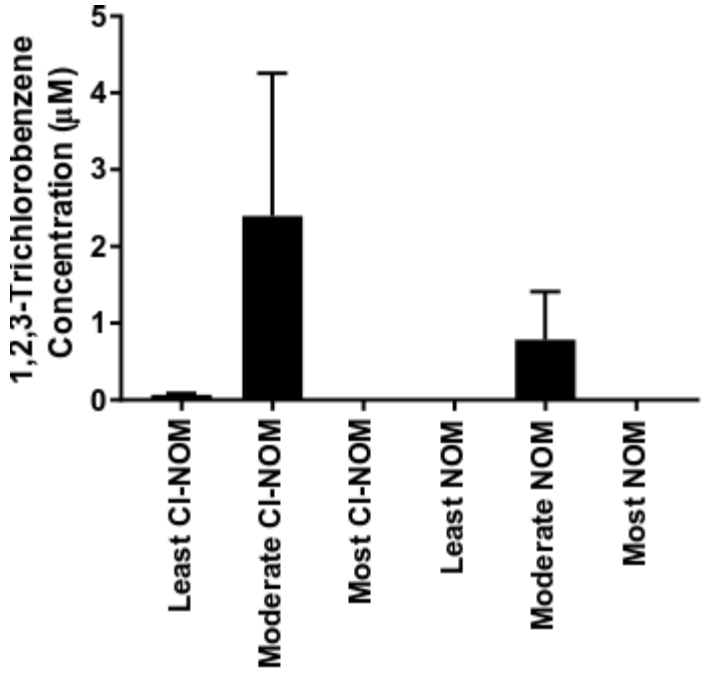

Figure 26. 1,2,3-trichlorobenzene concentration after 80 days of dechlorination of tetrachlorobenzene.

C.4 Tables with Spearman's and concentrations

C.4.1 Genera with Significant Growth in Cl-NOM Enrichments

Table C.2 Genera that grew significantly more in the at least one of the hydrophobic fractions of Cl-NOM enrichments compared to the NOM enrichments based on the Spearman's ρ for the least hydrophobic fractions. Log gene concentrations are listed for the five days sampled (copies/ml).

	Least Hydrophobic ClNOM							Least Hydrophobic NOM						
	ρ	***P*-value**	**1**	**17**	**50**	**83**	**113**	**ρ**	***P*-value**	**1**	**17**	**50**	**83**	**113**
Euryarchaeota														
Methanobacteria; Methanobacteriales; Methanobacteriaceae; unknown	0.62	0.05	0.00	0.00	0.89	3.14	3.22	0.00	0.00	0.00	0.00	0.00	1.39	1.43
Methanomicrobia; Methanosarcinales; Methanosarcinaceae; Methanosarcina	0.70	0.02	1.43	0.00	2.78	2.58	3.63	0.00	0.00	1.29	0.00	0.00	1.77	1.53
Methanomicrobia; YC-E6; unknown ; unknown	0.57	0.09	1.43	1.31	4.03	4.83	3.67	0.53	0.15	1.55	2.18	3.06	4.26	3.64
Actinobacteria														
Actinobacteria; Actinobacteria; Actinomycetales; Cellulomonadaceae; Actinotalea	0.26	0.56	2.97	1.39	2.34	2.24	2.53	0.63	0.05	1.46	3.03	3.41	3.72	3.46
Chloroflexi														
Anaerolineae; Anaerolineales; Anaerolinaceae; WCHB1-05	0.00	0.00	0.00	0.00	0.68	0.00	0.00	0.00	0.00	0.00	0.58	0.83	1.22	0.00
Firmicutes														
Bacilli; Lactobacillales; Carnoceae; unknown	0.00	0.00	0.00	0.63	0.00	1.68	0.00	0.30	0.53	0.64	0.58	0.00	1.06	1.59
Clostridia; SHA-98; D2; unknown	0.86	0.00	0.00	0.00	0.72	1.01	0.00	0.60	0.08	0.00	0.00	0.00	0.00	0.00
Clostridia; Clostridiales; unknown; unknown	0.84	3.5E-04	1.66	2.24	5.15	6.05	6.16	0.79	3.6E-03	2.82	3.91	4.70	5.47	4.94
Clostridia; Clostridiales; Tissierellaceae; Sedimentibacter	0.82	0.001	2.60	4.91	5.70	6.28	6.29	0.64	0.04	2.80	3.38	4.62	5.18	4.61
Clostridia; Clostridiales; Christensenellaceae; unknown	0.84	3.5E-04	2.97	0.89	4.73	5.91	6.11	0.77	4.1E-03	3.10	2.55	4.64	5.63	5.11
Clostridia; Clostridiales; Clostridiaceae; unknown	0.69	0.02	1.07	1.42	5.00	4.89	4.75	0.54	0.15	0.64	2.87	2.67	3.16	2.97
Clostridia; Clostridiales; Dehaloceae; unknown	0.80	0.001	0.78	3.51	5.08	5.55	5.39	0.66	0.04	0.80	1.32	4.52	5.07	4.15
Clostridia; Clostridiales; Dehaloceae; Dehalobacterium	0.93	2.9E-06	1.59	2.42	3.12	3.93	4.24	0.87	1.2E-04	2.36	1.61	3.40	5.64	6.03
Clostridia; Clostridiales; Euceae; Acetobacterium	0.61	0.06	2.72	2.37	2.17	3.47	3.66	0.00	0.00	2.51	2.60	2.31	2.12	2.72
Clostridia; Clostridiales; Euceae; unknown	0.00	0.00	0.00	0.00	0.00	1.01	0.00	0.00	0.00	0.81	0.00	0.00	0.00	0.00

	Least Hydrophobic ClNOM								Least Hydrophobic NOM					
Clostridia; Clostridiales; Gracilibacteraceae; unknown	0.76	3.7E-03	2.36	4.55	5.28	5.63	5.53	0.46	0.24	1.56	4.80	5.27	5.46	4.67
Clostridia; Clostridiales; Gracilibacteraceae; Gracilibacter	0.91	1.4E-05	2.34	3.62	4.64	5.50	5.75	0.77	4.1E-03	2.57	2.83	4.49	5.29	4.90
Clostridia; Clostridiales; Gracilibacteraceae; unknown	0.90	1.8E-05	0.00	2.41	3.16	3.93	4.15	0.79	3.6E-03	0.00	0.93	2.51	3.66	3.28
Clostridia; Clostridiales; Lachnospiraceae; unknown	0.90	1.8E-05	1.42	0.85	3.79	4.85	5.15	0.00	0.00	1.85	0.78	2.60	1.32	1.82
Clostridia; Clostridiales; Lachnospiraceae; Anaerostipes	0.79	1.6E-03	0.00	0.00	1.65	2.82	3.88	0.00	0.00	0.00	0.58	0.00	0.00	0.00
Clostridia; Clostridiales; Lachnospiraceae; Blautia	0.62	0.05	0.00	0.00	1.01	2.58	2.71	0.00	0.00	0.91	0.00	0.00	0.00	0.00
Clostridia; Clostridiales; Lachnospiraceae; Coprococcus	0.86	1.3E-04	1.92	2.82	5.15	6.15	6.24	0.45	0.26	2.11	4.65	4.39	4.72	4.37
Clostridia; Clostridiales; Lachnospiraceae; unknown	0.92	4.8E-06	0.00	0.00	2.90	4.34	4.73	-0.29	0.55	1.53	2.28	2.27	1.06	0.75
Clostridia; Clostridiales; Peptococcaceae; Dehalobacter	0.72	0.01	1.46	1.22	0.79	3.96	4.38	0.78	4.1E-03	1.46	0.00	2.25	4.57	4.40
Clostridia; Clostridiales; Peptococcaceae; WCHB1-84	0.81	1.2E-03	0.77	4.10	4.53	5.60	5.63	0.64	0.04	1.55	4.13	4.91	5.44	4.63
Clostridia; Clostridiales; Peptococcaceae; unknown	0.71	0.01	1.76	2.88	3.25	4.11	4.27	0.73	0.01	0.64	2.74	3.76	4.23	3.77
Clostridia; Clostridiales; Ruminococcaceae; unknown	0.82	7.9E-04	2.52	2.04	4.51	5.59	5.69	0.84	4.8E-04	2.26	2.54	4.10	4.95	4.48
Clostridia; Clostridiales; Ruminococcaceae; Oscillospira	0.81	1.2E-03	0.68	0.00	1.13	4.50	4.85	0.00	0.00	0.65	0.58	0.73	0.96	0.70
Clostridia; Clostridiales; Ruminococcaceae; Ruminococcus	0.00	0.00	0.68	0.00	0.00	1.43	2.86	0.00	0.00	0.65	0.00	0.00	0.00	0.00
Clostridia; Clostridiales; Veillonellaceae; BSV43	0.79	1.9E-03	1.86	2.28	4.82	5.54	5.49	0.69	0.03	1.90	2.54	2.91	3.97	4.60
Clostridia; Clostridiales; Veillonellaceae; Sporomusa	0.76	3.7E-03	2.90	2.82	5.37	6.38	6.40	0.68	0.03	2.81	3.24	4.38	4.95	4.74
Clostridia; Clostridiales; Veillonellaceae; unknown	0.67	0.03	0.00	1.84	2.41	2.18	3.39	0.73	0.01	0.00	0.00	0.59	0.82	2.63
Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; unknown	0.65	0.03	0.00	0.00	0.79	1.87	2.33	0.00	0.00	0.00	0.00	0.00	0.00	0.85
Proteobacteria														
Deltaproteobacteria; Desulfuromonadales; Geobacteraceae; Geobacter	0.80	1.4E-03	2.30	2.94	4.67	4.93	4.66	-0.46	0.24	2.99	2.47	2.27	2.32	1.54
Deltaproteobacteria; Myxococcales; Myxococcaceae; Anaeromyxobacter	0.87	9.7E-05	2.20	4.28	5.40	5.85	5.86	0.00	0.00	1.29	1.76	2.21	2.04	1.44

Table C.3 Genera that grew significantly more in the at least one of the hydrophobic fractions of Cl-NOM enrichments compared to the NOM enrichments based on the Spearman's ρ for the moderate hydrophobic fractions. Log gene concentrations are listed for the five days sampled (copies/ml).

	Moderate Hydrophobic ClNOM							Moderate Hydrophobic NOM						
	ρ	***P*-value**	**1**	**17**	**50**	**83**	**113**	**ρ**	***P*-value**	**1**	**17**	**50**	**83**	**113**
Euryarchaeota														
Methanobacteria; Methanobacteriales; Methanobacteriaceae; unknown	0.60	0.06	1.17	0.00	3.39	5.15	3.44	0.00	0.00	0.00	0.00	1.23	1.54	2.61
Methanomicrobia; Methanosarcinales; Methanosarcinaceae; Methanosarcina	0.44	0.21	0.00	0.70	0.00	4.20	1.18	0.74	0.01	0.00	0.00	0.69	3.26	3.79
Methanomicrobia; YC-E6; unknown ; unknown	0.18	0.67	3.20	1.46	1.48	4.92	2.49	0.91	3.7E-05	0.13	1.94	3.00	5.12	5.35
Actinobacteria														
Actinobacteria; Actinobacteria; Actinomycetales; Cellulomonadaceae; Actinotalea	0.45	0.20	0.00	0.00	1.72	2.57	1.50	0.68	0.03	0.00	3.14	3.54	3.05	4.14
Chloroflexi														
Anaerolineae; Anaerolineales; Anaerolinaceae; WCHB1-05	0.72	0.02	0.00	0.00	3.04	5.07	3.37	0.00	0.00	0.00	0.57	0.00	0.00	0.68
Firmicutes														
Bacilli; Lactobacillales; Carnoceae; unknown	0.65	0.05	0.00	3.82	4.02	4.63	4.22	0.00	0.00	0.73	0.00	0.40	0.00	0.00
Clostridia; SHA-98; D2; unknown	0.70	0.02	1.17	0.00	0.00	0.00	0.00	0.81	2.0E-03	0.00	0.00	0.00	0.00	0.00
Clostridia; Clostridiales; unknown; unknown	0.75	0.01	2.19	3.70	4.57	5.93	5.13	0.84	6.1E-04	0.83	2.97	4.72	5.37	4.97
Clostridia; Clostridiales; Tissierellaceae; Sedimentibacter	0.61	0.06	3.56	5.00	5.03	5.97	5.20	0.62	0.06	0.86	3.75	4.72	5.27	4.75
Clostridia; Clostridiales; Christensenellaceae; unknown	0.71	0.02	3.92	2.60	4.23	5.69	5.29	0.84	6.1E-04	1.34	1.98	4.53	5.66	5.53
Clostridia; Clostridiales; Clostridiaceae; unknown	0.38	0.29	2.71	2.27	3.28	4.00	3.71	0.51	0.20	1.57	1.85	2.85	3.85	3.30
Clostridia; Clostridiales; Dehaloceae; unknown	0.13	0.75	0.72	0.82	1.05	1.08	1.48	0.67	0.04	0.56	4.51	5.23	5.75	5.27
Clostridia; Clostridiales; Dehaloceae; Dehalobacterium	0.45	0.21	1.33	1.73	2.86	4.24	2.90	0.90	3.7E-05	0.29	1.35	3.12	5.52	6.13
Clostridia; Clostridiales; Euceae; Acetobacterium	0.71	0.02	3.40	2.27	5.79	6.74	5.90	0.00	0.00	1.06	2.23	1.41	3.55	0.68
Clostridia; Clostridiales; Euceae; unknown	0.71	0.02	0.00	0.66	3.61	4.77	3.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Clostridia; Clostridiales; Gracilibacteraceae; unknown	0.43	0.23	2.78	4.15	3.14	4.77	4.45	0.49	0.23	1.17	4.68	5.18	5.29	4.69

	Moderate Hydrophobic ClNOM							Moderate Hydrophobic NOM						
Clostridia; Clostridiales; Gracilibacteraceae; Gracilibacter	0.86	4.6E-04	0.72	3.80	4.17	5.77	5.43	0.80	3.0E-03	1.68	2.90	4.32	5.52	5.21
Clostridia; Clostridiales; Gracilibacteraceae; unknown	0.63	0.06	0.00	2.20	2.46	3.98	2.46	0.74	0.01	0.00	1.23	2.89	3.55	3.20
Clostridia; Clostridiales; Lachnospiraceae; unknown	0.55	0.10	0.72	0.00	2.94	2.45	2.56	0.48	0.23	0.96	2.19	2.62	2.49	2.77
Clostridia; Clostridiales; Lachnospiraceae; Anaerostipes	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Clostridia; Clostridiales; Lachnospiraceae; Blautia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Clostridia; Clostridiales; Lachnospiraceae; Coprococcus	0.46	0.20	3.69	2.71	3.24	4.49	4.85	0.59	0.09	1.06	3.69	4.02	4.51	4.04
Clostridia; Clostridiales; Lachnospiraceae; unknown	0.00	0.00	1.27	0.00	0.37	0.00	1.07	0.00	0.00	0.29	1.84	1.69	2.25	0.00
Clostridia; Clostridiales; Peptococcaceae; Dehalobacter	0.81	0.00	0.72	1.17	2.42	5.01	4.93	0.69	0.03	0.00	0.71	2.03	2.89	3.86
Clostridia; Clostridiales; Peptococcaceae; WCHB1-84	0.61	0.06	0.00	1.52	3.71	4.98	2.99	0.59	0.09	0.73	3.69	4.39	4.55	4.06
Clostridia; Clostridiales; Peptococcaceae; unknown	0.58	0.08	0.82	0.90	2.01	3.52	2.47	0.67	0.04	0.00	2.81	3.62	4.16	3.72
Clostridia; Clostridiales; Ruminococcaceae; unknown	0.79	4.1E-03	3.49	2.22	3.71	5.00	4.88	0.85	4.4E-04	1.30	2.10	2.90	4.71	5.16
Clostridia; Clostridiales; Ruminococcaceae; Oscillospira	0.37	0.31	0.72	0.00	0.37	1.47	2.08	0.00	0.00	-0.44	0.49	0.00	0.00	0.68
Clostridia; Clostridiales; Ruminococcaceae; Ruminococcus	0.00	0.00	1.27	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Clostridia; Clostridiales; Veillonellaceae; BSV43	0.17	0.67	3.51	2.19	2.61	5.09	2.63	0.62	0.06	1.39	2.37	2.68	4.39	4.11
Clostridia; Clostridiales; Veillonellaceae; Sporomusa	0.47	0.18	3.97	2.63	2.96	4.32	5.12	0.55	0.14	1.55	2.73	3.28	4.06	3.48
Clostridia; Clostridiales; Veillonellaceae; unknown	0.45	0.20	0.00	0.80	0.79	0.79	2.02	0.41	0.35	0.00	0.59	1.73	1.19	1.83
Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; unknown	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.13	0.00	0.00
Proteobacteria														
Deltaproteobacteria; Desulfuromonadales; Geobacteraceae; Geobacter	0.56	0.09	2.37	6.12	5.77	6.65	5.75	0.48	0.23	0.66	3.94	3.84	4.82	4.33
Deltaproteobacteria; Myxococcales; Myxococcaceae; Anaeromyxobacter	0.40	0.27	0.00	1.27	2.36	3.14	0.91	0.74	0.01	0.00	2.08	2.02	3.68	3.29

Table C.4 Genera that grew significantly more in the at least one of the hydrophobic fractions of Cl-NOM enrichments compared to the NOM enrichments based on the Spearman's ρ for the most hydrophobic fractions. Log gene concentrations are listed for the five days sampled (copies/ml).

	Most Hydrophobic ClNOM							Most Hydrophobic NOM						
	ρ	*P*-value	1	17	50	83	113	ρ	*P*-value	1	17	50	83	113
Euryarchaeota														
Methanobacteria; Methanobacteriales; Methanobacteriaceae; unknown	0.89	1.0E-05	0.00	0.00	1.71	3.62	3.99	0.00	0.00	0.00	0.00	0.00	1.39	1.43
Methanomicrobia; Methanosarcinales; Methanosarcinaceae; Methanosarcina	0.93	1.6E-07	0.00	0.89	3.56	4.23	4.42	0.00	0.00	1.29	0.00	0.00	1.77	1.53
Methanomicrobia; YC-E6; unknown ; unknown	0.87	1.9E-05	2.40	1.80	5.20	5.90	5.98	0.53	0.15	1.55	2.18	3.06	4.26	3.64
Actinobacteria														
Actinobacteria; Actinobacteria; Actinomycetales; Cellulomonadaceae; Actinotalea	-0.12	0.76	0.00	2.86	2.99	0.87	0.78	0.63	0.05	1.46	3.03	3.41	3.72	3.46
Chloroflexi														
Anaerolineae; Anaerolineales; Anaerolinaceae; WCHB1-05	0.00	0.00	0.60	0.61	0.00	0.69	0.76	0.00	0.00	0.00	0.58	0.83	1.22	0.00
Firmicutes														
Bacilli; Lactobacillales; Carnoceae; unknown	0.00	0.00	0.00	0.61	0.87	0.00	1.44	0.30	0.53	0.64	0.58	0.00	1.06	1.59
Clostridia; SHA-98; D2; unknown	0.83	1.6E-04	0.00	0.61	0.00	0.00	1.73	0.60	0.08	0.00	0.00	0.00	0.00	0.00
Clostridia; Clostridiales; unknown; unknown	0.87	1.9E-05	1.53	2.44	4.79	4.82	5.22	0.79	3.6E-03	2.82	3.91	4.70	5.47	4.94
Clostridia; Clostridiales; Tissierellaceae; Sedimentibacter	0.37	0.24	2.52	5.83	5.80	5.58	5.69	0.64	0.04	2.80	3.38	4.62	5.18	4.61
Clostridia; Clostridiales; Christensenellaceae; unknown	0.92	8.7E-07	2.60	2.77	4.56	4.99	5.56	0.77	4.1E-03	3.10	2.55	4.64	5.63	5.11
Clostridia; Clostridiales; Clostridiaceae; unknown	0.64	0.02	0.00	0.00	4.68	3.38	3.64	0.54	0.15	0.64	2.87	2.67	3.16	2.97
Clostridia; Clostridiales; Dehaloceae; unknown	0.35	0.27	0.65	4.71	5.33	4.41	4.41	0.66	0.04	0.80	1.32	4.52	5.07	4.15
Clostridia; Clostridiales; Dehaloceae; Dehalobacterium	0.68	0.01	1.43	2.38	3.37	3.55	3.47	0.87	1.2E-04	2.36	1.61	3.40	5.64	6.03
Clostridia; Clostridiales; Euceae; Acetobacterium	0.00	0.00	2.35	2.57	2.75	1.48	2.50	0.00	0.00	2.51	2.60	2.31	2.12	2.72
Clostridia; Clostridiales; Euceae; unknown	0.00	0.00	0.00	0.00	0.53	0.00	0.00	0.00	0.00	0.81	0.00	0.00	0.00	0.00
Clostridia; Clostridiales; Gracilibacteraceae; unknown	0.41	0.18	0.81	4.70	5.13	4.76	4.68	0.46	0.24	1.56	4.80	5.27	5.46	4.67
Clostridia; Clostridiales; Gracilibacteraceae; Gracilibacter	0.95	1.5E-08	1.35	4.30	4.57	5.07	5.52	0.77	4.1E-03	2.57	2.83	4.49	5.29	4.90
Clostridia; Clostridiales; Gracilibacteraceae; unknown	0.85	5.7E-05	0.00	2.74	3.49	3.57	3.76	0.79	3.6E-03	0.00	0.93	2.51	3.66	3.28
Clostridia; Clostridiales; Lachnospiraceae; unknown	-0.10	0.79	0.65	2.60	2.46	0.73	0.90	0.00	0.00	1.85	0.78	2.60	1.32	1.82
Clostridia; Clostridiales; Lachnospiraceae; Anaerostipes	0.00	0.00	0.60	0.00	0.78	1.53	0.00	0.00	0.00	0.00	0.58	0.00	0.00	0.00

	Most Hydrophobic ClNOM								Most Hydrophobic NOM					
Clostridia; Clostridiales; Lachnospiraceae; Blautia	0.00	0.00	0.00	0.00	0.53	0.00	0.00	0.00	0.00	0.91	0.00	0.00	0.00	0.00
Clostridia; Clostridiales; Lachnospiraceae; Coprococcus	0.89	5.1E-06	2.61	2.78	4.59	5.15	5.38	0.45	0.26	2.11	4.65	4.39	4.72	4.37
Clostridia; Clostridiales; Lachnospiraceae; unknown	0.72	4.4E-03	0.00	0.00	0.63	0.69	2.21	-0.29	0.55	1.53	2.28	2.27	1.06	0.75
Clostridia; Clostridiales; Peptococcaceae; Dehalobacter	0.90	3.7E-06	0.60	0.00	2.76	3.43	3.76	0.78	4.1E-03	1.46	0.00	2.25	4.57	4.40
Clostridia; Clostridiales; Peptococcaceae; WCHB1-84	0.61	0.03	0.00	4.06	4.00	4.21	4.65	0.64	0.04	1.55	4.13	4.91	5.44	4.63
Clostridia; Clostridiales; Peptococcaceae; unknown	0.87	1.9E-05	1.59	3.10	3.47	4.11	4.09	0.73	0.01	0.64	2.74	3.76	4.23	3.77
Clostridia; Clostridiales; Ruminococcaceae; unknown	0.99	0.00	0.00	2.70	4.83	5.13	5.53	0.84	4.8E-04	2.26	2.54	4.10	4.95	4.48
Clostridia; Clostridiales; Ruminococcaceae; Oscillospira	0.78	1.1E-03	0.00	0.00	0.87	1.67	3.15	0.00	0.00	0.65	0.58	0.73	0.96	0.70
Clostridia; Clostridiales; Ruminococcaceae; Ruminococcus	0.60	0.03	0.60	0.00	0.53	1.05	4.06	0.00	0.00	0.65	0.00	0.00	0.00	0.00
Clostridia; Clostridiales; Veillonellaceae; BSV43	0.64	0.02	2.45	2.67	4.14	4.78	4.82	0.69	0.03	1.90	2.54	2.91	3.97	4.60
Clostridia; Clostridiales; Veillonellaceae; Sporomusa	0.88	1.1E-05	2.69	2.95	3.52	3.55	5.20	0.68	0.03	2.81	3.24	4.38	4.95	4.74
Clostridia; Clostridiales; Veillonellaceae; unknown	0.00	0.00	0.00	3.16	2.85	1.72	2.31	0.73	0.01	0.00	0.00	0.59	0.82	2.63
Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; unknown	0.00	0.00	0.00	0.00	0.00	1.84	1.81	0.00	0.00	0.00	0.00	0.00	0.00	0.85
Proteobacteria														
Deltaproteobacteria; Desulfuromonadales; Geobacteraceae; Geobacter	0.66	0.02	0.75	2.90	4.46	4.09	3.60	-0.46	0.24	2.99	2.47	2.27	2.32	1.54
Deltaproteobacteria; Myxococcales; Myxococcaceae; Anaeromyxobacter	0.60	0.03	0.70	3.52	5.25	4.63	4.56	0.00	0.00	1.29	1.76	2.21	2.04	1.44

C.4.2 Genera with Significant Growth in Trichloroethene Enrichment

Table C.5 Genera that were significantly grew when correlated to cis-DCE formation based on the Spearman's ρ for the least hydrophobic fraction. Log concentrations of each gene are listed for the three days measured (copies/ml). Statistics could not be calculated for the most hydrophobic fraction of Cl-NOM or NOM because no cis-DCE was detected but the genera log concentratios are still listed.

	Least Hydrophobic Cl-NOM					Least Hydrophobic NOM				
	ρ	*P*-value	1	20	49	ρ	*P*-value	1	20	49
Euryarchaeota										
Methanomicrobia; unknown; unknown; unknown	0.00	0.00	0.00	1.22	0.00	0.00	0.00	0.00	2.45	0.00
Armatimonadetes										
SHA-37; unknown; unknown; unknown	0.81	0.02	0.95	4.49	5.43	0.00	0.00	0.00	0.00	0.00
SJA-176; RB046; unknown; unknown	0.00	0.00	0.00	0.00	1.31	0.00	0.00	0.00	0.00	0.00
Bacteroidetes										
Bacteroidia; Bacteroidales; unknown; unknown	0.79	0.03	3.43	6.20	6.34	-0.16	0.98	5.98	6.85	6.17
Bacteroidia; Bacteroidales; Porphyromonadaceae; Paludibacter	0.91	0.01	0.00	5.98	6.51	-0.02	0.98	2.73	5.55	5.03
Bacteroidia; Bacteroidales; SB-1; unknown	0.67	0.09	0.82	4.49	5.18	-0.17	0.98	5.07	5.58	5.33
Chloroflexi										
Anaerolineae; Anaerolineales; Anaerolinaceae; Anaerolinea	0.68	0.09	2.09	2.97	5.58	0.00	0.00	0.00	0.00	0.00
Anaerolineae; WCHB1-50; unknown; unknown	0.00	0.00	0.98	0.00	0.00	0.14	0.98	4.26	5.24	5.00
Firmicutes										
Bacilli; Bacillales; Planococcaceae; Rummeliibacillus	0.77	0.03	0.00	3.14	5.45	0.00	0.00	0.00	0.00	0.00
Bacilli; Lactobacillales; Carnobacteriaceae; unknown	0.63	0.13	0.82	5.43	6.07	-0.23	0.98	5.25	5.75	5.26
Bacilli; Lactobacillales; Carnobacteriaceae; Trichococcus	0.63	0.13	1.19	6.38	7.00	-0.16	0.98	6.16	6.62	6.18
Clostridia; Clostridiales; Eubacteriaceae; unknown	0.85	0.01	0.00	4.46	5.52	-0.05	0.98	5.44	6.24	5.97
Clostridia; Clostridiales; Peptococcaceae; Dehalobacter	0.94	0.00	4.10	5.97	6.83	-0.06	0.98	4.70	5.06	3.41
Clostridia; Clostridiales; Peptococcaceae; WCHB1-84	0.78	0.03	5.25	5.85	6.23	0.77	0.36	0.81	2.85	4.58
Clostridia; Clostridiales; Veillonellaceae; Pelosinus	0.78	0.03	4.71	5.32	5.73	0.63	0.91	3.37	2.90	4.61
Clostridia; SHA-98; D2; unknown	0.00	0.00	0.00	2.49	2.98	0.00	0.00	0.00	0.00	1.49

	Least Hydrophobic Cl-NOM					Least Hydrophobic NOM				
Proteobacteria										
Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; unknown	0.74	0.05	1.15	6.77	7.15	0.29	0.98	5.16	6.18	6.26
Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; unknown	0.78	0.03	0.00	4.21	4.75	0.00	0.00	0.81	2.45	1.18
Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingomonas	0.81	0.02	4.62	5.54	5.76	-0.08	0.98	2.79	5.14	4.73
Betaproteobacteria; Burkholderiales; unknown; unknown	0.37	0.46	3.02	4.29	4.29	-0.60	0.91	3.61	3.13	1.18
Deltaproteobacteria; Myxococcales; unknown; unknown	0.00	0.00	0.00	1.22	1.31	0.00	0.00	0.00	0.00	0.00
Tenericutes										
Mollicutes; RF39; unknown; unknown	0.81	0.02	0.00	4.96	5.48	0.00	0.00	0.00	0.00	0.00
Verrucomicrobia										
Pedosphaerae; Pedosphaerales; unknown; unknown	0.00	0.00	0.87	1.49	0.00	0.00	0.00	0.00	0.00	1.18

Table C.6 Genera that were significantly grew when correlated to cis-DCE formation based on the Spearman's ρ for the moderate hydrophobic fraction. Log concentrations of each gene are listed for the three days measured (copies/ml). Statistics could not be calculated for the most hydrophobic fraction of Cl-NOM or NOM because no cis-DCE was detected but the genera log concentratios are still listed.

	Moderate Hydrophobic Cl-NOM					Moderate Hydrophobic NOM				
	ρ	*P*-value	1	20	49	ρ	*P*-value	1	20	49
Euryarchaeota										
Methanomicrobia; unknown; unknown; unknown	0.76	0.04	1.85	1.28	4.04	0.00	0.00	0.65	0.00	0.00
Armatimonadetes										
SHA-37; unknown; unknown; unknown	0.00	0.00	0.00	0.00	1.35	0.00	0.00	0.00	0.00	0.00
SJA-176; RB046; unknown; unknown	0.84	0.01	0.00	4.72	5.03	0.00	0.00	0.88	0.00	0.00
Bacteroidetes										
Bacteroidia; Bacteroidales; unknown; unknown	0.58	0.17	2.71	7.23	6.87	0.38	0.62	1.84	6.83	6.27
Bacteroidia; Bacteroidales; Porphyromonadaceae; Paludibacter	0.00	0.00	0.00	2.66	1.29	0.75	0.21	0.65	6.63	6.70
Bacteroidia; Bacteroidales; SB-1; unknown	0.90	0.00	0.90	6.55	6.99	0.88	0.05	0.00	3.53	5.44
Chloroflexi										
Anaerolineae; Anaerolineales; Anaerolinaceae; Anaerolinea	0.95	0.00	0.00	4.40	5.15	-0.67	0.38	2.91	4.71	0.00
Anaerolineae; WCHB1-50; unknown; unknown	0.94	0.00	0.00	3.05	4.90	0.00	0.00	0.00	0.00	0.00
Firmicutes										
Bacilli; Bacillales; Planococcaceae; Rummeliibacillus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacilli; Lactobacillales; Carnobacteriaceae; unknown	0.78	0.04	0.86	3.49	4.82	0.19	0.80	0.75	5.15	3.36

Bacilli; Lactobacillales; Carnobacteriaceae; Trichococcus	0.87	0.01	0.74	5.22	5.61	0.37	0.63	0.00	6.23	5.54
Clostridia; Clostridiales; Eubacteriaceae; unknown	0.97	0.00	1.02	4.15	4.44	0.15	0.82	3.09	5.82	5.38
Clostridia; Clostridiales; Peptococcaceae; Dehalobacter	0.97	0.00	3.79	4.71	6.94	0.79	0.15	3.47	6.65	7.12
Clostridia; Clostridiales; Peptococcaceae; WCHB1-84	0.14	0.78	4.82	4.86	4.84	0.28	0.74	3.30	0.00	2.66
Clostridia; Clostridiales; Veillonellaceae; Pelosinus	0.86	0.01	4.79	5.52	5.82	0.49	0.61	4.02	3.38	4.77
Clostridia; SHA-98; D2; unknown	0.97	0.00	1.93	4.55	5.55	0.62	0.46	2.27	4.71	5.12
Proteobacteria										
Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; unknown	0.61	0.14	4.54	5.60	5.54	0.24	0.76	4.13	5.82	5.63
Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; unknown	0.00	0.00	1.70	0.00	0.00	0.00	0.00	1.59	1.39	0.00
Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingomonas	0.16	0.76	3.01	3.07	3.28	0.24	0.76	4.02	3.14	3.12
Betaproteobacteria; Burkholderiales; unknown; unknown	0.90	0.00	2.92	4.03	4.28	0.80	0.15	3.31	2.81	4.56
Deltaproteobacteria; Myxococcales; unknown; unknown	0.94	0.00	0.96	4.17	4.56	0.20	0.78	0.00	4.22	3.76
Tenericutes										
Mollicutes; RF39; uknown; unknown	0.71	0.06	1.09	5.45	5.44	0.00	0.00	1.97	0.00	0.00
Verrucomicrobia										
Pedosphaerae; Pedosphaerales; unknown; unknown	0.89	0.00	1.60	2.90	5.03	0.79	0.15	3.56	4.97	5.32

Table C.7 Genera that were significantly grew when correlated to cis-DCE formation based on the Spearman's ρ for the most hydrophobic fraction. Log concentrations of each gene are listed for the three days measured (copies/ml). Statistics could not be calculated for the most hydrophobic fraction of Cl-NOM or NOM because no cis-DCE was detected but the genera log concentratios are still listed.

	Most Hydrophobic Cl-NOM			Most Hydrophobic NOM		
	1	20	49	1	20	49
Euryarchaeota						
Methanomicrobia; unknown; unknown; unknown	0.00	0.00	0.00	0.00	0.00	0.00
Armatimonadetes						
SHA-37; unknown; unknown; unknown	0.00	1.54	1.42	2.78	3.18	3.00
SJA-176; RB046; unknown; unknown	0.00	0.00	0.00	0.00	1.68	1.56
Bacteroidetes						
Bacteroidia; Bacteroidales; unknown; unknown	2.67	4.91	4.49	4.20	5.48	4.86
Bacteroidia; Bacteroidales; Porphyromonadaceae; Paludibacter	0.00	2.10	5.64	0.86	1.40	1.56
Bacteroidia; Bacteroidales; SB-1; unknown	0.00	1.18	0.00	0.00	1.45	0.00
Chloroflexi						
Anaerolineae; Anaerolineales; Anaerolinaceae; Anaerolinea	1.80	1.63	1.45	0.00	2.93	3.15
Anaerolineae; WCHB1-50; unknown; unknown	0.00	1.40	0.00	0.00	0.00	0.00
Firmicutes						
Bacilli; Bacillales; Planococcaceae; Rummeliibacillus	0.00	0.00	0.00	0.00	0.00	0.00
Bacilli; Lactobacillales; Carnobacteriaceae; unknown	1.16	0.00	0.00	1.33	2.65	1.36
Bacilli; Lactobacillales; Carnobacteriaceae; Trichococcus	1.94	0.00	1.61	0.00	2.75	0.00
Clostridia; Clostridiales; Eubacteriaceae; unknown	0.00	0.00	2.68	1.22	1.43	2.97
Clostridia; Clostridiales; Peptococcaceae; Dehalobacter	4.42	5.63	5.78	5.22	5.49	5.82
Clostridia; Clostridiales; Peptococcaceae; WCHB1-84	4.19	4.95	4.58	5.40	5.50	4.49
Clostridia; Clostridiales; Veillonellaceae; Pelosinus	3.93	4.47	2.95	4.28	1.60	4.08

	Most Hydrophobic Cl-NOM			Most Hydrophobic NOM		
Clostridia; SHA-98; D2; unknown	1.77	0.00	3.67	1.61	0.00	0.00
Proteobacteria						
Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; unknown	3.76	5.84	5.73	4.66	5.55	5.75
Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; unknown	0.84	2.77	2.42	0.76	1.44	2.71
Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingomonas	3.99	4.91	4.63	4.69	4.72	3.06
Betaproteobacteria; Burkholderiales; unknown; unknown	3.31	4.20	4.22	4.05	3.16	4.03
Deltaproteobacteria; Myxococcales; unknown; unknown	0.00	1.34	1.16	0.00	1.17	1.36
Tenericutes						
Mollicutes; RF39; uknown; unknown	0.00	1.60	3.11	0.00	0.00	1.36
Verrucomicrobia						
Pedosphaerae; Pedosphaerales; unknown; unknown	0.68	1.24	2.26	0.00	0.00	1.25

C.4.3 Genera with Significant Growth in Tetra-chlorobenzene Enrichments

Table C.8 Genera that were significantly grew when correlated with 1,2,3-triCB formation based on the Spearman's ρ for the most hydrophobic fraction. Log concentrations of each gene are listed for the two days measured (copies/ml).

		Least Cl-NOM		Least NOM		Mod Cl-NOM		Mod NOM		Most Cl-NOM		Most NOM	
	P-value	1	50	1	50	1	50	1	50	1	50	1	50
Acidobacteria													
Acidobacteriia; Acidobacteriales; Koribacteraceae; Koribacter	0.0044	2.2	3.0	1.2	6.2	4.1	1.2	5.1	1.7	1.6	6.9	3.8	3.1
Bacteroidetes													
Bacteroidia; Bacteroidales; unknown; unknown	0.0005	3.4	5.4	6.0	6.7	2.7	4.0	1.8	5.9	2.7	6.0	4.2	5.0
Bacteroidia; Bacteroidales; SB-1; unknown	0.0061	0.8	5.0	5.1	5.6	0.9	1.4	0.0	5.3	0.0	5.5	0.0	2.7

		Least Cl-NOM		Least NOM		Mod Cl-NOM		Mod NOM		Most Cl-NOM		Most NOM	
Firmicutes													
Clostridiales; Gracilibacteraceae; Gracilibacter	0.0044	5.3	6.6	6.2	7.0	5.5	6.5	6.0	7.1	5.1	7.2	6.1	6.6
Clostridiales; Gracilibacteraceae; unknown	0.0012	3.4	4.6	3.6	4.9	3.5	4.4	3.9	4.9	3.1	5.1	4.0	4.6
Clostridiales; Ruminococcaceae; unknown	0.0006	5.3	6.5	6.1	7.0	5.9	6.2	5.9	7.0	5.0	7.1	5.9	6.7
Clostridia; Clostridiales; Syntrophomonadaceae; Syntrophomonas	0.0002	2.4	4.9	5.2	6.3	5.8	2.7	4.9	5.3	4.0	6.3	4.4	3.5
Clostridia; SHA-98; D2; unknown	0.0002	0.0	0.0	0.0	5.2	1.9	0.0	2.3	0.0	1.8	5.5	1.6	0.0
Planctomycetes													
Phycisphaerae; Pla1; unknown; unknown	0.0041	0.0	0.0	0.0	4.8	0.0	1.3	1.9	1.7	0.8	5.1	1.1	0.0
Planctomycetia; Pirellulales; Pirellulaceae; unknown	0.0406	1.7	1.2	2.7	4.2	2.8	0.0	3.3	3.5	0.0	4.4	3.8	1.4
Proteobacteria													
Epsilonproteobacteria; Campylobacterales; Campylobacteraceae; Sulfurospirillum	0.0303	0.0	1.2	4.4	5.9	5.2	5.5	5.0	5.0	4.1	6.1	2.7	5.4
Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	0.0059	4.0	5.2	7.0	6.8	5.4	5.0	5.0	7.5	4.3	6.2	4.6	5.2
Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; unknown	0.0147	0.9	1.2	5.4	5.2	3.9	4.1	4.0	6.0	1.7	4.9	3.7	3.0