A COMMON AND PREVIOUSLY UNKNOWN ECOLOGICAL NICHE: THE HALORESPIRATION OF NATURAL ORGANOCHLORINES IN TERRESTRIAL SOIL AND SEDIMENT

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Although I was interested in Environmental Engineering since reading an encyclopedia article on the subject in middle school, the culmination of my education towards a Ph.D. has been cultivated by the people around me. Although the decision to obtain my undergraduate degree in Civil Engineering at Oklahoma State University was straightforward, the continuation of my education would not have happened without the encouragement of others. First, I would like to thank the Environmental Engineering faculty at Oklahoma State University, specifically Dee Ann Sanders and Greg Wilber, who showed me the joy and rewards of academia and encouraged me to set my goals high and earn a doctorate degree. Their guidance and counseling has helped me realize the potential in myself. Secondly, I would like to thank my friends who always challenged me to be a better student.

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Most importantly, I thank my wife, Jessica Sparks, who believed in me every step of the way and who has continued to make sacrifices which have allowed me to continue my education. My Ph.D. could not have been completed without her constant support.

DEDICATION

This dissertation is dedicated to my daughter Marabel. For the first six and a half months of her life, she has had to unwillingly share her dad's attention with this dissertation. She has brought a lot of joy in my life and I love her very much.

ABSTRACT

The phylum *Chloroflexi* contains several isolated bacteria that have been found to dechlorinate a diverse array of halogenated anthropogenic chemicals. These bacteria use organohalogens, such as polychlorinated biphenyls and trichloroethene, as their terminal electron acceptors and are called halorespirers. While the distribution and role of halorespiring *Chloroflexi*, as well as halorespirers in other phyla such as the *Firmicutes*, have been relatively well characterized in contaminated systems and laboratory cultures, their distribution and role in uncontaminated terrestrial environments, where abundant natural organohalogens could function as potential electron acceptors, has not been studied. This dissertation focuses on the distribution and role of halorespiring *Chloroflexi* in uncontaminated environments and investigates the ability of natural organochlorines to serve as terminal electron acceptors for halorespiring organisms.

In the third chapter of this thesis, soil samples from a range of uncontaminated sites (116 total, including 6 sectioned cores) were analyzed for the number of putatively halorespiring *Chloroflexi* 16S rRNA genes present. *Chloroflexi* populations were detected in all but 13 samples. The concentrations of organochlorine ([organochlorine]), inorganic chloride, and total organic carbon were obtained for 67 soil core sections. The number of putatively halorespiring *Chloroflexi* 16S rRNA genes positively correlated to [organochlorine]/TOC while the number of *Bacteria* 16S rRNA genes did not. Putatively halorespiring *Chloroflexi* were also observed to increase in number with a concomitant accumulation of chloride when cultured with an enzymatically produced mixture of organochlorines in the laboratory. This research demonstrates that putatively

halorespiring *Chloroflexi* are widely distributed as part of uncontaminated terrestrial ecosystems, they are correlated to the fraction of TOC present as organochlorines, and they appear to grow while dechlorinating organochlorines. These findings suggest halorespiring *Chloroflexi* may play an integral role in the biogeochemical chlorine cycle.

In the fourth chapter of this thesis, the geochemical parameters that impact the abundance of putatively halorespiring *Chloroflexi*, other than the amount of natural organochlorines present, was studied in more depth. Across the U.S. Upper Midwest, a natural sulfate gradient exists in lake water and lake sediment, providing an opportunity to explore whether sulfur impacts the presence or abundance of halorespiring *Chloroflexi*. Sixty-eight lake sediment samples were taken across this gradient and putatively halorespiring *Chloroflexi* were detected in nearly all of the samples (62) by quantitative polymerase chain reaction. Their quantities in the environment were quite high, ranging from 3.5×10^4 to 8.5×10^{10} copies 16S rRNA genes/g dry sediment and averaging 4.1% of the total *Bacteria* in the sediment. A total of 26 geochemical parameters were measured in the sediment samples, including the concentration of trace elements such as cobalt and zinc and general environmental parameters such as the NO_3^- concentration and pH; nevertheless, only two parameters correlated to the number of putatively halorespiring *Chloroflexi*: the number of *Bacteria* present and the concentration of total dissolved sulfur present. Sulfur, presumably present as sulfite and sulfide in the reduced lake sediments, appeared to impact putatively halorespiring *Chloroflexi* abundance at concentrations above 1 mM in the porewater. The impact of sulfur was corroborated with a terminal restriction fragment length polymorphism method targeting putatively halorespiring *Chloroflexi* in these environments, where the relative percentage of

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In the fifth chapter of this thesis, the ability of natural dechlorinating communities to use specific natural compounds as their terminal electron acceptor was tested. Two chlorinated xanthones, 2,7-dichloroxanthone and 1,3-dihydroxyl-5,7-dichloroxanthone were added to batch reactors and the dechlorination ability of bacterial communities from three different soils and two sediments was tested. The bacterial communities from two soils and one lake sediment from uncontaminated sites and a dioxin-contaminated marine sediment successfully dechlorinated both of these compounds. In all of these batch reactors the dechlorination of 1,3-dihydroxyl-5,7-dichloroxanthone occurred at a higher rate and with a shorter lag time than the dechlorination of 2,7-dichloroxanthone. One bacterial community, from an uncontaminated soil from a pine-dominated forest, was unable to dechlorinate either chlorinated xanthone, indicating that although this dechlorination ability was common, but not ubiquitous in the environment. Denaturing gradient gel electrophoresis and sequencing was used to determine which microbial populations thrived in the chlorinated xanthone-amended cultures compared to the unamended controls. A total of 12 sequences were obtained, predominantly belonging to the *Firmicutes* phylum. Although none of these sequences were closely related to the known halorespiring organisms, eight of the twelve sequences closely matched (>97%

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CHAPTER 1. Introduction

Over the next 30 years, the U.S. EPA has estimated that 350,000 contaminated sites in the United States will require remediation at a projected cost of 250 billion dollars (U.S. EPA, 2004). Halogenated pollutants are one of the most common sources of contamination at these sites (U.S. EPA, 2004). The cost to remediate Superfund sites alone is projected to be $19 billion, and of these sites, 69% are contaminated with halogenated volatile organics, 27% with polychlorinated biphenyls (PCBs), and 26% with other halogenated semi-volatile organic compounds (U.S. EPA, 2004). Additionally, of the twelve pollutants the EPA has classified as causing the most significant risk to human health and safety, nine are chlorinated (U.S. EPA, 2006a). Scientific research that leads to technologies to safely and affordably remediate chlorinated compounds is of great importance for human and ecological health.

In contaminated environments several microorganisms, called (de)halorespirers, have been identified that can reduce toxic chlorinated organics to less- or non-toxic compounds using these pollutants as electron acceptors for growth and energy (Adrian et al., 2000; Cutter et al., 2001; Cupples et al., 2003; Yoshida et al., 2005; Yan et al., 2006; Ahn et al., 2007):

$$H^+ + 2e^- + R\text{-}Cl \rightarrow R\text{-}H + Cl^-$$

Although halorespirers have been shown to be marginally effective for bioremediation at some contaminated sites (Imfeld et al., 2008; Scheutz et al., 2008), the potential that these microorganisms hold remains to be fully realized. Halorespirers are found in many phylogenetic groups, but those within the phylum *Chloroflexi* have received the most

attention because of their strict requirement for halogenated organic compounds for energy generation (Maymó-Gatell et al., 1997; Adrian et al., 2000; Fennell et al., 2004; Smidt and de Vos, 2004; Sung et al., 2006). The development and evolution of the enzymes responsible for halorespiration, called (reductive) dehalogenases, has been uncertain. Genetic analysis has indicated that the genes encoding for these enzymes are derived from a common ancestor prior to the introduction of anthropogenic chemicals (Hölscher et al., 2004) and thus naturally-occurring substrates for these enzymes must exist. These natural substrates may stimulate the growth of halorespiring *Chloroflexi*, which if applied at contaminated sites, may lead to the more complete remediation of chlorinated anthropogenic contaminants.

In natural systems, the chlorine cycle has been gaining recognition as important and complex. The amount of chlorinated organic matter in soil has been found to often surpass the amount of inorganic chloride (Myneni, 2002; Öberg, 2002; Gribble, 2003), and the number of identified natural chlorinated organic compounds discovered has been increasing each year, currently numbering more than 4,400 (Vetter and Gribble, 2007). Xanthones are a large family of compounds that are synthesized by plants, bacteria and fungi (Tomasek and Crawford, 1986) and are a major fraction of chlorinated organic matter in soils (Myneni, 2008). More than 180 xanthones with hydroxyl-, methyl-, isoprenyl-, methoxyl-, carboxyl-, and chloro- substituents are known (Tomasek and Crawford, 1986) and 38 xanthones with between 1 and 4 chlorines have been identified (Elix and Bennett, 1990; Elix and Crook, 1992). Thus, chlorinated xanthones are a potential source of natural electron acceptors for halorespirers in soil systems. Although the production and presence of chlorinated compounds has been studied (Leri et al.,

2006; Rohlenová et al., 2009), the dechlorination of natural chlorinated organics has not. Understanding the cycling of natural chlorinated organics may be fundamental to understanding the natural attenuation of chlorinated contaminants.

1.1 Objectives

Although the dechlorination of selected anthropogenic compounds by selected microorganisms has been well-established, neither the dechlorination of organochlorines as a natural ecological niche, nor the dechlorination of organic matter as a biogeochemical process has been established. In this dissertation, this ecological niche is strongly supported. Because of the pervasiveness of organochlorines in the natural environment, the process of halorespiring natural organochlorines is likely common, akin to the well-studied biogeochemical processes of fermentation, sulfate-reduction, and nitrate-reduction, and should be accepted in this context. **Chapter 2** presents a comprehensive literature review of what is currently known about halorespiration and organochlorines. **Chapter 3** presents research establishing the common detection of putatively halorespiring *Chloroflexi* in natural terrestrial systems, the quantitative correlation of these *Chloroflexi* to natural organochlorines in the environment, and links the growth of these *Chloroflexi* to the presence and dechlorination of organochlorines in batch cultures. **Chapter 4** presents research into the abundance and diversity of three subphyla of *Chloroflexi*, including those that contain putative halorespirers, across a gradient of geochemically diverse lake sediments. **Chapter 5** focuses on the halorespiration of two chlorinated xanthones, which are natural compounds never produced commercially, and the putative halorespirers involved in the dechlorination of

chlorinated xanthones are explored. With these discoveries we move closer to understanding the natural chlorine cycle and being able to manipulate it to remediate the thousands of contaminated sites that exist.

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CHAPTER 2. Literature Review

2.1 *Chloroflexi*

The phylum *Chloroflexi* is a mix of deeply branching bacteria from a diverse array of both extreme and common ecosystems. The root of *Chloroflexi* is debated as perhaps being phylogenetically close to the last universal common ancestor of all *Bacteria* (Tang et al., 2011; Sutcliffe, 2011). The phylum *Chloroflexi* has been estimated to dominate the microbial community of some sea-floor sediments and has been found to make up 12% and 16% of the community in the B horizon of temperate grasslands and alpine meadows, respectively (Inagaki et al., 2003; Costello and Schmidt, 2006; Will et al., 2010; Blazejak and Schippers, 2010). *Chloroflexi* have also been attributed to compose of 1-30% of the community in the activated sludge of municipal wastewater treatment plants and have been identified as important to the structure of flocs (Kragelund et al., 2011). In activated sludge, increased abundances of *Chloroflexi* have been associated with acidification and foaming episodes (Akuzawa et al., 2011; Kragelund et al., 2011). Much of the *Chloroflexi* present in these environments have been found to form deeply branching lineages unrelated to any currently isolated strains of *Chloroflexi*.

The *Choroflexi* phylum contains up to 16 proposed classes (Costello and Smidt, 2006), though isolated strains only currently represent six classes (FIG. 2.1): *Anaerolineae*, *Caldilineae*, *Dehalococcoidetes*, *Thermomicrobia*, *Chloroflexi*, and *Ktedonobacteria*. Isolated members of *Anaerolineae* are gram-negative, strictly anaerobic, non-spore-forming, and either mesophilic or thermophilic and have all been cultured fermenting sugars from food processing wastewater treatment plants (Yamada et

al., 2006). The isolated members of the class *Caldilinea* have been cultured from hot springs and are thermophilic facultative anaerobe with metabolisms consisting of the fermentation of various sugars and organic acids (Sekiguchi et al., 2003; Gregoire et al., 2011). The members of the class *Chloroflexi* consist of facultatively-anaerobic, mesophilic and thermophilic, filamentous, and usually photosynthetic bacteria (Hanada et al., 1995; Tang et al., 2011). The *Thermomicrobia* are thermophilic, obligate aerobic, chemoheterotrophs (Sekiguchi et al., 2003; Hugenholtz and Stackebrandt, 2004). *Ktedonobacter* are thermophilic, filamentous, spore-forming, gram-positive aerobic heterotrophs (Cavaletti et al., 2006; Yabe et al., 2010; Yabe et al., 2011). Finally, the isolates of the class *Dehalococcoidetes* have thus far been found to be obligate anaerobic halorespirers (Maymó-Gattell et al., 1997; Adrian et al., 2000; Cutter et al., 2001; Cupples et al., 2003; He et al., 2003; He et al., 2005; Sung et al., 2006b; May et al., 2008; Yan et al., 2009; Moe et al., 2009; Cheng and He, 2009). Other proposed classes within the *Chloroflexi* phylum contain microbes from a diverse range of environments, both "extreme," such as uranium mine pits, undersea mud volcanoes, and hot springs, and more common, such as seawater, soils, and sediments (Costello and Schmidt, 2006). Interestingly, the phylum contains a diverse array of uncultured clones distantly related from any isolated strains. The research in this thesis focuses primarily on the class *Dehalococcoidetes*, but attention is also given to the classes *Anearolinea* and *Caldilinea* because of the high frequency of clones from these classes that are found in halorespiring communities (Santoh et al., 2006; Kittelmann and Friedrich, 2008a).

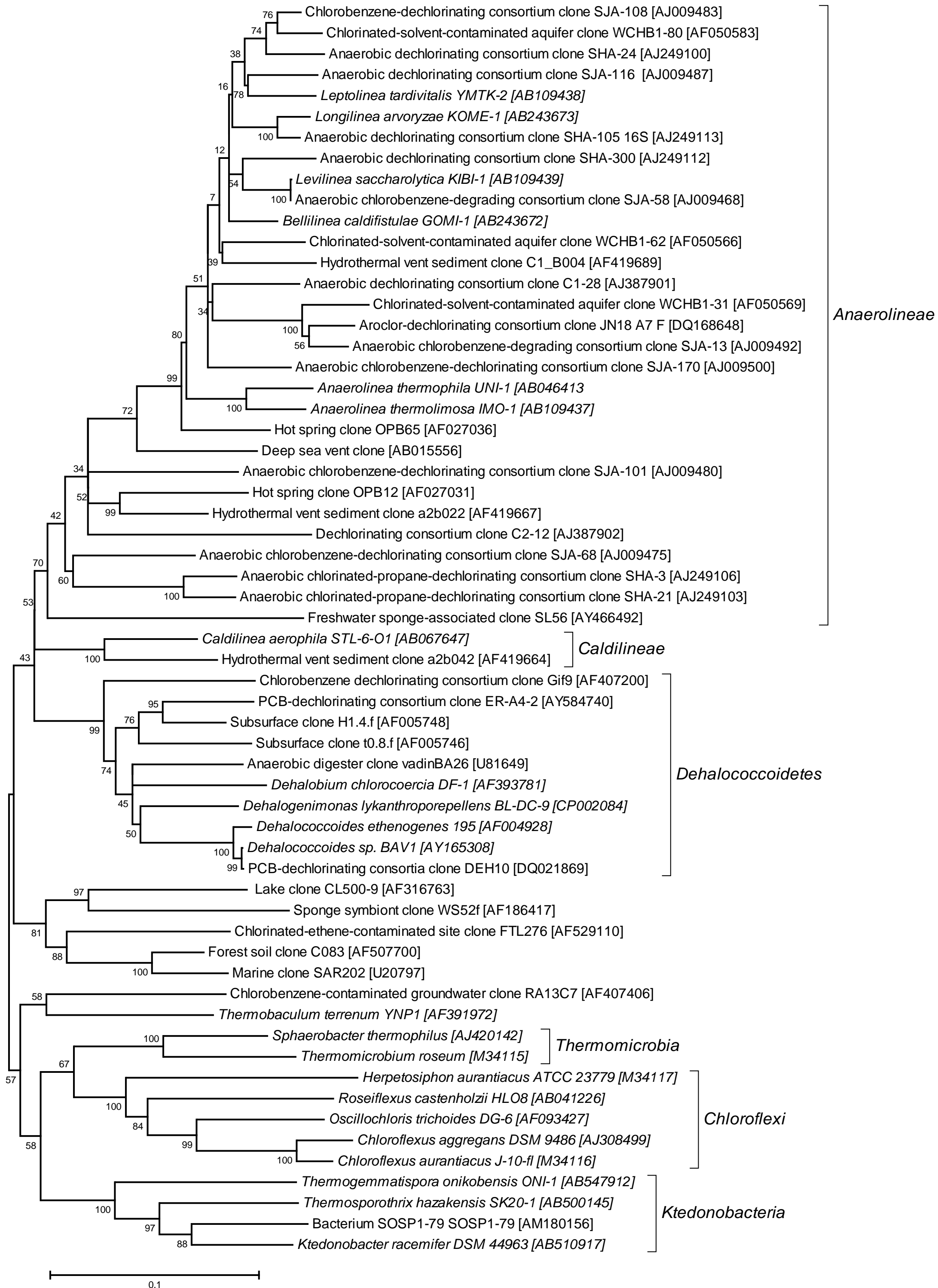

FIG. 2.1. Phylogenetic analysis of *Chloroflexi* and classes as proposed by Yamada et al., (2006) and Yabe et al., (2010). Isolated species of halorespirers have thus far clustered in the *Dehalococcoidetes* class, although numerous putatively halorespiring clones have also clustered in the *Anaerolineae* class.

2.2 *Dehalococcoides*

Strains of the genus *Dehalococcoides* are the best-characterized halorespiring *Chloroflexi*, as a strain from this genus was the first halorespiring *Chloroflexi* to be isolated (Maymó-Gatell et al., 1997). *Dehalococcoides*-like species appear to be widely, though not ubiquitously, present in anthropogenically-contaminated systems. In a survey of chloroethene-contaminated aquifers in North America and Europe, *Dehalococcoides*-like species were detected at 21 of 24 sites (Hendrickson et al., 2002), and the spatial heterogeneous distribution of indigenous halorespiring communities at contaminated sites have been well-documented and characterized (Müller et al., 2004; DePrato et al., 2007; Scheutz et al., 2008; Imfeld, et al., 2008). Generally, *Dehalococcoides*-like species and dechlorination activity are unevenly distributed within contaminant plumes (Lendvay et al., 2003; Fennell et al., 2004). Although pure strains are strictly anaerobic (Amos et al., 2008), dechlorination and *Dehalococcoides*-like species have been detected within close proximity to aerobic environments and plants as well (Kassenga et al., 2004; Hiraishi et al., 2005; Yoshida et al., 2005; Tu et al., 2011). The common detection but uneven distribution of *Dehalococcoides*-like species in the environment is not well understood.

Thus far, eight strains of *Dehalococcoides* have been isolated. The first strain, *Dehalococcoides ethenogenes* 195, was isolated from anaerobic digester sludge (Freedman and Gossett, 1989; Maymó-Gatell et al., 1997). Three strains (VS, BAV1, and GT) were isolated from contaminated aquifers (Cupples et al., 2003, He et al., 2003, Sung et al., 2006b). Strains MB was isolated from marine sediment, strains CBDB1 and DCMB5 were isolated from contaminated river sediments, and strain FL2 was isolated from an uncontaminated river sediment (Adrian et al., 2000; He et al., 2005; Bunge et al.,

2008; Cheng et al., 2009). All of these strains, with the exception of CBDB1 and DCMB5, were enriched and isolated using chlorinated ethenes as electron acceptors (Maymó-Gatell et al., 1997, Cupples et al., 2003, He et al., 2003; He et al., 2005; Sung et al., 2006b; Cheng et al., 2009), strain CBDB1 was isolated with chlorinated benzenes (Adrian et al., 2000), and strain DCMB5 was isolated on both chlorinated benzenes and chlorinated dioxins (Bunge et al., 2008). *Dehalococcoides* strains CBDB1, BAV1, FL2, GT, and *D. ethenogenes* 195 each exhibit unique characteristics with regard to their ability to dechlorinate different chlorinated ethenes and/or with regard to preferentially dechlorinating trichloroethene to *cis*-dichloroethene or *trans*-dichloroethene (Maymó-Gatell et al., 1997; He et al., 2003; He et al., 2005; Sung et al., 2006b; Marco-Urrea et al., 2011). In addition to the chlorinated ethenes, benzenes, and dioxins from which the *Dehalococcoides* strains have been enriched, *Dehalococcoides* strains have been found to halorespire chlorinated ethanes (He et al., 2003; Grostern and Edwards, 2006), chlorinated propanes (He et al., 2003), chlorinated phenols (Adrian et al., 2007), polychlorinated biphenyls (Yan et al., 2006; Bedard et al., 2007; Adrian et al., 2009), chlorinated furans (Fennell et al., 2004), chlorinated naphthalene (Fennell et al., 2004) and polybrominated diphenyl ethers (Robrock et al., 2008). Remarkably, none of these strains have been shown to be able to use non-halogenated electron acceptors in the laboratory, and the five currently sequenced genomes (*D. ethenogenes* 195, CBDB1, GT, VS, and BAV1) of *Dehalococcoides* indicates that *Dehalococcoides* are not capable of utilizing non-halogenated electron acceptors (Kube et al., 2005; Seshadri et al., 2005, McMurdie et al., 2009). Sequencing has, however, confirmed a unique abundance of

reductive dehalogenase genes present in these organisms (Kube et al., 2005; Seshadri et al., 2005, McMurdie et al., 2009).

The growth yields of the isolated strains of *Dehalococcoides* on various chlorinated substrates varies between 0.16-3.0 g of protein per mol chloride released or ~10^{12}-10^{14} cells per mol chloride released (Adrian et al., 2007; Tas et al., 2011; Marco-Urrea et al., 2011). All isolated strains are strictly anaerobic and require hydrogen or acetate as the electron donor, acetate as the carbon source, and as stated above, chlorinated compounds as the electron acceptor (Adrian et al., 2000; He et al., 2003; He et al., 2005; Maymó-Gatell et al., 1997; Smidt and de Vos, 2004).

Although the connection between the *Dehalococcoides*-like organisms and the dechlorination of anthropogenic compounds in laboratory cultures is well-established, weak correlations in studies of dechlorination of anthropogenic compounds in the environment suggests the existence of other halorespiring microorganisms and other electron acceptors. For example, in a study of sediments contaminated with 1,2-dichloroethane, the abundance of *Dehalococcoides*-like species did not correlate with 1,2-dichloroethane dechlorination (van der Zaan et al., 2009). In another study of the dechlorination of chlorinated benzenes, the abundance of *Dehalococcoides*-like species in contaminated river sediment did not correlate significantly with the amount of hexachlorobenzene *in situ* (Tas et al., 2009). In batch cultures *Dehalococcoides*-like organisms from fresh sediment grew two orders of magnitude before the dechlorination of amended chlorobenzenes was detected, suggesting the presence of electron acceptors alternative to the contaminated organochlorines present (Tas et al., 2011). In the same batch cultures, the dechlorination of hexachlorobenzene occurred at times without the

concomitant growth of *Dehalococcoides*-like organisms, also suggesting that other halorespirers may have been contributing to its dechlorination (Tas et al., 2011). Similarly, in a study of the halorespiration of polybrominated diphenyl ethers, *Dehalococcoides*-like organisms were not present in every sample exhibiting halorespiration, though more deeply branching organisms from the phylum *Chloroflexi* were detected via cloning in every case (Lee and He, 2010). This disconnect between the presence and growth of *Dehalococcoides* and the halorespiration of the chlorinated contaminants suggests that much is unexplained concerning halorespiration in the environment.

2.3 Other Halorespiring and Putatively-Halorespiring *Chloroflexi*

Although the isolated *Dehalococcoides* species form a tight group with nearly-identical 16S rRNA gene sequences, the other isolates from the *Dehalococcoidetes* class, despite having similarly restrictive metabolic capacities, are deeply branching (Dojka et al., 1998; Rossetti et al., 2003; Santoh et al., 2006; Schlötelburg et al., 2000; von Wintzingerode et al., 1999; May et al., 2008). Two isolates of this group, *Dehalobium* DF-1 and *o*-17 have 91% 16S rRNA gene identity to each other and approximately 87-88% and 89% 16S rRNA gene identity, respectively, to the cultured *Dehalococcoides* species (Bedard 2008; May et al., 2008). Two other isolates that reductively dechlorinate chlorinated propanes, *Dehalogenimonas lykanthropropellens* BC-DC-8 and BC-DC-9, also form a distinct lineage within the *Dehalococcoides*-like group, with 89-90% 16S rRNA gene sequence similarity to *o*-17, DF-1, and the cultured *Dehalococcoides* species

(Yan et al., 2009). Like the *Dehalococcoides* spp., all four of these other *Dehalococcoidetes* isolates have been shown thus far to be obligate halorespirers and are similarly limited in the electron donors that they can use (Hiraishi, 2008; May et al., 2008; Yan et al., 2008).

In addition to the *Dehalococcoidetes* class, the class *Anaerolineae*, as well as some of the proposed classes lacking isolates, also contain a high frequency of clones from dechlorinating enrichment cultures and contaminated sites (Kittelmann and Friedrich, 2008a, Watts et al., 2005; Tas et al., 2010). Although this may be coincidental., particularly because the isolated *Anaerolineae* have fairly generic carbohydrate and acid fermentative metabolisms (Yamada et al., 2006; Sekiguchi et al., 2003), a possibility exists that halorespirers are also present in these classes. In addition, one must recognize that the growing number of isolates in the class *Anaerolineae* is a result of the intensive enrichment of these organisms in a single laboratory from the starchy wastewater of food processing facilities (Yamada et al., 2006), which may bias our understanding of the metabolism of these organisms. Indeed, isotopic probing has found that a diverse array of deeply branching *Anaerolineae* are active in the halorespiration of low concentrations of perchloroethene *(*Kittelmann and Friedrich, 2008a), indicating that the presence of these organisms in halorespiring cultures may be more than just coincidence. Likewise, the current exclusivity (among *Chloroflexi*) of isolated halorespirers from *Dehalococcoidetes* is possibly a result of our limited enrichment techniques using high doses of common anthropogenic contaminants as electron acceptors, particularly the chlorinated ethenes. The possibility of nonhalorespiring organisms inside of the *Dehalococcoidetes* class, is also possible, though not yet supported. Only from the continued isolation and

sequencing of *Chloroflexi* can more cohesive descriptions of these groups of bacteria be elucidated.

2.4 Halorespiration from microbes outside of *Chloroflexi*

Several halorespirers have been isolated and characterized from phyla distinct from the *Chloroflexi*. Strains of *Clostridium*, *Dehalobacter* and *Desulfitobacterium,* of the phylum *Firmicutes*, and strains of *Anaeromyxobacter*, *Desulfomonile*, *Desulfuromonas*, *Geobacter*, and *Sulfurospirillum* of the phylum *Proteobacteria* can also dechlorinate some chlorinated hydrocarbons (Shelton and Tiedje, 1984b; Holliger et al., 1993; Krumholz et al., 1996; Dennie et al., 1998; Chang et al., 2000; Luitjen et al., 2003; Sung et al., 2006a; Suyama et al., 2001; Sanford et al., 2002; Futagami et al., 2007; Yoshida et al., 2009; Nonaka et al., 2006). With the exception of the *Dehalobacter*, these bacteria differ from the *Dehalococcoidetes* in that they have a broad array of metabolic capabilities beyond halorespiration (Futagami et al., 2007; Nonaka et al., 2006). The sequencing of a halorespiring *Desulfitobacterium* also elucidated the presence of only two dehalogenase genes, few in comparison to the number found in the *Dehalococcoidetes* isolates (Nonaka et al., 2006; Futagami et al., 2007). Nonetheless, the halorespirers and putative-halorespirers of these phyla have been largely understudied.

2.5 Reductive Dehalogenase Genes

Genome sequencing has indicated that some *Dehalococcoides* strains contain as many as 32 reductive dehalogenase (rdh) genes (Hölscher et al., 2004; Kube et al., 2005). Only four rdh genes from *Dehalococcoides* strains (*pceA*, *bvcA*, *tceA*, *vcrA*) have been

specifically studied, and thus, the majority of rdh genes identified have unknown substrate specificities (Waller et al., 2005). In addition to the multitude of rdh genes that exist, the few well-characterized rdh enzymes studied have been found to have wide substrate ranges (Fung et al., 2007). For example, TceA from *D. ethenogenes* 195 not only contributes to the dechlorination of trichloroethylene to ethene, but has also been found to dechlorinate a wide range of chlorinated and brominated alkanes and alkenes (Magnuson et al., 2000). Interestingly, several rdh genes are also simultaneously transcribed during the dechlorination of a single chlorinated substrate and multiple chlorinated substrates can induce transcription of the same rdh gene (Waller et al., 2005). These characteristics imply that *Dehalococcoides* species may have evolved to halorespire a rich diversity of halogenated compounds simultaneously.

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

Because anthropogenic chlorinated compounds have only existed for a short period of time, the development and evolution of rdh genes has been a subject of inquiry. Many rdh genes are located in regions that indicate horizontal gene transfer in both

strains 195 and CBDB1 (Kube et al., 2005; Regeard et al., 2005). Also, locations and orientations of orthologous rdh genes located in both CBDB1 and strain 195 vary widely, indicating that these genes are highly plastic (Kube et al., 2005). Still, nearly every rdhB gene is unique and evolutionarily connected to its corresponding rdhA gene (Kube et al., 2005). Genetic analysis has concluded that rdh genes in isolated *Dehalococcoides* species are all true homologs of each other and derived from a common ancestor (Hölscher et al., 2004). Because of the short time that anthropogenic compounds have existed, the duplication, divergence, and adaptation of rdh genes must be a result of older, natural causes (Hölscher et al., 2004).

2.6 Natural Chlorinated Organic Matter

The natural chlorine cycle has been receiving increasing attention as a multifaceted biogeochemical cycle. Thousands of natural chlorinated compounds are known to exist in nature (Öberg 2002; Gribble 2003). In addition, the transformation of chloride into organochlorine has been shown to be common, resulting in organochlorine levels often exceeding those of chloride in soil (Myneni 2002; Leri et al., 2006). There are more than 4,400 identified organohalogens that are produced by plants, marine organisms, insects, bacteria, fungi, and mammals (Gribble 1994; Rohlenová et al., 2009), many of which closely resemble anthropogenic pollutants (Vetter and Gribble, 2007). Indeed, in marine environments, naturally-occurring organohalogens are produced by a variety of species, and in certain sponges, up to 12% of the sponge dry weight can be accounted for by organobromines including bromoindoles, bromophenols and bromopyrroles (Gribble 1999; Turon et al., 2000; Ahn et al., 2003). In these systems

some natural organohalogens have been shown to bioaccumulate in higher organisms in a manner similar to the bioaccumulation of anthropogenic compounds (Teuten et al., 2005; Vetter and Gribble, 2007).

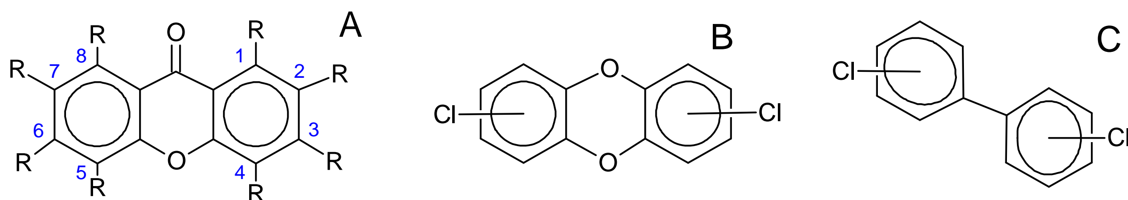

FIG. 2.2 The chemical structure of xanthones (A) are shown, as are the structures of PCBs (B) and dioxins (C). For xanthones, carbons are numbered and the most common subgroups are chloro-, methyl-, methoxyl-, hydroxyl-, and hydrogen.

Organochlorines account for the major chlorine species in weathering plant materials (Myneni, 2002). The fraction of chlorine present as aromatic or aliphatic organochlorines increases as fresh plant material becomes senescent and begins to humify (Myneni, 2002). It has been demonstrated that in terrestrial systems the chloroperoxidase enzymes, found in a variety of plants and fungi, can chlorinate natural organic matter with both aliphatic and aromatic—including phenolic—moieties (Reina et al., 2004). These enzymes also chlorinate aliphatic and aromatic structures during the breakdown of large molecular weight lignin molecules (Ortiz-Bermúdez et al., 2003).

Xanthones (Fig. 2) are a large family of compounds that are synthesized by plants, bacteria and fungi (Tomasek and Crawford, 1986). Although unsubstituted xanthone is not known to exist in nature, more than 180 xanthones with hydroxyl-, methyl-, isoprenyl-, methoxyl-, carboxyl-, and chloro- substituents are known (Tomasek and Crawford, 1986). Thirty-eight xanthones with between 1 and 4 chlorines as well as various methoxyl-, methyl-, and dihydroxyl- substitutions have been identified (Elix and

Bennett, 1990; Elix and Crook, 1992). Thus, chloroxanthones could serve as a major natural source of electron acceptors for halorespirers in uncontaminated environments.

2.7 Halorespiration of Natural Organochlorines

The hypothesis that *Dehalococcoides*-like organisms use natural organochlorines as their electron acceptors has been discussed in recent literature (Adrian et al., 2007; Bunge et al., 2008; Hiraishi 2008; Kittelmann and Friedrich, 2008a; Kittelmann and Friedrich 2008b; Tas et al., 2011). Research concerning this hypothesis, however, has been indirect and has comprised of the dechlorination of anthropogenic compounds that are also naturally produced. Laboratory cultures of *Dehalococcoides* strains 195 and CBDB1 have been shown to grow on 2,3-dichlorophenol and 2,3,4-trichlorophenol and are also capable of dechlorinating several other chlorophenols (Adrian et al., 2007). Additionally, 2,3-dichlorophenol has been shown to induce transcription of several rdh genes in *D. ethenogenes* 195, and the same rdh used to dechlorinate tetrachloroethene to trichloroethene is thought to be responsible for its dechlorination (Fung et al., 2007). Because chlorinated phenols can be produced naturally (Ortiz-Bermúdez et al., 2003), *Dehalococcoides* species may use them in uncontaminated systems.

A mixed indigenous bacterial culture from a sea sponge, found to contain *Dehalococcoides*-like bacteria as well as other putative halorespirers, was also shown to reduce several synthetic brominated phenols (Ahn et al., 2003). Because brominated phenolic compounds can be naturally produced by sea sponges (Gribble, 1999), this study associated natural halorespiring communities in marine systems to naturally occurring organohalogens.

Another study found that mixed cultures containing *Dehalococcoides*-like microorganisms from uncontaminated sediment in the North Sea could degrade tetrachloroethene to *trans*- and *cis*-dichloroethene (Kittelmann and Friedrich, 2008b). Because evidence exists for the natural production of tetrachloroethene by marine algae (Abrahamsson et al., 1995), it could also be considered a putative natural substrate for *Dehalococcoides*-like organisms in marine systems. Chlorinated ethenes, however, do not have a known natural source in terrestrial systems outside of extreme geological events, such as volcanic activity (Gribble, 1992), and are therefore unlikely electron acceptors for *Dehalococcoides*-like organisms in uncontaminated terrestrial systems.

Therefore, although these studies link halorespiration to natural chlorinated or brominated compounds, their focus was constrained to specific halorespiring strains and/or specific compounds, and thus halorespiration in natural systems as an ecological niche could only be speculated. Additionally, any association between halorespirers and halogenated compounds in uncontaminated soils of *terrestrial* systems has not been tested and much remains to be discovered and learned about these environments.

2.8 Biostimulation and bioaugmentation of halorespirers with alternative electron acceptors

Study into halorespiration as a natural niche has implications for environmental engineering and could lead to new technologies for bioremediation. The biostimulation of halorespirers using alternative electron acceptors, often called priming, holds promise for the remediation of toxic chlorinated compounds. Because the isolates of

Dehalococcoidetes only use halogenated electron acceptors for growth, alternative electron acceptors are likely to be limited to other halogenated compounds. Dibromobiphenyls and several chlorinated aromatics have been shown to stimulate the dechlorination of PCBs (Bedard et al., 2006; Bedard et al., 1998; Deeward and Bedard, 1999; Krumins et al., 2009), and several chlorinated aromatic compounds were found to stimulate the dechlorination of dioxins (Ahn et al., 2007; Ahn et al., 2008). One barrier to the use of biostimulation in the environment is that these halogenated compounds are anthropogenic and toxic themselves, and cannot therefore be administered *in situ*. The discovery of a *naturally occurring* compound or groups of compounds that could serve as electron acceptors for the growth of halorespirers may be the missing key to the feasible application of biostimulation at contaminated sites, particularly at those sites where the contaminant of interest is only sparingly soluble.

Bioaugmentation, the addition of exogenous microorganisms with particular metabolic abilities into a contaminated system, has also been shown to be a potentially effective strategy for the enhancement of bioremediation, both for PCBs (Yan et al., 2006a; Ahn et al., 2008; Winchell and Novak, 2008; Krumins et al., 2009) and for chlorinated ethenes (Harkness et al., 1999; Major et al., 2002; Adamson et al., 2003; Ritalahti et al., 2005). Unfortunately, concerns exist that augmented strains will exhibit reduced fitness or the distribution of augmented cultures through a contaminated site will not be optimal, which has hampered the application of this technology (Adamson et al., 2003; Lee et al., 2010). The development of robust halorespiring cultures derived from the halorespiration of natural organochlorines may help these cultures to maintain their original fitness, and thus increase the applicability of bioaugmentation.

2.9 Summary

In summary, although the putatively halorespiring *Chloroflexi*, as well as other halorespiring *Bacteria*, have been well-studied in regards to their ability to use anthropogenic contaminants as their terminal electron acceptors, the natural role and niche of these organisms have only been speculative. These organisms have not yet been studied in uncontaminated environments where they could use natural organochlorines as their terminal electron acceptors. By studying their natural environments, though, insights into their in situ physiology could be gained, which could lead to new approaches in the way we use these organisms for bioremediation. By determining the abundance of these bacteria in natural environments and whether they correlate to natural organochlorines, halorespiring microorganisms can be established as integral to natural microbial communities where they may have profound impacts on the chlorine and carbon cycles. Additionally, the geochemical pressures on these bacteria can be studied, which may lead to insights on what geochemical conditions, if any, may impact their abundance and diversity which would have implications for bioremediation potential and for carbon and chlorine cycling as well. And finally, by determining what natural organochlorines can be directly used as their terminal electron acceptors, new insights might be gained to what chemicals these bacteria evolved to halorespire which could lead to new technologies for biostimulation and bioremediation.

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CHAPTER 3. Natural niche for halorespiring *Chloroflexi*

3.1 Introduction

Chloroflexi are a deeply branching and diverse phylum containing isolates that are aerobic and anaerobic thermophiles, filamentous anoxygenic phototrophs, and anaerobic halorespirers (Hanada et al., 1995; Maymó-Gatell et al., 1997; Sekiguchi et al., 2003; Hugenholtz and Stackebrandt, 2004). *Chloroflexi* have been estimated to dominate the microbial community of some sea-floor sediments and also can make up 12% and 16% of the community in the B-horizon of temperate grasslands and alpine meadows, respectively (Inagaki et al., 2003; Costello and Schmidt, 2006; Will et al., 2010). Much of the *Chloroflexi* present in these environments have been found to form deeply branching lineages unrelated to any isolated strains of *Chloroflexi*. In addition, there is a lack of physiological data regarding the niche of these high-abundance *Chloroflexi*.

The *Chloroflexi* phylum contains several isolates that have been shown to be obligate halorespirers. These isolates include the genus *Dehalococcoides*, and more recently, *Dehalobium chlorocoercia* DF-1, strain *o*-17, and *Dehalogenimonas lykanthroporepellens* strains BL-DC-8 and BL-DC-9 (Maymó-Gatell et al., 1997; Fagervold et al., 2005; May et l., 2008; Yan et al., 2009). Although the *Dehalococcoides* isolates have nearly identical 16S rRNA sequence similarity, *Dehalobium*, strain *o*-17, and *Dehalogenimonas* are more distantly related, with 89-91% 16S rRNA gene sequence identity to each other, and approximately 87-90% 16S rRNA gene sequence identity to the cultured *Dehalococcoides* species (Bedard et al., 2008; May et al., 2008; Yan et al., 2009). Members of the genus *Dehalococcoides* have been found to dechlorinate a wide range of persistent organic contaminants and are thus thought to be promising for bioremediation applications (Adrian et al., 2000; Fennell et al., 2004; He et al., 2005;

Maymó-Gatell et al., 1997). All cultured halorespiring *Chloroflexi* have been shown thus far to be obligate halorespirers and share similar limited metabolic capabilities with respect to nutrients and electron donors (Hiraishi, 2008; May et al., 2008; Yan et al., 2009). In addition to the isolated strains, several uncultivated *Chloroflexi* have been linked with the reductive dechlorination of polychlorinated biphenyls (PCBs), chlorobenzenes, chloroethenes, chloroethanes, brominated phenols, and polybrominated diphenyl ethers (Dojka et al., 1998; von Wintzingerode et al., 1999; Schlötelburg et al., 2000; Ahn et al., 2003; Santoh et al., 2006; Yan et al., 2006; Ahn et al., 2007; van der Zaan et al., 2009; Lee and He, 2010) and many of these uncultivated *Chloroflexi* form distinct lineages from the isolated halorespirers, indicating that the halorespiring *Chloroflexi* may be even more deeply branching and diverse. The known halorespirers and the uncultured bacteria that have been associated with reductive dechlorination have been previously referred as *Dehalococcoides*-like bacteria (Hiraishi et al., 2005; Yan et al., 2006; Hiraishi, 2008) or *Dehalococcoides*-like *Chloroflexi* (Adrian 2009). Although the genus *Dehalococcoides* is the most studied, the diversity of these halorespiring and putatively-halorespiring organisms may span several subphyla within the *Chloroflexi*; therefore, I refer herein to these organisms as putatively-halorespiring *Chloroflexi*.

Although the connection between the putatively-halorespiring *Chloroflexi* and the dechlorination of anthropogenic contaminants in laboratory cultures is well established, additional evidence suggests that other electron acceptors, such as natural organochlorines, exist. For example, in a study of sediments contaminated with 1,2-dichloroethane (1,2-DCA), the abundance of *Dehalococcoides* did not correlate with the presence or absence of 1,2-DCA dechlorination (van der Zaan et al., 2009). In another

study of the halorespiration of chlorinated benzenes, the abundance of *Dehalococcoides* in contaminated river sediment did not correlate significantly with the amount of hexachlorobenzene *in situ* (Tas et al., 2009). Furthermore, *Dehalococcoides*-like organisms from fresh sediment in that study grew two orders of magnitude in batch cultures before dechlorination of amended chlorobenzenes was detected (Tas et al., 2011). This disconnect between the presence and growth of *Dehalococcoides* and the halorespiration of known chlorinated contaminants suggests that much is unexplained concerning halorespiration in the environment.

In uncontaminated systems, naturally occurring organohalogens could potentially serve as electron acceptors for halorespiring bacteria. In marine environments, natural organobromine compounds are produced by a variety of bacterial species and include bromoindoles, -phenols and -pyrroles, among other molecules (Gribble, 1999; Turon et al., 2000). Natural organobromine is ubiquitous in marine sediments and appears to be degraded during the breakdown of organic matter as part of a biogeochemical bromine cycle (Leri et al., 2010). Reducing conditions are believed to promote reductive debromination of natural organobromine in the sedimentary environment (Müller et al., 1996; Ahn et al., 2003; Biester et al., 2006). Indeed, the hypothesis that bacteria indigenous to seafloor sediments may halorespire brominated phenols has been supported in the recent literature (Futagami et al., 2009).

The natural chlorine cycle has also received increasing attention as a multifaceted biogeochemical process. Plants, marine organisms, insects, bacteria, fungi, and mammals produce thousands of natural organochlorines, and many of these organochlorines closely resemble anthropogenic compounds (Gribble, 1994; Oberg, 2002; Gribble, 2003; Vetter

and Gribble, 2007). In terrestrial environments, the transformation of chloride into organochlorine compounds occurs in part, via the activity of the chloroperoxidase enzyme (Reina et al., 2004), resulting in organochlorine levels often exceeding those of chloride in surface soils (Öberg, 2002; Ortiz-Bermúdez et al., 2003). With soil depth, chlorine speciation changes from predominantly organic to inorganic, suggesting that the natural organochlorine in soil organic matter may undergo biogeochemical dechlorination processes as well (Lei and Myneni, 2010*)*. The hypothesis that halorespiring *Chloroflexi* may use natural organochlorines as electron acceptors in uncontaminated environments has been discussed in recent literature (Adrian et al., 2007; Bunge et al., 2008; Hiraishi, 2008; Kittelmann and Friedrich, 2008; Kittelmann and Friedrich, 2008), though the association between putatively-halorespiring *Chloroflexi* in natural terrestrial environments and natural organochlorine levels has not been investigated.

In this study, soils from pristine areas with different vegetative covers (uncontaminated grasslands and forests) were investigated for the presence of putatively halorespiring *Chloroflexi*. Additionally, soil cores from the New Jersey Pine Barrens were assessed for the number of putatively halorespiring *Chloroflexi* present, natural organochlorine content, and a correlation between these parameters. Finally, the growth of putatively halorespiring *Chloroflexi* and the accumulation of chloride were measured in batch reactors fed an enzymatically-(chloroperoxidase) produced mixture of organochlorines.

3.2 Methods

Soil collection. Both grab samples and soil cores were collected for analysis from sites with no known history of anthropogenic contamination. Grab samples were collected between 3 cm and 5 cm below the surface, generally in the upper A horizon of the soil, from four separate Minnesota State Parks (20 samples), three nature parks in Oklahoma (12 samples), a regional park in California (1 sample), and a national forest in Oregon (1 sample). Pairs of adjacent soil cores were collected from the Brendan Byrne State Forest within the New Jersey Pine Barrens in both September 2006 and January 2008, for a total of 6 pairs. Each pair came from a maple-, oak-, or pine-dominated area. One core of each pair was used for organochlorine analysis and the other was used for microbial analysis. Soil cores were 30 cm in depth with the exception of the maple-dominated core sampled September 2006 for microbial analysis, which was 14 cm deep. All soil cores for microbial analysis were split into 2-cm sections, providing a total number of 82 core samples.

Grab samples were collected with scoopulas and spoons washed with 95 percent ethanol between sampling to avoid microbial cross-contamination. Samples were packaged individually in glass jars or plastic bags, placed immediately on ice, and shipped or transported to the laboratory within 24 hours. All samples were frozen at -70°C upon arrival. Soil cores were collected in butyrate plastic sleeves. The cores for microbial analyses were shipped on ice within 24 hours of collection. Upon arrival the cores were immediately transferred into an anaerobic glove bag (Coy Laboratory Products) where the sections were cut with a cast-cutter, separated with ethanol-washed scoopulas, and frozen at -70°C until analysis.

DNA extraction. For genomic DNA extraction, each soil core section or grab sample was homogenized with a mortar and pestle washed and rinsed with 95 percent ethanol. DNA was extracted with the FastDNA Spin Kit for Soil (MP Biomedical) with one modification; the DNA-containing Binding Matrix was washed two times with 1.0 mL of 6.0 M BioUltra-grade guanidine thiocyanate solution (Sigma-Aldrich) to remove soil humics (Jaatinen et al., 2004). For the grab samples, 50 µL of water was used for last step of the extraction with no further cleanup. For the cores, 150 µL of water was used during the last step of the extraction, which was further cleaned using the PowerClean DNA Clean-Up kit (MoBio Laboratories). DNA was also extracted from sediments contaminated with PCBs (from Baltimore Harbor, Maryland, Fox River, Wisconsin, and Hudson River, New York) or dioxins (Palos Verdes Harbor, California), and trichloroethene (TCE)-contaminated aquifer material (New York) to allow the comparison of the DNA in uncontaminated samples to that in contaminated samples and to serve as a positive control for the microbial analyses.

Each round of DNA extractions was performed along with one sample of autoclaved soil extracted in an identical manner to serve as a negative control. In addition, three separate sub-samples of three samples (the 24-26 cm section of the soil core from an oak-dominated area taken January 2008, a grab sample with pine cover taken from Mille Lacs-Kathio State Park, MN, and the TCE-contaminated aquifer material, New York) were extracted to determine reproducibility and variability of the DNA extraction. The standard deviations of the qPCR results (see below) for both *Chloroflexi* and *Bacteria* 16S rRNA gene copies of the subsamples were less than the standard deviations obtained for replicate thermocycler runs of the same sample.

qPCR of putatively halorespiring *Chloroflexi* and *Bacteria*. Putatively halorespiring *Chloroflexi* 16S rRNA genes and *Bacteria* 16S rRNA genes were quantified for each sample using quantitative polymerase chain reaction (qPCR). Each qPCR reaction totaled 25 µL using iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories), 25 µg Bovine Serum Albumin (Roche Diagnostics), 300 nM forward primer, 300 nM reverse primer, and 1 µL of undiluted DNA extract or standard. An ABI 7000 Thermocycler (Applied Biosystems) with 7000 System Software was used with a thermocycler protocol of 50°C for 2 minutes, 95°C for 3 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds. A melting curve analysis was performed after each complete run to ensure that primer-dimers were not amplified and the amplification was specific.

For qPCR to enumerate putatively halorespiring *Chloroflexi* 16S rRNA genes, two primers, Dhc1154F (5’-CAC ACA CGC TAC AAT GGA CAG AAC-3’) and Dhc1286R (5’-GAT ATG CGG TTA CTA GCA ACT CCA AC-3’), were designed using PrimerExpress software based on the *Dehalococcoides* genome. Previously published primers for *Dehalococcoides* (Smits et al., 2004; Yoshida et al., 2005) were found to be nonspecific for the samples of this study. The concentration of both the reverse and forward primers and the annealing/extension temperature were optimized for specificity using melting curve analysis and gel electrophoresis. The *Chloroflexi* isolate *Herpetosiphon aurantiacus* was used as a negative control on qPCR assays. For *Bacteria* 16S rRNA gene quantification, Eub341F (5’-CCT ACG GGA GGC AGC AG-3’) and Eub534R (5’-ATT ACC GCG GCT GCT GGC-3’), were used under the same conditions described above (Muyzer et al., 1993).

Both *Bacteria* and putatively halorespiring *Chloroflexi* qPCR runs used the same set of standards. Standards were made from a frozen glycerol stock of an *E. coli* clone containing a plasmid with the complete 16S rRNA gene from *Dehalococcoides* sp. strain BAV1. Clones were grown overnight and plasmids were extracted using the QIAprep Spin MiniPrep Kit (Qiagen) following the microcentrifuge protocol. Plasmid concentration was measured using Hoechst dye 33258 and a fluorometer with dilutions of calf thymus DNA as standards. The plasmid extract was serially diluted to achieve 12 standards containing between 10 and 2×10^9copies of plasmid per µL. For the putatively halorespiring *Chloroflexi* 16S rRNA gene quantification, all standards were log-linear. For *Bacteria* 16S rRNA gene quantification, standards were log-linear between 2×10^5 and 2×10^9 copies per µL. The 16S rRNA genes were quantified in triplicate for each sample of DNA extract, allowing the standard deviation of the qPCR assay to be calculated. The detection limits were 500 gene copies/g soil for *Chloroflexi* and 10^7 gene copies/g soil for *Bacteria*.

Quality assurance and primer specificity verification. A clone library (42 total clones) was used to verify the specificity of the qPCR primers. Thirteen unique 16S rRNA sequences were obtained from eight samples. Although only 86 bp were amplified by the two primers as a result of the qPCR method, the close phylogenetic relationship of these clones to other putatively-halorespiring *Chloroflexi* (see Appendix A) supports the specificity of our qPCR method. Using the program MatGat 2.1 (Campanella et al., 2003), amplified sequences ranged from 77 to 100% sequence identity to the obligately halorespiring *Dehalococcoides*. With the exception of the single sequence that was identical to *Dehalococcoides* sp. BAV1, BLAST searches found that the amplified

sequences were most similar to uncultured unclassified bacteria and uncultured *Chloroflexi* from rhizospheric bacterial communities, freshwater and marine sediments, anaerobic sludge digesters, and contaminant-dechlorinating consortia (≥97 percent sequence identity). These primers, however, do contain mismatches with the more recently discovered *Dehalogenimonas* and *Dehalobium* sequences, and are therefore likely to exclude some halorespiring *Chloroflexi* from the analysis.

Organochlorine and inorganic chloride analysis on soil cores. The cores for organochlorine and inorganic chloride analysis were separated into sections with a small power saw. These sections were 2 cm in depth for the first 10 cm of the core, and 4 to 6 cm in depth for the remaining core lengths. These divisions were made because of the decrease in variability of soil characteristics at deeper depths. Each section was analyzed as previously described (Leri et al., 2006), with some modifications. Freeze-dried soil samples were pulverized and compressed into pellets in a matrix of ~50% by weight (polyacrylic acid), sodium salt. Chlorine 1*s* X-ray absorption near-edge structure (XANES) spectra were acquired at beamline X15B at the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory, Upton, NY, USA. X-ray analysis was performed in the beamline's specialized hutch box under He using a Ge fluorescence detector. Sample pellets were mounted on Kapton tape and exposed to the incoming X-ray beam at a 45° angle. Sample fluorescence was measured over an energy range of 2800 to 2880 eV using a 0.25 eV step size near the chlorine K-absorption edge and 0.5-2.0 eV step sizes above and below the edge. Chlorine 1*s* XANES spectra were processed and analyzed as described previously (Leri et al., 2006). Chlorine data was obtained for eight grab samples, however, the high mineral content of several grab samples prohibited

the acquisition of quantitative, reliable, and reproducible chlorine speciation data. In addition, the chlorine concentrations of the maple core collected in January 2008 could not be analyzed with minimal error because of X-ray beam instability at the synchrotron, resulting in poor calibration curves. Organochlorine concentrations ([organochlorine]) and inorganic chloride concentrations were therefore only obtained along the depths of 5 of the 6 soil cores. Error bars reported below indicate standard errors from multiple scans of the same sample, when performed. The minimum detection limit for total chlorine speciation was 0.03 mmol/kg of soil.

Total organic carbon analysis in soil cores. Total organic carbon (TOC) was measured in the soil core segments at the West Virginia University Division of Plant and Soil Sciences (Morgantown, WV). The samples were subjected to dry combustion on a LECO TruSpec CHN 2000 and run interspersed with blanks, as well as EDTA standards to ensure consistent measurements.

Enzymatic synthesis of organochlorines. The chlorination of organic matter was performed with a chloroperoxidase enzyme (from *Caldariomyces fumago*, Sigma-Aldrich) using an adaptation of a previously described method (Niedan et al., 2000; Ortiz-Bermúdez et al., 2003; Reina et al., 2004). Soil collected from Father Hennepin State Park in Minnesota (pine cover) was extracted with an accelerated solvent extractor (ASE 350, Dionex) using a mixture of 50:50 acetone and hexane. The extract was split evenly by volume into two flasks, and each was blown down to dryness and resuspended in 165 mL phosphate buffer (0.1 M K_2PO_4, 20 mM KCl). The two flasks were treated identically throughout the chlorination process (described briefly below), with the exception that the chloroperoxidase enzyme was only added to one of the flasks, allowing

the organic matter in the other flask to be used as a non-chlorinated control. While both flasks were stirring, 600 units of chloroperoxidase enzyme were added to one of them. This was immediately followed by the addition of 150 µL of 0.1 M hydrogen peroxide to both flasks every 20 minutes for 1 hour. The reaction mixtures were left overnight and the addition of chloroperoxidase and hydrogen peroxide (or only hydrogen peroxide for the control) was repeated every day for four days. The pH of the reactors was maintained at 3.0-3.5 throughout the process. After the reaction was complete, the mixture was purified on a C18 column and extracted with sequential extractions of acetone, a mixture of 50:50 acetone and hexane, and hexane for use in the batch experiments described below.

Samples were taken from each flask at the beginning and end of the chlorination reaction for chloride analysis; between 7.3 and 19.4 mM chloride was consumed in the reaction with chloroperoxidase and no loss of chloride occurred in the flasks to which no chloroperoxidase was added.

Batch enrichments. Batch reactors were used to test the hypothesis that putatively-halorespiring *Chloroflexi* from uncontaminated environments would grow and concomitantly dechlorinate organochlorines produced in a manner similar to naturally derived organochlorines (*e.g.*, generated enzymatically via the action of the chloroperoxidase enzyme). Three sets of reactors, each in triplicate, were set-up. One set received three additions of enzymatically produced organochlorines ("organochlorine amended"); a second set received two additions of the extracted organic matter to which no chloroperoxidase enzyme was added, and one addition of the enzymatically produced organochlorines ("organic matter control"); a final set received no amendments

("unamended control"). For the amendments, the organochlorine or nonchlorinated organic matter extracts from the C18 column (above) were respectively split into three equal parts by volume for the triplicate reactors. For the first amendment of organochlorines or the nonchlorinated organic extract (amendment 1), the amendment was added to empty 160-mL serum bottles and the solvent was blown down to dryness. The reactors were then moved into an anaerobic glovebag with a 3% H_2/97% N_2 headspace (Coy) and the following was added: 130 mL mineral media (Shelton and Tiedje, 1984) reduced with 2 µM titanium citrate, 10 mM potassium acetate, 1 mL of vitamin solution (Wolin et al., 1963) and 5 grams of soil from the New Jersey Pine Barrens (Maple cover). For subsequent amendments 2 and 3, the amendment was added to new reactor bottles, blown down to dryness, and the entire content of the previous corresponding reactor was transferred to the new bottle in the glovebag. Potassium acetate (10 mM) was then added and the volume of each reactor mixture was brought up to 140 mL with fresh reduced mineral media. The unamended controls were treated the same, except they received no amendment of organochlorines or organic extract. The pH of the reactors was maintained at 7.0-7.5 with H_2PO_4 and NaOH. Samples for chloride and qPCR were taken throughout the experiment as previously described (Yan et al., 2006). The DNA from the samples was extracted with the PowerSoil DNA Isolation Kit (MoBio Laboratories) and qPCR for putatively-halorespiring *Chloroflexi* and *Bacteria* was performed as described above. Error bars represent the standard errors between triplicate reactors of the means of duplicate measurements for qPCR. Chloride was analyzed as described below; error bars represent the standard errors between triplicate reactors.

Ion chromatography. Chloride concentrations were quantified via ion chromatography on a Metrohm 761 Compact Ion Chromatograph (Metrohm US Inc). Samples were centrifuged for 5 minutes at 10,000×g to settle particulates and the supernatant was diluted 100 fold in Milli-Q water. A volume of 1.4 mL of diluted sample was injected onto a Metrosep A Supp 5 column. An isocratic method was used with an eluent (3.2 mM Na_2CO_3; 1.0 mM $NaHCO_3$) flowrate of 0.7 mL/min. The detection limit was 0.2 mM.

Calculation of growth yield. The growth yield of putatively-halorespiring *Chloroflexi* 16S rRNA genes per mol of chloride was calculated using the equation $Y=N \times V/C$, where N is the maximum number of *Chloroflexi* 16S rRNA genes per mL detected in a given reactor, V is the volume of the reactor (140 mL), and C is the concentration of chloride that accumulated in the reactors at the time of maximum *Chloroflexi* numbers.

Statistical analysis. StataIC 10.1 software was used to analyze the relationship of the number of *Chloroflexi* 16S rRNA genes/g soil and the number of *Bacteria* 16S rRNA genes/g soil to [organochlorine]/TOC, inorganic chloride, depth, and TOC. The nonparametric Spearman's rank coefficient correlation was used to determine whether a correlation between any two variables existed. The Wilcoxon rank-sum test was used to investigate the significance on the variables *Chloroflexi*, *Bacteria*, TOC, and [organochlorine]/TOC due to the difference in tree cover type of the soil cores. A linear regression model was used to examine the relative contribution and significance on the number of *Chloroflexi* 16S rRNA genes/g soil from the variables [organochlorine]/TOC, depth, and whether the core was from the oak-dominated forest. Between the 5 soil cores

for which paired microbial and chemical data were available, a total of 67 samples were available for analysis. If the value of a parameter was at the detection limit ("non detect"), the value of the detection limit itself was used for statistical analysis. Replicate statistical analyses were performed in which the value of the detection limit was replaced by either half or ten percent of the detection limit; the statistical significance of the results did not change as a result.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences derived from the verification of qPCR method have been deposited in the GenBank database under the accession numbers EU912597 to EU912609.

3.3 Results and Discussion

Abundance of putatively halorespiring *Chloroflexi* in natural soils. All 34 grab samples from uncontaminated grasslands and forests tested positive for putatively halorespiring *Chloroflexi*, with numbers ranging from 9.4×10^3 to 4.2×10^7 16S rRNA gene copies/g soil and ranging from 4.7×10^{-6} to 3.6×10^{-3} *Chloroflexi*/*Bacteria* 16S rRNA genes (Table 3.1). Different cover types (grass, hardwood, pine, and cedar) did not have a statistically significant affect on *Chloroflexi* or *Bacteria* abundance. Numbers of putatively halorespiring *Chloroflexi* 16S rRNA genes in the soil cores ranged from below the detection limit ($<5 \times 10^2$) to 1.5×10^7 gene copies/g soil (corresponding to a *Chloroflexi*/*Bacteria* 16S rRNA genes of $<6.75 \times 10^{-7}$ to 2.5×10^{-2}) (FIG. 3.1). For comparison, the 5 contaminated samples (PCB-, dioxin-, or TCE-contaminated) contained 5.0×10^4 to 1.1×10^7 *Chloroflexi* 16S rRNA gene copies/g soil (corresponding to 1.5×10^{-3} to 4.2×10^{-2} of *Chloroflexi*/*Bacteria* 16S rRNA genes). There was no

statistically significant difference between samples collected from contaminated and uncontaminated sites with respect to the number of *Chloroflexi* 16S rRNA genes present or the percent of the total community of *Bacteria* that consisted of *Chloroflexi*.

Correlation of putatively halorespiring *Chloroflexi* to natural organochlorines. If these widespread putatively halorespiring *Chloroflexi* are using natural organochlorine as an electron acceptor for growth, as has been hypothesized (Adrian et al., 2007; Bunge et al., 2008; Hiraishi, 2008; Kittelmann and Friedrich, 2008; Kittelmann and Friedrich, 2008), the number of these organisms present in a given sample should correlate to [organochlorine] while the number of *Bacteria* present should not. [Organochlorine] was observed to correlate linearly with TOC (R^2=0.66, t=11.2, P<0.001); this result was expected, as TOC is the precursor of organochlorines (Leri et al., 2006). Therefore, to factor out the covariance of [organochlorine] with TOC, [organochlorine]/TOC was used to investigate further correlations with putatively halorespiring *Chloroflexi*. The nonparametric Spearman's rank test was used to determine whether such a correlation existed for the 67 soil core sections in which paired microbial and organochlorine data were available. The number of *Chloroflexi* 16S rRNA genes/g soil in these samples correlated to [organochlorine]/TOC (ρ=0.31, P=0.012), but did not correlate with depth, TOC, or [chloride] (ρ=0.10, P=0.40,ρ=-0.16, P=0.18, and ρ=-0.03, P=0.82, respectively). Indeed, it was not expected that putatively halorespiring *Chloroflexi* would correlate to [chloride] (a product of halorespiration) or depth (implying dissolved oxygen concentrations) because the soils investigated in this study were well-drained, resulting in a lack of chloride accumulation and an environment that was not predictably more reduced with depth. TOC was not expected to correlate with

Chloroflexi, as non-chlorinated organic matter should not exert a direct selective pressure for the growth of halorespirers. The number of *Bacteria*, however, were expected to correlate to TOC and were not expected to correlate to [organochlorine]/TOC; this was observed ($\rho=0.82$, $P<0.001$ for TOC and $\rho=-0.15$, $P=0.22$ for [organochlorine]/TOC). There was also a negative correlation between the number of *Bacteria* present and depth ($\rho=-0.66$, $P<0.001$), although this may be a factor of the association of depth to TOC ($\rho=-0.56$, $P<0.001$). There was no observed correlation between *Bacteria* and [chloride] ($\rho=0.12$, $P=0.34$).

TABLE 3.1. Quantification of putatively-halorespiring *Chloroflexi* in the grab samples from both uncontaminated and contaminated sites

Soil Cover	Location	*Chloroflexi* 16S rRNA genes/g soil	*Chloroflexi* 16S rRNA genes/*Bacteria* 16S rRNA genes
Grass	Redbud Valley NP, OK[a]	$(2.4\times10^{7}) \pm (4.3\times10^{6})$[b]	$(1.2\times10^{-3}) \pm (2.4\times10^{-4})$
	Afton SP[c], MN	$(4.2\times10^{7}) \pm (1.1\times10^{7})$	$(3.6\times10^{-3}) \pm (1.1\times10^{-3})$
	Interstate SP, MN	$(4.0\times10^{5}) \pm (1.0\times10^{5})$	$(5.6\times10^{-4}) \pm (1.4\times10^{-4})$
Hardwood	Redbud Valley NP, OK	$(9.9\times10^{6}) \pm (2.5\times10^{6})$	$(3.7\times10^{-4}) \pm (1.1\times10^{-4})$
	Redbud Valley NP, OK	$(1.1\times10^{5}) \pm (2.3\times10^{4})$	$(8.4\times10^{-5}) \pm (1.7\times10^{-5})$
	Ray Harral NP, OK[d]	$(2.0\times10^{7}) \pm (3.5\times10^{6})$	$(9.0\times10^{-4}) \pm (1.6\times10^{-4})$
	Ray Harral NP, OK	$(8.6\times10^{6}) \pm (6.9\times10^{4})$	$(2.9\times10^{-4}) \pm (1.4\times10^{-5})$
	Ray Harral NP, OK	$(8.9\times10^{5}) \pm (1.6\times10^{5})$	$(2.4\times10^{-4}) \pm (4.5\times10^{-5})$
	Ray Harral NP, OK	$(1.2\times10^{6}) \pm (1.4\times10^{5})$	$(2.3\times10^{-4}) \pm (3.1\times10^{-5})$
	Afton SP, MN	$(4.5\times10^{6}) \pm (2.6\times10^{5})$	$(4.5\times10^{-4}) \pm (5.6\times10^{-5})$
	Afton SP, MN	$(7.4\times10^{6}) \pm (7.7\times10^{5})$	$(2.1\times10^{-4}) \pm (3.4\times10^{-5})$
	Interstate SP, MN	$(9.0\times10^{6}) \pm (6.9\times10^{5})$	$(1.4\times10^{-4}) \pm (5.9\times10^{-5})$
	Interstate SP, MN	$(5.5\times10^{5}) \pm (1.2\times10^{5})$	$(5.4\times10^{-4}) \pm (1.2\times10^{-4})$
	Father Hennepin SP, MN	$(3.1\times10^{5}) \pm (7.0\times10^{4})$	$(9.7\times10^{-5}) \pm (2.2\times10^{-5})$
	Tilden Regional Park, CA	$(3.3\times10^{5}) \pm (1.0\times10^{4})$	$(4.3\times10^{-4}) \pm (2.0\times10^{-5})$
Pine	McClellan-Kerr WMA[e], OK	$(3.0\times10^{7}) \pm (6.8\times10^{6})$	$(2.2\times10^{-3}) \pm (6.1\times10^{-4})$
	Afton SP, MN	$(4.4\times10^{6}) \pm (9.2\times10^{5})$	$(4.7\times10^{-6}) \pm (2.5\times10^{-6})$
	Afton SP, MN	$(1.2\times10^{7}) \pm (5.2\times10^{5})$	$(8.9\times10^{-4}) \pm (2.4\times10^{-4})$
	Interstate SP, MN	$(3.3\times10^{6}) \pm (1.3\times10^{5})$	$(3.3\times10^{-5}) \pm (3.0\times10^{-5})$
	Interstate SP, MN	$(1.7\times10^{6}) \pm (7.5\times10^{4})$	$(5.3\times10^{-4}) \pm (9.0\times10^{-5})$
	Interstate SP, MN	$(8.2\times10^{5}) \pm (1.3\times10^{5})$	$(6.3\times10^{-4}) \pm (1.1\times10^{-4})$
	Mille Lacs-Kathio SP, MN	$(2.3\times10^{5}) \pm (9.4\times10^{4})$	$(1.1\times10^{-4}) \pm (4.5\times10^{-5})$
	Mille Lacs-Kathio SP, MN	$(5.1\times10^{5}) \pm (8.6\times10^{4})$	$(2.1\times10^{-4}) \pm (3.5\times10^{-5})$
	Banning SP, MN	$(1.7\times10^{4}) \pm (3.2\times10^{3})$	$(1.5\times10^{-5}) \pm (3.0\times10^{-6})$
	Father Hennepin SP, MN	$(3.3\times10^{5}) \pm (2.2\times10^{4})$	$(1.4\times10^{-4}) \pm (1.6\times10^{-5})$
	Father Hennepin SP, MN	$(2.2\times10^{5}) \pm (1.1\times10^{4})$	$(8.8\times10^{-5}) \pm (6.0\times10^{-6})$
	Father Hennepin SP, MN	$(5.3\times10^{5}) \pm (2.1\times10^{5})$	$(2.6\times10^{-4}) \pm (1.1\times10^{-4})$
	Father Hennepin SP, MN	$(3.4\times10^{6}) \pm (1.1\times10^{6})$	$(1.5\times10^{-4}) \pm (5.1\times10^{-5})$
	Mount Hood National Forest, OR	$(9.4\times10^{3}) \pm (1.5\times10^{3})$	$(2.0\times10^{-5}) \pm (3.3\times10^{-6})$
Cedar	Ray Harral NP, OK	$(6.4\times10^{6}) \pm (1.2\times10^{6})$	$(4.9\times10^{-4}) \pm (1.0\times10^{-4})$
	Ray Harral NP, OK	$(2.5\times10^{7}) \pm (9.7\times10^{6})$	$(1.8\times10^{-3}) \pm (7.1\times10^{-4})$
	Ray Harral NP, OK	$(6.2\times10^{6}) \pm (1.4\times10^{6})$	$(1.8\times10^{-4}) \pm (4.8\times10^{-5})$
	Ray Harral NP, OK	$(2.3\times10^{6}) \pm (8.6\times10^{5})$	$(1.1\times10^{-4}) \pm (5.2\times10^{-5})$
	Afton SP, MN	$(1.4\times10^{7}) \pm (5.0\times10^{6})$	$(1.1\times10^{-3}) \pm (4.0\times10^{-4})$
Contaminated	Solvent-contaminated aquifer, NY	$(4.7\times10^{5}) \pm (2.3\times10^{4})$	$(1.2\times10^{-2}) \pm (7.2\times10^{-4})$
	Hudson River, NY	$(5.0\times10^{4}) \pm (6.9\times10^{3})$	$(1.5\times10^{-3}) \pm (2.5\times10^{-4})$
	Baltimore Harbor, MD	$(1.1\times10^{5}) \pm (3.8\times10^{4})$	$(1.7\times10^{-3}) \pm (6.9\times10^{-4})$
	Fox River, WI	$(1.1\times10^{7}) \pm (5.9\times10^{5})$	$(4.2\times10^{-2}) \pm (2.4\times10^{-3})$
	Palos Verdes Harbor, CA	$(1.1\times10^{6}) \pm (4.5\times10^{5})$	$(5.3\times10^{-3}) \pm (2.4\times10^{-4})$

[a] Redbud Valley Nature Preserve, Catoosa, OK
[b] Standard deviation
[c] State Park
[d] Ray Harral Nature Park, Broken Arrow, OK
[e] Wildlife Management Area

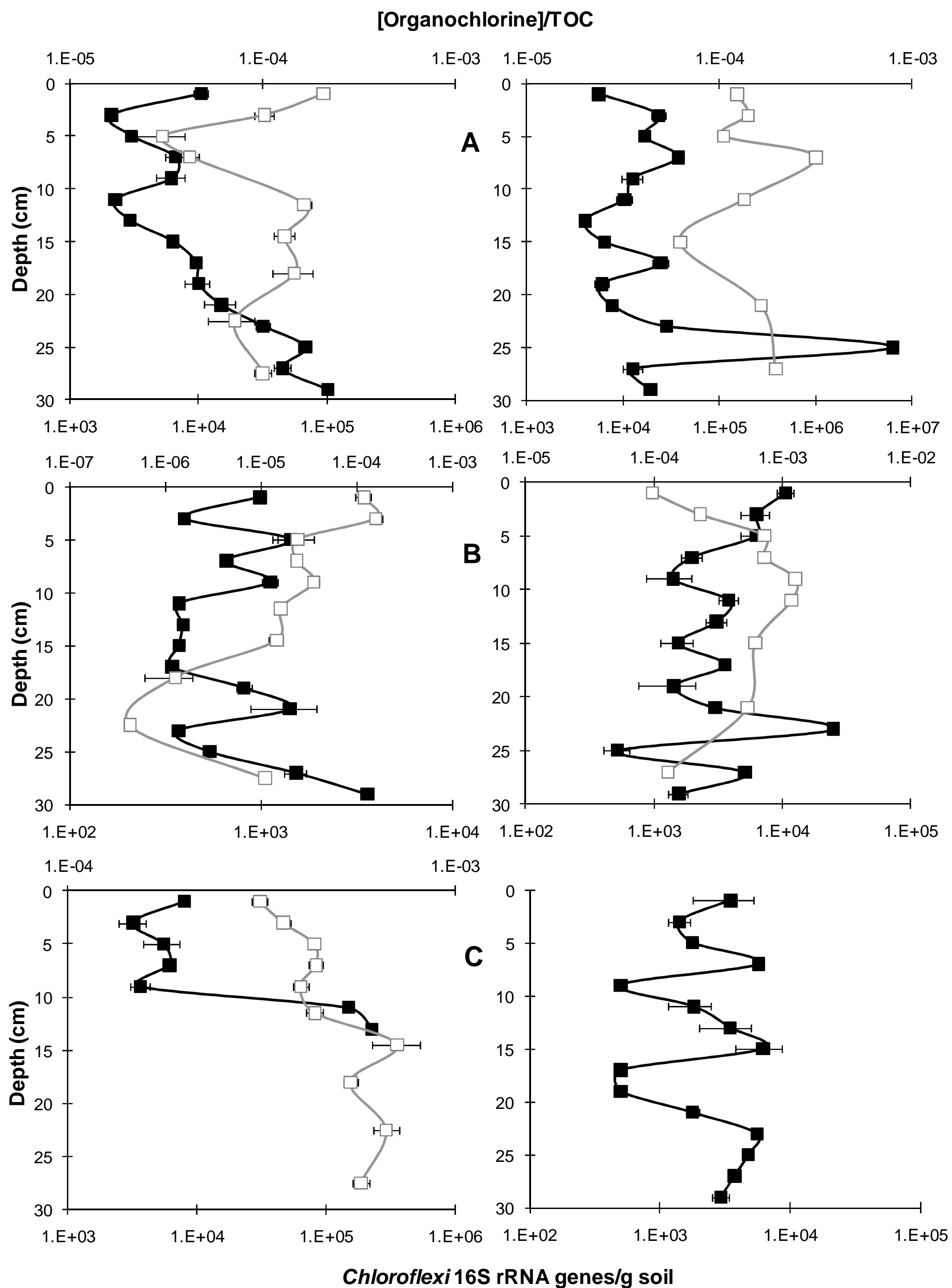

FIG. 3.1. The distribution of putatively-halorespiring *Chloroflexi* (solid symbols) and total [organochlorine]/TOC (open symbols) in the soil cores taken from dominantly oak (A), pine (B), and maple (C) forests in September 2006 (left) and January 2008 (right).

To further investigate the association of *Chloroflexi* and the environmental parameters measured in this study, a linear regression model was developed. The two variables depth and [organochlorine]/TOC are not statistically correlated with each other (Spearman's ρ=-0.14, P=0.11); therefore, these two parameters may be included in a linear regression model as independent variables. Additionally, the variable "vegetative cover" may be included by giving a particular cover type a value of 1 and the other cover types values of 0. In this case, "pine cover" was investigated; it is also independent from the variables depth and [organochlorine]/TOC. In a linear regression model with these three variables (FIG. 3.2), the correlation coefficients were 0.025, 0.33, and -0.88 for depth, log([organochlorine]/TOC), and "pine cover," respectively; all were statistically significant with respect to their correlation with log(*Chloroflexi*) (t=3.29, P=0.002, t=3.62, P<0.001, and t=-6.6, P<0.001, respectively). This linear regression analysis again supported the statistical association between the number of *Chloroflexi* 16S rRNA genes and [organochlorine]/TOC, but also highlighted an unexplained and quite strong association between tree cover and *Chloroflexi* 16S rRNA genes. The strength of the correlation with pine cover indicates that factors in addition to [organochlorine]/TOC affect the number of *Chloroflexi* 16S rRNA genes/g of soil. Different tree species have been observed to produce different organic exudates, including ones that are able to induce aerobic PCB-degraders (Leigh et al., 2006); therefore, certain tree species, such as oak and maple, may produce organochlorines that are more bioavailable, more oxidized, or otherwise more favorable for reduction and energy generation in halorespiring *Chloroflexi*. Because the variable of tree cover is not controlled, however, other factors not measured in this study could have affected the abundance of putatively halorespiring

Chloroflexi in the two cores from pine cover as well. Indeed, no significant correlation between the number of putatively halorespiring *Chloroflexi* and vegetation type was observed in the grab samples (above).

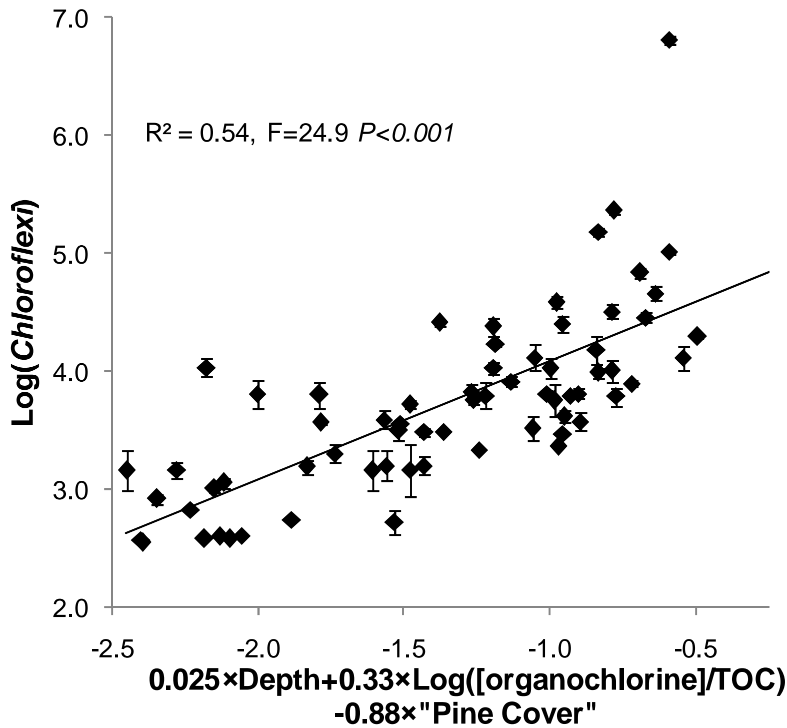

FIG. 3.2. Linear regression fit of the variable Log(*Chloroflexi*) and a model including the independent variables depth (cm), [organochlorine]/TOC, and "pine cover" (samples with pine cover are given a value of 1 and maple and oak cover are given a value of 0).

Growth of putatively halorespiring *Chloroflexi* in batch enrichments. The increase in putatively halorespiring *Chloroflexi* 16S rRNA genes was measured in batch reactors to which enzymatically-produced organochlorines, organic matter, or nothing was added (FIG. 3.3). The organochlorine amended reactors were amended three times with enzymatically-produced organochlorines. For the organic matter control reactors, the first and third amendments were made with the organic extract, whereas the second amendment was with the enzymatically-produced organochlorines. For each amendment

of enzymatically produced organochlorines the number of *Chloroflexi* 16S rRNA genes increased by 2.9±0.3 to 4.0±0.2 orders of magnitude for the triplicate reactors. The organic extract increased the number of *Chloroflexi* 16S rRNA genes, but by a significantly lower amount (0.89±0.2 and 1.7±0.3 orders of magnitude). For the unamended controls, there was an initial increase in the number of *Chloroflexi* 16S rRNA genes (0.55±0.1 orders of magnitude) and no statistically significant increase thereafter. The number of *Bacteria* 16S rRNA genes was also measured with qPCR and did not increase significantly during these experiments.

The organic extract and the soil used to inoculate the reactors would have contained any natural organochlorines already present in the soil, and therefore, some growth of *Chloroflexi*, at least initially, was expected in all of the reactors. Indeed, this increase in growth was observed. Nevertheless, the growth of *Chloroflexi* (rate and total amount of genes present) was statistically greater (Student t-test, $P<0.05$) in the reactors to which the enzymatically-produced organochlorines were added, indicating that terrestrial organochlorines do serve as a growth substrate for putatively-halorespiring *Chloroflexi*. Furthermore, the increase in *Chloroflexi* in the organic matter control reactors when amended with the enzymatically chlorinated organic matter (amendment 2) indicates that the results of the organic control amendments were not a result of alternative factors of the reactors.

The accumulation of chloride was also measured in the batch reactors. After amendment with the enzymatically-produced organochlorine, the chloride increase was between 2.4±0.5 mM and 2.7±0.4 mM (FIG. 3.3). In the organic matter and unamended controls, the increase in chloride was not significantly different from zero. Again, this

indicates that the growth of the putatively-halorespiring *Chloroflexi* on the amended organochlorines was significant and resulted in dechlorination at a level that was detectable via chloride production.

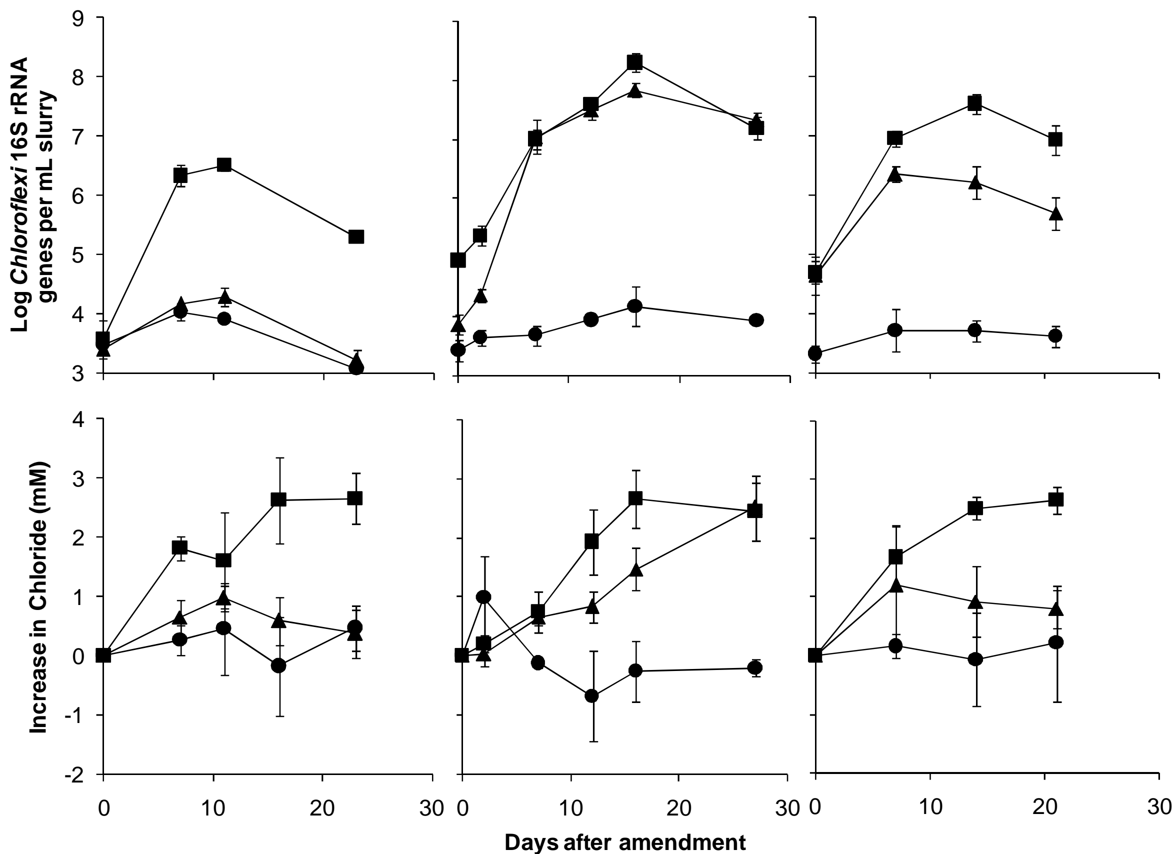

FIG. 3.3. The increase of 16S rRNA gene sequences of putatively halorespiring *Chloroflexi* (top) and increase in chloride concentrations (bottom) during amendments 1 (left), 2 (center) and 3 (right). Symbols are: organochlorine amended (■),organic matter control (▲) (received organochlorines only for amendment 2 and organic matter for amendments 1 and 3), and unamended (●). The amount of organic matter used for each of the amendments was equivalent, regardless of whether it was treated with chloroperoxidase. Error bars are the standard error between mean values of triplicate reactors.

Recent literature has supported the concept that halorespiration of natural organohalides occurs in the environment. For example, laboratory cultures of two different species of *Dehalococcoides* have been shown to be capable of growing on

several chlorinated phenols as electron acceptors (Adrian et al., 2007) and one chlorinated phenol has been shown to induce transcription of several reductive dehalogenase genes (Fung et al., 2007). Because chlorinated phenols can be produced naturally (Ortiz-Bermúdez et al., 2003), it was conjectured that they represent at least one class of naturally occurring compounds that *Dehalococcoides* may use in uncontaminated environments. Another study found that mixed cultures containing *Dehalococcoides*-like microorganisms from uncontaminated sediment in the North Sea could degrade tetrachloroethene to *trans*- and *cis*-dichloroethene (Kittelmann and Friedrich, 2008). Because evidence exists for the natural production of tetrachloroethene by marine algae (Abrahamsson, et al., 1995), tetrachloroethene could be considered a natural substrate for *Dehalococcoides*-like organisms in marine systems. Our study, however, is the first to find direct evidence of a natural niche for halorespiring *Chloroflexi* by showing that putatively halorespiring *Chloroflexi* are widespread in uncontaminated terrestrial environments, they correlate to the quantity of natural organochlorine compounds present in these uncontaminated samples, and they grow in the presence of enzymatically produced organochlorines while releasing chloride.

Interestingly, no lag in growth was observed in our batch experiments, suggesting that these *Chloroflexi* may constitutively dechlorinate enzymatically produced organochlorines for energy generation. Also interesting is the observation that these organisms grew rather quickly, reaching a maximum population 11-16 days after the amendment of organochlorine. The growth yield of these putatively-halorespiring *Chloroflexi* was estimated to be 3×10^{11} to 1×10^{13} copies 16S rRNA genes/mol chloride, which is similar to the ~10^{12}-10^{14} cells/mol chloride determined for *Dehalococcoides*

isolates respiring anthropogenic contaminants (Adrian et al., 2007; Marco-Urrea et al., 2011; Tas et al., 2011). This data is promising because if the organisms that respire natural organochlorines can also dechlorinate compounds such as PCBs, halorespirers could be quickly grown to a high density on natural organochlorines *ex situ*, after which they could be added to contaminated dredge spoils, or even contaminated sediment (Krumins et al., 2009; Winchell and Novak, 2008). The environment is complicated, however, and multiple parameters not measured in this study may also affect the number of *Chloroflexi* present in a given uncontaminated environment; this was observed via the negative association between the number of *Chloroflexi* and "pine cover". Furthermore, the specific organochlorines that were dechlorinated during *Chloroflexi* growth were not determined in this study. Research of this nature would help in not only understanding the niche of these important organisms more fully, but could also aid in the development of technologies for the remediation of anthropogenic contaminants, such as PCBs.

3.4 References

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CHAPTER 4. Geochemical pressures on halorespiring *Chloroflexi* in lake sediments

4.1 Introduction

The *Chloroflexi* phylum contains several deeply branching lineages of halorespiring organisms, and putative halorespirers may span several classes (Maymó-Gatell et al., 1997; Watts et al., 2005; Kittelmann and Friedrich, 2008a). The *Chloroflexi* class *Dehalococcoidetes* has been a subject of fairly intense study over the past 10 years because of the role that these organisms appear to play in the dechlorination and subsequent detoxification of anthropogenic contaminants (e.g. Bedard, 2008). Indeed, the isolates of the class *Dehalococcoidetes* have all been found to be obligate anaerobic halorespirers (Maymó-Gattell et al., 1997; Adrian et al., 2000; Cutter et al., 2001; Cupples et al., 2003; He et al., 2003; He et al., 2005; Sung et al., 2006; May et al., 2008; Yan et al., 2009; Moe et al., 2009; Cheng and He, 2009). Strains of the genus *Dehalococcoides* and closely related populations are the best-characterized group of *Dehalococcoidetes* and halorespiring *Chloroflexi* in general, as they were the first to be isolated (Maymó-Gatell et al., 1997). Thus far, *Dehalococcoides* species have been found to dechlorinate chlorinated ethenes (Maymó-Gatell et al., 1997; He et al., 2003; He et al., 2005; Sung et al., 2006), chlorinated ethanes (Grostern and Edwards, 2006), chlorinated benzenes (Adrian et al., 2000), chlorinated phenols (Adrian et al., 2007), polychlorinated biphenyls (Yan et al., 2006; Bedard et al., 2007; Adrian et al., 2009), chlorinated dioxins (Fennel et al., 2004), chlorinated furans (Fennell et al., 2004), chlorinated naphthalene (Fennell et al., 2004) and polybrominated diphenyl ethers (Robrock et al., 2008). These organisms, or those closely related phylogenetically, have also been found in contaminated sites where they are presumed to have a niche dechlorinating pollutants (Hendrickson et al., 2002; Müller et al., 2004; Scheutz et al., 2008; Imfeld et al., 2008).

It has recently been hypothesized that organisms in the *Dehalococcoidetes* class and potentially other halorespiring *Chloroflexi* play a larger role in ecosystems as part of the natural chlorine cycle (Adrian et al., 2007 Bunge et al., 2008; Hiraishi, 2008; Kittelmann and Friedrich, 2008a; Kittelmann and Friedrich, 2008b; Chapter 3). In addition, through the dechlorination of chlorinated organics, they may also facilitate carbon cycling. In fact, chlorinated organic compounds have been observed to be a particularly recalcitrant fraction of natural organic carbon (Winterton 2000; Redon et al., 2011), which suggests that these dechlorinating organisms may play an important role in modulating carbon breakdown. My recent work has shown that in uncontaminated environments the quantity of 16S rRNA gene sequences related to *Dehalococcoides* species correlated to the fraction of organic carbon that is chlorinated (Chapter 3). In addition, when fed enzymatically chlorinated organic matter in the laboratory, these same organisms grew (Chapter 3). Nevertheless, little is known about the halorespiring *Chloroflexi* or the environmental and geochemical parameters that stimulate or inhibit their activity and/or growth.

A natural sulfur gradient exists across the lakes of the U.S. Upper Midwest. This gradient has been studied in depth previously (Dean and Gorham, 1976; Gorham et al., 1982; Gorham et al., 1983) and provides a natural geographic zone within which to test the hypothesis that sulfur impacts the growth and diversity of putatively halorespiring *Chloroflexi.* Sulfite and sulfide have been observed to inhibit reductive dechlorination activity in the laboratory (Magnuson et al., 1998; He et al., 2005; Rysavy et al., 2005; May et al., 2008), and may therefore be important for controlling the activity of halorespiring *Chloroflexi* in the environment. Furthermore, these lakes also provide an

opportunity to test the hypothesis that trace metals, such as iron and cobalt, may be important for the enrichment of diverse halorespiring *Chloroflexi*. Dehalogenase enzymes contain cobalt corrinoids (e.g. Johnson et al., 2009) and iron is important in sequestering potentially inhibitory sulfide (Swider and Mackin, 1989); therefore, both of these trace metals may be important for the enrichment of halorespiring *Chloroflexi*. As anthropogenic activities alter the sulfur content of various water bodies, this information may become important for understanding natural chlorine, and potentially carbon, cycling. It is for these reasons that I sought to explore the geochemical niche of the *Chloroflexi* class *Dehalococcoidetes* along with a slightly larger group of *Chloroflexi* that may also contain halorespirers.

4.2 Methods

Sampling. Sediment and lake water samples were gathered between November 22 and November 24, 2010. Longitude and latitude of sampling locations are indicated in appendix B. The lakes chosen for sampling had significant historical data available (see Dean and Gorham, 1976; Gorham et al., 1982; Gorham et al., 1983). During sampling, ambient air temperatures ranged from -18°C to -5°C and lake water temperatures ranged from 0.2^{o}C to 6°C. Sampling holes were chipped with a shovel for lakes with ice cover. Sediment and lake water was gathered 5 to 25 m from the shore. A Hydrolab DS5X (Hach) was placed at a lake depth of 1.5-2.5 m for temperature, pH, specific conductivity, turbidity, dissolved oxygen, and depth measurement. Sediment samples and cores were gathered with a plastic coring apparatus. Grab samples were gathered at a sediment depth of 0-3 cm and transferred into aluminum containers. Sediment cores between 12 and 30

cm deep were gathered at four lakes and kept in the plastic core sleeve until arrival in the laboratory, at which point they were partitioned into 2 cm length sections. Lake water was gathered into 1-L plastic bottles at a depth of 0.2 meters below the surface of the lake. Sediment samples, and lake water samples were kept on ice until arrival in the laboratory. Upon arrival in the laboratory, sediment samples were homogenized and large rocks were removed. For microbial analysis, 0.5 g sediment was transferred into bead-beating tubes and frozen at -20°C. Between 1 and 1.5 mL of porewater was transferred into 1.7 mL microcentrifuge tubes with a pasteur pipette and frozen at -20°C. A subsection of sediment was transferred into weigh boats for percent solids and total organic matter determination.

Chemical Analysis. Anion concentrations (F^-, SO_4^{-2}. PO_4^{-3}, Br^-, Cl^-, NO^-_3) of lake water and porewater were quantified via ion chromatography on a Metrohm 761 Compact Ion Chromatograph (Metrohm US Inc). A Metrosep A Supp5 column was used for ion separation. An isocratic method was used with an eluent (3.2 mM Na_2CO_3; 1.0 mM $NaHCO_3$) and flowrate of 0.7 mL/min. Milli-Q water was used as rinsing solution and 100 mM H_2SO_4 was used as regenerant solution. Samples were collected from grab samples and sectioned core samples with a Pasteur pipette and stored in microcentrifuge tubes at -20°C until further analysis. Samples were filtered through Acrodisc 32 mm syringe filters with 0.45 micron Supor membranes (Pall Corporation, Port Washington, NY) prior to being injected onto the column. Samples were diluted with Milli-Q water. Because porewater processing involved exposure to oxygen (pipetting, filtration, and dilution with oxygenated water), all reduced forms of dissolved sulfur (sulfide and sulfite) are assumed to be oxidized as sulfate. Percent solids were calculated

by oven drying at 105°C and total organic matter was determined by loss on ignition (LOI) at 550°C. All 20 grab sediment samples and selected sections of the sediment cores from Sand Lake and Gladstone Lake were sent to the Research Analytical Laboratory at the University of Minnesota for quantification of P, K, Ca, Mg, Na, Al., Fe, Mn, Zn, Cu, B, Pb, Ni, Cr, Cd, S, and Co by inductively coupled plasma atomic emission spectroscopy (ICP-AES).

DNA Extraction, quantitative polymerase chain reaction, and terminal restriction fragment length polymorphism. DNA was extracted using the PowerSoil DNA kit (MoBio Laboratories) according to the recommended procedure. For quantitative polymerase chain reaction (qPCR), the number of putatively halorespiring *Chloroflexi* 16S rRNA genes was determined using the qPCR method previously described (Chapter 3) with one exception: the forward primer was replaced with a newly designed primer Chl1150F (5'-GGG CTA CAC ACA CGC TAC AAT GG-3') to better-capture a wider fraction of putatively halorespiring *Chloroflexi*. The number of *Bacteria* 16S rRNA genes was determined as previously described (Chapter 3). Standards were prepared as described previously (Chapter 3). and were serially diluted to make 9 standards containing between 100 and 10^{10} copies of 16S rRNA genes per reaction. For putatively halorespiring *Chloroflexi* 16S rRNA gene quantification, all standards were log-linear. For *Bacteria* 16S rRNA gene quantification, standards were log-linear between 10^{5} and 10^{10} copies per reaction. The detection limits were 10^{4} gene copies/g wet sediment for *Chloroflexi* and 10^{7} gene copies/g wet sediment for *Bacteria*. Additionally, a previously published method was used for quantification of *Dehalobacter* (Smits et al., 2004). Standards from an *E. coli* clone containing the 16S RNA gene from

Dehalobacter restrictus strain DSN 29455, were prepared using the Hoechst Dye method with calf thymus DNA in known concentrations. Eight standards were used ranging from 100 gene copies per reaction to 10^9 gene copies per reaction. Melting curve analysis was performed for all qPCR reactions to screen for non-specific amplification and primer/dimer formation. Each sample was quantified in triplicate for each sample of DNA extract and the mean was used in the analysis.

To test the specificity of the qPCR amplification for putatively halorespiring *Chloroflexi*, clone libraries were constructed from the amplification products of three samples: the 2-4 cm depth section of the sediment core from Sand Lake, SD, the 2-4 cm depth section of the sediment core from Long Lake, MN, and the grab sediment section from East Stump Lake, ND. Out of 48 clones sequenced, 45 unique sequences were obtained. These sequences were aligned with known halorespiring *Chloroflexi* and with putatively halorespiring clones obtained from NCBI's BLAST database (see Appendix B).

To ensure that inhibition was not occurring with the qPCR method, serial dilutions (10 and 100 fold) of the DNA extracts for ten samples (grab samples from Salt Lake, Waubay Lake, Lake Parmley, Richmond Lake, Sand Lake, Dry Lake, East Stump Lake, Devil's Lake, Elbow Lake, and Free People's Lake) were prepared and tested via qPCR for *Bacteria* and putatively halorespiring *Chloroflexi.* The calculated amounts of both *Chloroflexi* and *Bacteria* 16S rRNA genes/g sediment did not change significantly (less than ±10%) as a result of dilution; thus, inhibition resulting from compounds in the DNA extract was not a factor in our work.

For terminal restriction fragment length polymorphism (TRFLP), *Chloroflexi* 16S rRNA genes were amplified with two primers designed for this study: Chl553F (5'-CCG GCT TAA CCG GGA CG WGT-3') and Chl1150R (5'-CCA TTG TAG CGT GTG TGT AGC CC-3'). PCR reactions (30 µL) contained1 × reaction buffer (Promega), 1 mM $MgCl_2$, 2.5 µg BSA, 0.2 mM each dNTP, 0.5 mM each primer, 1.5 U *Taq* DNA polymerase, and 1 µL of DNA extract. The thermocycling procedure contained an initial denaturing step at 95°Cfor 5 min, 30 cycles of 95°C for 45 s, 58°C for 30 s, 72°C for 1 min, and final extension step of 72°C for 5 min. PCR amplification products were screened on 1% agarose gels for proper size (600 bp) to ensure specificity of amplification, and PCR products were cleaned using the GeneClean II kit (MP Biomedical). Cleaned product was used as template for a second PCR amplification, performed as before except with the Chl1150R primer labeled with carboxyflourescin. PCR products were cleaned again and digested with 2U of each Taq$^{\alpha}$I, RsaI, BamHI, and 1× Buffer 4 (New England Biolabs) at 37°C for 2 h, followed by 65°C for 1 h, and 80°C for 20 min for deactivation. Fragment analysis was performed by the Biomedical Genomics Center (University of Minnesota) on an ABI 3730xl capillary instrument with GeneMapper Software. MapMarker 1000 was used as a size standard. Results of the TRFLP were analyzed with PeakScanner software v1.0 (Applied Biosystems). Peaks above 50 FU and between 50 bp and 1000 bp in length were used in the analysis and peaks with peak areas less than 0.5% of the total peak area of the sample were removed from analysis. Peaks were manually binned with Microsoft Xcel.

To match the terminal restriction fragments (TRFs) from the TRFLP analysis to the *Dehalococcoidetes* class or other *Chloroflexi* classes, clone libraries were constructed

from the amplification products of Dry Lake, Sand Lake, and Leech Lake (see Appendix B). From these three clone libraries, a total of 11 TRFs were identified out of 29 total. Smaller clone libraries from an additional four samples (grab samples from Salt Lake, Long Lake, Devils Lake, and Ball Club Lake) produced a classification of 4 additional TRFs, for a total of 15 TRFs out of 29. These 15 TRFs represented between 50 and 99% of the total peak area for each sample with an average of 86% for all samples. A total of 8 TRFs grouped within the *Dehalococcoidetes* class while 7 grouped outside of this class.

Statistical Analysis. Statistical analysis was performed with Stata/IC 10.1 software. Spearman's rank coefficient test was used to compare relationships between parameters. A *P* value greater than 0.05 was considered significant.

4.3 Results and Discussion

As mentioned above, the twenty lakes in this study were chosen for the natural sulfur gradient present in lakes across the U.S. Upper Midwest. Generally, lake sulfate concentrations were lower than those indicated in historical data (Gorham et al., 1983; Table 4.1). This is expected and common in the U.S., where atmospheric deposition of sulfur has been decreasing as a result of air pollution policies enacted in the Clean Air Acts (Kahl et al., 2004; Mitchell and Likens, 2011). Several lakes have much less sulfate than the historical record (Waubay Lake (SD), Devil's Lake (ND), and East Stump Lake (ND)) and four had not decreased in sulfate over this period (Dry Lake, Lake Parmley, Richmond Lake, and Sand Lake, all SD), perhaps as a result of the extensive use of sulfur compounds in agricultural fertilizer in the area (e.g. Chien et al., 2011 and references therein).

Table 4.1. The quantity of chloride and sulfate in lake water in this study compared to historical data

Lake	This Study Chloride (mM)	This Study Sulfate (mM)	Historical* Chloride (mM)	Historical* Sulfate (mM)
Salt Lake, MN	18.003	102.8	50.4	104.4
Dry Lake, SD	0.472	20.9	0.79	0.853
Waubay Lake, SD	0.454	5.3	4.681	48.88
Lake Parmley, SD	2.195	6.0	2.327	1.7005
Richmond Lake, SD	2.055	6.3	1.912	1.9085
Sand Lake SD	2.064	10.0	1.664	1.869
Devil's Lake, ND	3.531	11.7	30.256	51.515
Free People's Lake ND	11.454	31.1	20.896	31.2
Elbow Lake, ND	1.010	0.58	0.959	0.905
East Stump Lake ND	10.647	41.7	321.51	620.35
Long Lake, MN	0.244	0.020	0.025	0.0325
Lake Itasca, MN	0.021	0.0003	0.03	0.0275
Leech Lake, MN	0.093	0.066	0.054	0.0445
Winnebigoshish, MN	0.115	0.029	0.034	0.055
Moose Lake, MN	0.049	0.025	0.023	0.0545
Ball Club Lake, MN	0.071	0.027	0.017	0.069
Pelican Lake, MN	0.110	0.019	0.031	0.0425
Gladstone Lake, MN	0.094	0.004	0.01	0.03
Nokay Lake, MN	0.049	0.034	0.021	0.0465
Lake Mille Lacs, MN	0.215	0.068	0.071	0.074

* Data from Gorham *et al.,* 1982.

Putatively halorespiring *Chloroflexi* were found in nearly all of the samples (62 out of 68 total samples, see Appendix B). In addition, their quantities in the environment were quite high, ranging from 3.5×10^4 to 8.5×10^{10} copies 16S rRNA genes/g dry sediment, and averaging 4.1% of the total *Bacteria* in the sediment (see Appendix B). This is in contrast to the results that we obtained in soil samples (Chapter 3), in which the percentage of putatively halorespiring *Chloroflexi* averaged 0.04%. This difference is likely attributable to the nature of the samples—saturated anaerobic lake samples versus soil samples with a much higher oxygen content, as well as the slightly broader target of

the forward primer used in the qPCR method (see Methods). These results corroborate our previous findings that the putatively halorespiring *Chloroflexi* are a consistent member of uncontaminated ecosystems and could therefore be an important part of chlorine and carbon cycling. Interestingly, *Dehalobacter*-like species, members of the *Firmicutes* and relatively under-studied halorespirers, were also detected widely (31 out of 68 samples), but at much lower concentrations (1.5×10^4 to 1.5×10^6 copies 16S rRNA genes/g dry sediment; see Appendix B).

Although 26 geochemical parameters were measured in this study, including trace elements such as cobalt and zinc and general environmental parameters such as NO_3^- concentration and pH, only two parameters significantly correlated to the number of putatively halorespiring *Chloroflexi* present: the number of *Bacteria* present (measured as 16S rRNA genes/g sediment) and the concentration of total dissolved sulfur (measured as sulfate with IC analysis). The correlation between putatively halorespiring *Chloroflexi* and *Bacteria* suggests that, as observed in many studies of *Dehalococcoides* (e.g. Seshadri et al., 2005; He et al., 2007), the putatively halorespiring *Chloroflexi* may have a nutritional dependence on other organisms. Alternatively, there may be habitat characteristics that are generally favorable or unfavorable for growth, resulting in niches in which both the putatively halorespiring *Chloroflexi* and *Bacteria* in general thrive.

As mentioned above, the number of putatively halorespiring *Chloroflexi* also negatively correlated to the concentration of total dissolved sulfur. In fact, although the number of putatively halorespiring *Chloroflexi* and *Bacteria* correlated, the percent of the bacterial community that was made up of the putatively halorespiring *Chloroflexi* was also significantly influenced by the dissolved sulfur concentration (measured as aqueous

sulfate with ion chromatography after oxidation) (FIG. 4.1), suggesting that there was a more pronounced impact of sulfur on the putatively halorespiring *Chloroflexi* than on the entire bacterial community. Using Spearman's rank test, the impact of sulfur on *Chloroflexi*/*Bacteria* was significant (ρ=-0.46, P<0.001). Upon inspection of the data (FIG. 4.1), sulfur actually appeared to negatively affect the abundance of *Chloroflexi* only at concentrations above 1 mM in the porewater or 0.1 mM in the lake water column, but no significant correlation exists with the data points above or below these amounts. This suggests that there was an inhibitory effect of sulfur, presumably present as sulfite and sulfide in the reduced lake sediments, at about 1 mM. This is similar to what has been observed in the laboratory where sulfide has been observed to inhibit halorespiration at concentrations of 5-10 mM and sulfite has been observed to inhibit at concentrations of 0.5-2 mM (Magnuson et al., 1998; He et al., 2005; May et al., 2008). Because chloride and sulfur strongly correlate to each other (Spearman's' ρ=0.92, P<0.001), it must be verified that it is indeed sulfur rather than chloride that is the parameter of interest. When sulfur is normalized by the total soluble anion concentration (the sum of sulfate, bromide, chloride, phosphate, and nitrate) the statistical relationships between *Chloroflexi* and sulfur retain their significance and direction (ρ=-0.2762 P=0.02). If chloride is similarly normalized, however, the relationship between *Chloroflexi* and chloride is positive and statistical significance is loss (ρ=0.14 P=0.23). This suggests that sulfur is indeed the causal parameter, and not chloride.

Individually, the metals cobalt, chromium, copper, iron, and nickel were positively correlated to the percentage of the community composed of putatively halorespiring *Chloroflexi*, though not significantly (Spearman's ρ=0.20, 0.43, 0.123,

0.275, and 0.16, respectively). Thus, it does not appear likely that these metals significantly alter the geochemical pressures of sulfur on the putatively halorespiring *Chloroflexi*.

The abundance of *Dehalobacter*-like species did not correlate with any of the geochemical parameters measured.

In low sulfur lakes, the quantity of both *Bacteria* (16S rRNA genes) and the putatively halorespiring *Chloroflexi* (16S rRNA genes) correlated strongly to the organic matter content of the samples (measured as mass loss on ignition at 550°C) (Spearman's $\rho=0.77$, $P<0.001$, $\rho<0.74$ $P<0.001$, FIG. 4.2). This correlation did not exist in the samples containing high (>1 mM) sulfur.

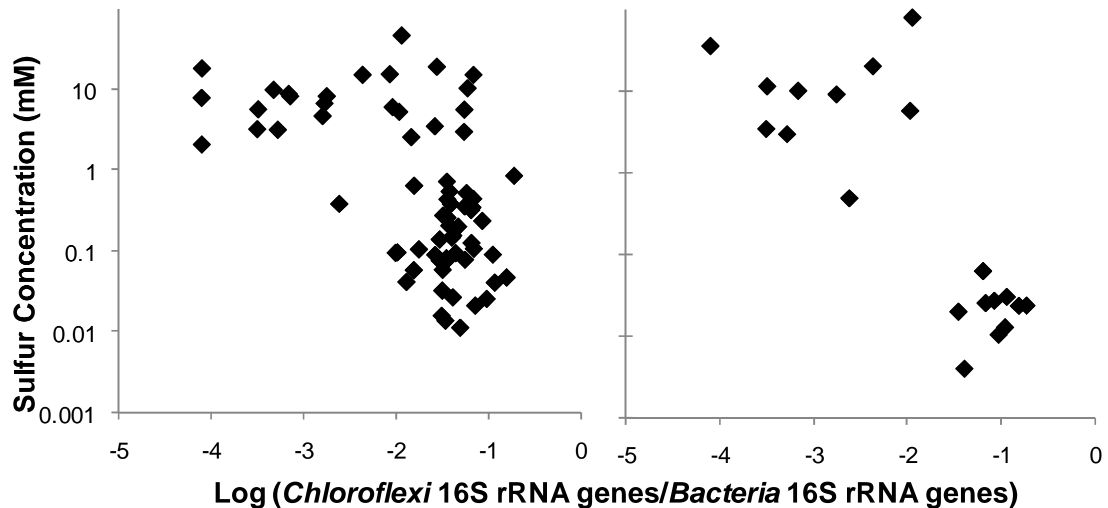

FIG. 4.1. The amount of *Chloroflexi*/*Bacteria* 16S rRNA genes in sediments (grab and cores) are negatively impacted at higher amounts of sulfur in porewater (left) and the amount of *Chloroflexi*/*Bacteria* 16S rRNA genes in the shallow grab sediment samples are negatively associated with higher sulfur levels in lake water (right).

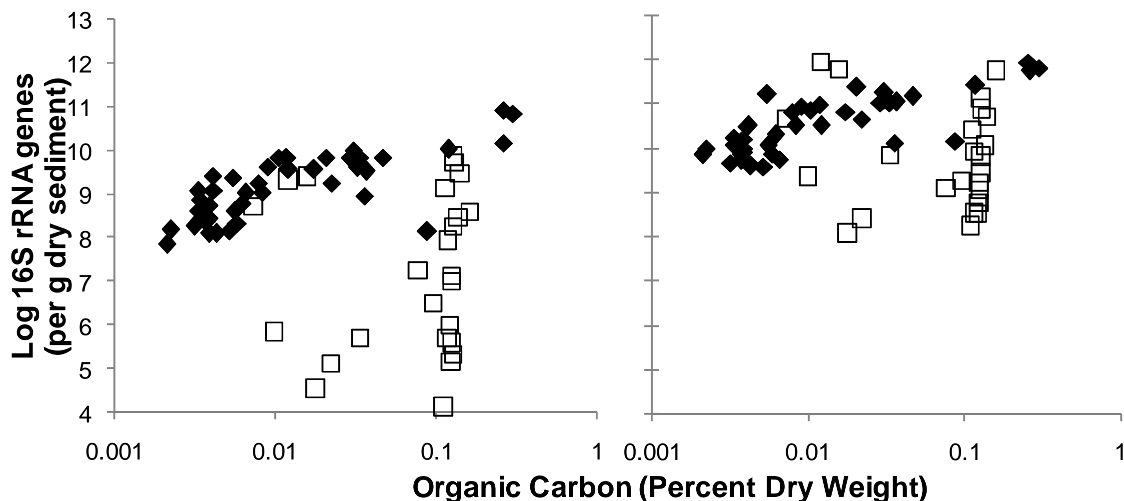

FIG. 4.2. The amount of *Chloroflexi* 16S rRNA genes (left) and *Bacteria* 16S rRNA genes (right) in the sediment correlates to organic carbon in low sulfur lake sediments (<1.0 mM porewater sulfur, black diamonds), but does not in higher sulfur lakes (>1.0 mM porewater sulfur, white squares).

To probe the relationship between sulfur and putatively halorespiring *Chloroflexi* further, TRFLP, augmented by cloning (see Appendix B), was performed to determine how the diversity of the targeted *Chloroflexi* were impacted by sulfur in the lake samples. With the TRFLP method used, halorespirers, and particularly those in the *Dehalococcoidetes* class were targeted. Because of this, if halorespirers were common in a given sample, the fraction of TRFs belonging to the *Dehalococcoidetes* class would be large; in contrast, if halorespirers were relatively rare in a sample, the fraction of *Dehalococcoidetes* amplified would be much less. As suggested by the overall correlation between putatively halorespiring *Chloroflexi* and sulfur, the percent of TRFs falling into the *Dehalococcoidetes* class was strongly and statistically affected by sulfur, with low sulfur lakes containing both a higher number of *Dehalococcoidetes* members (TRFs) and a greater percentage of the TRFs made up by *Dehalococcoidetes* members (FIG. 4.3). The average number of TRFs from *Dehalococcoidetes* was statistically higher (Student t-

test $P<0.001$) in lower sulfur sediments than higher sulfur sediments (an average of 4.5 TRFs and 3.1 TRFs, respectively). As expected based on these strong and consistent correlations, the relative percentage of *Dehalococcoidetes* was found to correlate to the amount of *Chloroflexi* 16S rRNA genes/g dry sediment in all of the sediment samples ($\rho=0.58$, $P<0.001$), indicating that the qPCR method was a good measurement for the relative distribution of putatively halorespiring *Chloroflexi*.

As mentioned above, although the number of geochemical parameters measured in this study was extensive, no other parameter was found to significantly affect the relationship between the number of putatively-halorespiring *Chloroflexi* in high or low sulfur lake sediments, and no parameter was found to affect the diversity of *Dehalococcoidetes* other than sulfur. Additionally, no significant differences in the biogeochemical parameters or relationships occurred with depth in the sediment cores collected in this study. As is always possible, parameters not measured in this study may be important. Although all samples in this study were taken from anaerobic sediments, the exact reductive potential could not be measured. Furthermore, the quantity of organochlorine present in the sediment samples could not be measured.

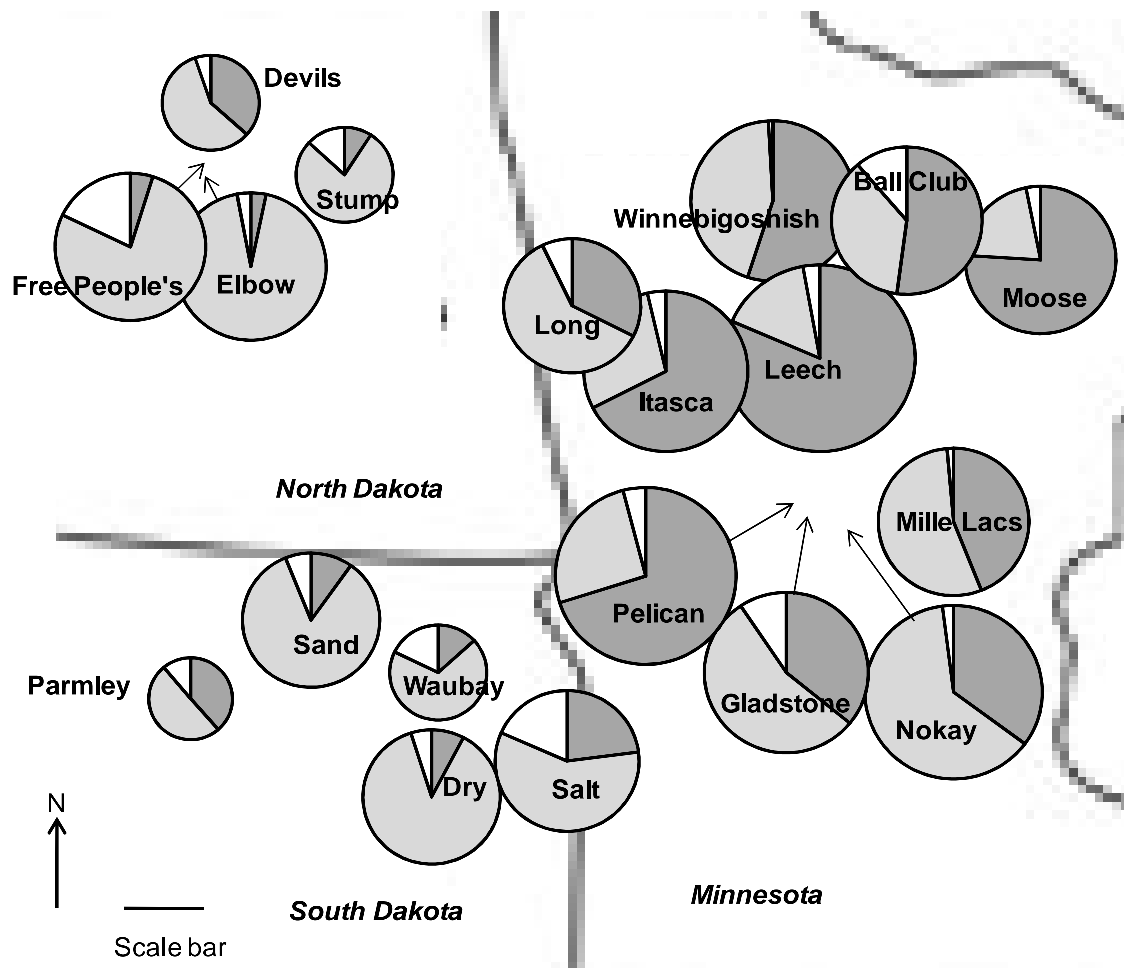

FIG 4.3. The quantity of putatively halorespiring *Chloroflexi* determined by qPCR is shown by the size of the circle, with the scale bar shown being equal to 5 logarithmic units *Chloroflexi* 16S rRNA per g dry sediment, and the percentage of TRFs determined to be *Dehalococcoidetes* (dark grey), other *Chloroflexi* (light grey), and undetermined (white) by TRFLP analysis is shown by the pie charts. Positions of circles, or arrows (when present), are approximate location of lake. For Richmond Lake (not shown), no 16S rRNA genes were detected in TRFLP analysis and the detection of putatively halorespiring *Chloroflexi* was 3.5×10^{4} 16S rRNA genes per g sediment (just above detection limit).

Although halorespiring *Chloroflexi* likely play an important role in the cycling of chlorine and are beneficial for bioremediation applications, little is known about their natural physiology. Our results indicate that sulfur has an important, and perhaps controlling, effect on the abundance of these organisms. Because natural organochlorines

also tend to be recalcitrant (Winterton et al., 2000; Redon et al., 2011), sulfur may also impact carbon cycling. Indeed, although *Bacteria* correlated to organic carbon in low sulfur lakes, this was not the case in samples with high sulfur concentrations. In addition, the decrease in *Bacteria* in these higher sulfur lake sediments occurred concomitant with a larger decrease in *Chloroflexi*. Again, these findings point to sulfur as a potentially controlling factor in the natural cycling of chlorine and perhaps carbon. Although the quantity of atmospheric sulfur precipitation in the United States and Europe has been declining, it has been increasing in developing countries such as China (Kahl et al., 2004; EIA, 2008; Mitchell and Likens, 2011). Furthermore, the use of sulfur compounds in fertilizers has been on the rise (e.g. Chien et al., 2011 and references therein). These anthropogenic pressures may therefore increase sulfur in particular environments, not only impacting natural attenuation in contaminated sites, but also impacting chlorine cycling in uncontaminated environments as well. Further research is needed, particularly on the effect of geochemical parameters on reductive dehalogenase genes in the environment, and on the wider role of other halorespiring organisms, such as those in the *Firmicutes* phylum, in the chlorine cycle.

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CHAPTER 5. Dechlorination of two chlorinated xanthones in anaerobic enrichment cultures

5.1 Introduction

Several strains of *Bacteria* are capable of the reductive dehalogenation of toxic anthropogenic organohalogens. These strains form in whole or in part the genera *Clostridium*, *Dehalobacter* and *Desulfitobacterium* of the phylum *Firmicutes*, the *Anaeromyxobacter*, *Desulfomonile*, *Geobacter*, *Desulfuromonas, Desulfoluna* and *Sulfurospirillum* of the phylum *Proteobacteria*, and *Dehalococcoides*, *Dehalogenimonas*, *Dehalobium*, and the '*o*-17' group of the phylum *Chloroflexi* (Shelton and Tiedje, 1984b; Holliger et al., 1993; Krumholz et al., 1996; Maymó-Gatell et al., 1997; Dennie et al., 1998; Chang et al., 2000; Wu et al., 2002a; Wu et al., 2002b; Luitjen et al., 2003; Sung et al., 2006; Moe et al., 2009; Ahn et al., 2009). These bacteria have been found to dechlorinate a diversity of chlorinated aliphatic compounds, such as chlorinated ethenes and propanes, as well as a diversity of chlorinated aromatic compounds, such as chlorinated biphenyls, chlorinated dioxins, and chlorinated phenols (Maymó-Gatell et al., 1997; Schlötelburg et al., 2000; Adrian et al., 2007; Bunge et al., 2008; May et al., 2008). Because of their ability to use anthropogenic pollutants as terminal electron acceptors, dehalogenating bacteria are used for *in situ* bioremediation applications (*e.g.* Imfeld et al., 2008; Scheutz et al., 2008); nevertheless, bioremediation can be incomplete or slow, particularly with more complex or hydrophobic contaminants such as polychlorinated biphenyls or dioxins (Bedard, 2003). The failure of *in situ* bioremediation in such cases is often blamed on a lack of labile electron acceptors, which limits the growth of these halorespiring organisms. Further research into alternative electron acceptors that have the ability to increase the population size and activity of dehalogenating bacteria so that they

can then degrade the target (toxic and anthropogenic) electron acceptors, has therefore been identified as a potential way to improve bioremediation.

Because anthropogenic organohalogens have been only recently produced, researchers have speculated that naturally occurring organohalogens can be used by dehalogenating bacteria, and in fact, are the original physiological electron acceptors for these organisms. In support of such speculation are our recent findings that putative halorespirers were widely present in uncontaminated natural environments (Chapter 3, Chapter 4). In addition, a dechlorinating organism, *Desulfoluna spongiiphila*, has been isolated from a marine sponge (Ahn et al., 2009). Because some chlorophenols, polybrominated diphenyl ethers, and trichloroethene can be produced naturally (Abrahamsson et al., 1995; Gribble, 1999; Ortiz-Bermúdez et al., 2003), these compounds may be examples of naturally occurring terminal electron acceptors for halorespirers (Ahn et al., 2003; Adrian et al., 2007; Kittelmann and Friedrich, 2008). Unfortunately, all of these potential natural electron acceptors are also anthropogenically produced and toxic. Nevertheless, if a non-toxic natural electron acceptor for halorespirers could be identified, it could provide a means to enrich these organisms for more complete or more rapid bioremediation of contaminated sites.

As described above, although research on naturally occurring electron acceptors for dehalogenating bacteria has focused on compounds that are also anthropogenically-produced and toxic, thousands of natural chlorinated compounds are known to be produced in nature (Gribble, 2003; Öberg, 2002). There are more than 4400 identified natural halogenated compounds that are produced by plants, marine organisms, insects, bacteria, fungi, and mammals (Gribble, 1994; Rohlenová et al., 2009). One class of

natural organochlorines is the xanthones, which are synthesized by plants, bacteria, and fungi (Tomasek and Crawford, 1986). Interestingly, although never manufactured and therefore not anthropogenic, the basic structure of xanthones is similar to that of PCBs and dioxins (FIG. 5.1). Thirty-eight xanthones with between 1 and 4 chlorines have been identified in natural systems (Elix and Bennett, 1990; Elix and Crook, 1992). In addition to chlorine, naturally occurring xanthones contain hydroxyl-, methyl-, isoprenyl-, methoxyl-, and carboxyl-, groups (Tomasek and Crawford, 1986; Elix and Bennett, 1990; Elix and Crook, 1992). Thus, chloroxanthones could serve as a natural, and presumably non-toxic, source of electron acceptors for halorespirers in uncontaminated environments, providing an opportunity to potentially exploit these compounds for bioremediation purposes as well. The goal of this paper was therefore to test whether chloroxanthones can indeed serve as a terminal electron acceptor for dechlorinators in the natural environment and to determine whether the dechlorinators supported by chloroxanthones are similar to previously identified putative halorespirers from the *Chloroflexi* phylum, thus suggesting that they may be more amenable for use in biostimulation.

5.2 Methods

Batch Reactors. Batch reactors were used to determine the ability of five different soils and sediments to dechlorinate chlorinated xanthones. Two chlorinated xanthones, 2,7-dichloroxanthone and 1,3-dihydroxyl-5,7-dichloroxanthone were purchased from Specs and Princeton Biomolecular Research, Inc, respectively. Soils used to test for dechlorination activity were from a maple dominated forest from the Pine Barrens in New Jersey (Maple), a pine dominated forest from the Pine Barrens in New

Jersey (Pine), Tilden Regional Park in California (Tilden), and sediments came from Leech Lake in Minnesota (Leech) and Palos Verdes Harbor in California (PV). The soils and Leech Lake sediment are not known to be contaminated with any anthropogenic chlorinated compounds, but the Palos Verdes Harbor sediment has been historically contaminated with dioxins. These soils and sediments were collected for previous studies (Tao et al., 2006b; Chapter 3, Chapter 4) and stored in an anaerobic glovebag (Coy) with 3%/97% H_2/N_2 headspace. Batch reactors contained 130 mL of mineral media (Shelton and Tiedje, 1984b) reduced with 2 µM titanium citrate, 10 mM potassium acetate, 1 mL of vitamin solution (Wolin et al., 1963) and 5 g of soil or sediment. For each soil or sediment, triplicate batch reactors were amended with chlorinated xanthones and triplicate batch reactors were not amended and acted as controls on the microbiological community for each reactor. One set of autoclaved batch reactors, in triplicate, was also set-up and maintained identically with the addition of autoclaving and an amendment of 50 mM sodium azide. Thus a total of 11 sets of triplicate reactors were set up and maintained. The Tilden, PV, and autoclave control reactors were maintained for 698 days; the Pine reactors were maintained for 557 days, and the Leech and Maple reactors were maintained for 118 days. The Tilden, Pine, and PV reactors were initially inoculated with 10 µM of 2,7-dichloroxanthone and multiple amendments of 2,7-dichloroxanthone and 1,3-dihydroxy-5,7-dichloroxanthone thereafter. The Maple and Leech reactors were initially inoculated with 100 µM of each 2,7-dichloroxanthone and 1,3-dihydroxy-5,7-dichloroxanthone without any subsequent amendment. The two chlorinated xanthones were amended in a solution of acetone (100 mM) or as dry powder. After amendment with dry chlorinated xanthones, the reactors were placed on a shaker table for ten days to

better incorporate the xanthones into the soil matrix. Otherwise, the reactors were stored in an anaerobic glovebag. Reactors were sampled identically as previously describe (Yan et al., 2006b).

Analytical Analysis of Xanthones. Samples taken for chemical analysis were extracted with an ASE 350 accelerated solvent extractor (Dionex). Samples were mixed with 10 g oven-baked (550°C) Ottawa sand and air-dried for 48 hours prior to extraction. The extraction process was then carried out under the EPA guidelines for accelerated solvent extraction of dioxins (EPA Method 3545A). The samples were extracted with a 50:50 mix of acetone:hexane in three 15 minute static intervals at 100°C and 1600 psi. The amount of solvent extract was then reduced with roto-evaporation and transferred to a vial where the remaining extract was blown-down to dryness under a stream of nitrogen. Anhydrous sodium sulfate was added to the vial, and 10 mL of hexane was added to resuspend the extract. The exact amount of hexane was determined by weight. An aliquot of nonderivatized sample was then transferred to autosampler vials for analysis. The chlorinated xanthones, and the unchlorinated products xanthone, and 1,3-hydroxylxanthone were analyzed with gas chromatography equipped with an electron capture detector (ECD) with external standards. Standards were also analyzed with gas chromatography equipped with a mass spectrometer (GC-MS) to verify elution times, and samples exhibiting dechlorination were analyzed with GC-MS to screen for peaks contained expected mass spectra of the singly chlorinated products (the M+ peaks 230 and 232 for 2-chloroxanthone and 262 and 264 for 1,3-dihydroxy-7-chloroxanthone and 1,3-dihydroxyl-5-chloroxanthone). Xanthone (unsubstituted) was purchased from Sigma, and 1,3-dihydroxylxanthone was purchased from Princeton Biomolecular Research, Inc.

Five standards was made in hexane for each compound and ranged from 1.0-10 ng/mg, 0.1-10 ng/mg, 0.1-10 ng/mg, and 1-20 ng/mg for 2,7-dichloroxanthone, 1,3-dihydroxyl-5,7-dichloroxanthone, xanthone, and 1,3-dihydroxylxanthone, respectively. All standards were found to be in the linear range. Samples with concentrations above the range of standards were diluted in hexane and reanalyzed. The temperature program for the gas chromatography was 40°C for 2 min, a ramp of 10°C/min to 180°C, a ramp of 15°C/min to 270°C and 15 min at 270°C for a total run time of 37 min. Both the GC-ECD and GC-MS was equipped with an HP-5 (Agilent) column. Glassware and autosampler vials used for analytical analysis were silanized, as recoveries from non-silanized glassware were poor. Derivatizations of compounds were attempted with trimethylchlorosilane and N-trimethylsilylimidazole but both derivatization products were found to be unstable. Recoveries of 2,7-dichloroxanthone, 1,3-dihydroxyl-5,7-dichloroxanthone, xanthone, and 1,3-dihydroxylxanthone were each individually tested as follows. Five vials (21 mL) with 15 mL of slurry identical in composition to batch reactors above were autoclaved. Different amounts of chemical were added to each of the five vials for the concentrations expected in this study (1 µM, 5 µM, 10 µM, 20 µM, and 100 µM) and these reactors were placed on a shaker table overnight. The reactors were then sampled as above and these samples were extracted and treated as above. The amount of chemical quantified with GC-ECD was then divided by the amount of chemical added. The recoveries for 2,7-dichloroxanthone, 1,3-dihydroxyl-5,7-dichloroxanthone, xanthone, and 1,3-dihydroxylxanthone measured in this study were 92 ± 1.3%, 89 ± 6.3%, 95 ± 3.1%, and 96 ± 2.4%, respectively (mean ± SE of the five concentrations). Detection limits were 0.1

μM for 2,7-dichloroxanthone, and 1 μM for 1,3-dihydroxyl-5,7-dichloroxanthone, xanthone, and 1,3-dihydroxylxanthone.

QPCR, DGGE, and Sequencing. Samples for DNA analysis from the batch reactors were obtained as previously described (Chapter 3) and DNA was extracted with the PowerSoil DNA kit (MoBio Laboratories). Quantitative PCR (qPCR) was performed as described previously (Chapter 3) to measure the number of putatively halorespiring *Chloroflexi*. Additionally, the DNA extracts from four time points for the Tilden, PV, Maple, and Leech reactors were subjected to denaturing gradient gel electrophoresis (DGGE). Each time point contained samples from two reactors amended with chlorinated xanthones and two reactors that were unamended controls. DGGE was performed similarly as described previously (LaPara et al., 2000; Yan et al., 2006b). Briefly, genomic DNA was diluted 1:100 fold and amplified with primers 338F (5'-ACT CCT ACG GGA GGC AGC AG-3') (Lane et al., 1991) and 518R (5'-ATT ACC GCC GCT GCT GG-3') attached with a GC clamp (Muyzer et al., 1993). PCR amplicons were analyzed on a 30-55% denaturing gradient gel and electrophoresis was performed on a D-Code apparatus (BioRad) in a 0.5 × TAE buffer. Electrophoresis was performed at 20 V for 15 min, followed by 200 V until the loading dye migrated to the bottom of the gel (at least 3 hours). Gels were stained with SYBR Green I (Molecular Probes) and visualized on an EC3 Bioimaging system (Ultra-Violet Products). Bands that arose in the chloroxanthone-amended reactors but not in the unamended reactors were excised. Excised bands were moved to microcentrifuge tubes with ethanol-washed tweezers and 20 μL of DNAse/RNAse-free water was added. Gel slices were then left overnight, and the supernatant was diluted 1:100 fold and subjected to repeated rounds of PCR, DGGE,

and excision until the fragment appeared clear of other fragments. The final excised fragments were then subject to PCR as above except without the GC-clamp. PCR fragments were cleaned with the GeneClean II Kit (MP Biomedical) and submitted to the BioMedical Genomics Center (University of Minnesota) for analysis with an ABI Prism 3730xl DNA Analyzer.

Statistical Analysis. To determine if dechlorination was significant, a 1-tailed Student T-test was used to compare concentrations of the chlorinated xanthones between amended reactors and controls or between two time points for the amended reactors. A *P* value less than 0.05 was considered significant. Calculation of dechlorination rates was performed by taking the difference in concentrations between different sampling points in time and dividing by the number of days between those sampling points.

5.3 Results

Five different soils and sediments were used as inocula to determine the ability of indigenous bacterial communities to dechlorinate chlorinated xanthones. The soils came from Tilden Regional Park in California (Tilden), a Pine-dominated forest in the New Jersey Pine Barrens (Pine), and a Maple-dominated forest in the New Jersey Pine Barrens (Maple). The sediments came from a Leech Lake in Minnesota (Leech) and Palos Verdes Harbor in California (PV). Both 2,7-dichloroxanthone and 1,3-dihydroxyl-5,7-dichloroxanthone were dechlorinated in the Tilden, PV, Leech, and Maple batch reactors with the concomitant production of daughter products (FIG. 5.2, FIG. 5.3). No significant decrease in chlorinated xanthones occurred in the autoclaved reactors (maintained for 698 days) or the Pine reactors (maintained for 557 days) and the dechlorination products

xanthone and 1,3-dihydroxylxanthone were not detected at any time in those reactors (FIG. 5.4).

For the Tilden reactors, there was an initial lag time of 61 days prior to the dechlorination of 2,7-dichloroxanthone;for the PV reactors, this lag time was 76 days. Upon amendment with 1,3-dihydroxyl-5,7-dichloroxanthone, there was no lag time prior to its dechlorination in the Tilden reactors, while the PV reactors had a lag time of 73 days before the hydroxylated chloroxanthone was dechlorinated. For the Maple and Leech reactors, the lag time prior to dechlorination of both chloroxanthones (the hydroxylated compound and the non-hydroxlyated 2,7-dichloroxanthone) was less than 20 days. This short lag time might have been a result of the higher initial load of the compounds (100 µM compared to 10 µM) in these reactors or the simultaneous amendment of both compounds.

Initial dechlorination rates varied, with relatively slow initial dechlorination in the Tilden (11 nmol/day and 28 nmol/day for 2,7-dichloroxanthone and 1,3-dihydroxy-5,7-dichloroxanthone dechlorination, respectively) and PV reactors (4 nmol/day and 13 nmol/day for2,7-dichloroxanthoneand1,3-dihydroxy-5,7-dichloroxanthonedechlorination, respectively) and more rapid dechlorination in the Maple (0.30 µmol/day and 0.58 µmol/day for 2,7-dichloroxanthone and 1,3-dihydroxy-5,7-dichloroxanthone dechlorination, respectively) and Leech (0.20 µmol/day and 0.56 µmol/day for 2,7-dichloroxanthone and 1,3-dihydroxy-5,7-dichloroxanthone dechlorination, respectively) reactors. The rate of dechlorination for 1,3-dihydroxy-5,7-dichloroxanthone was generally greater than the rate of dechlorination for 2,7-dichloroxanthone, though the rate

of dechlorination for 2,7-dichloroxanthone did increase with additional chloroxanthone amendment to between 0.25 and 0.5 μmol/day.

The concentrations of xanthone and 1,3-dihydroxylxanthone also increased in these reactors during periods of rapid chloroxanthone dechlorination; indeed, the initial production of these products was nearly stoichiometric with respect to the loss of the parent compounds. Subsequent loss of the dechlorinated products was observed, however, suggesting that these compounds were anaerobically metabolized over time. No singly chlorinated xanthones were detected in this experiment, suggesting that either the detection limit on the GC-MS was too high or the dechlorination of the singly-chlorinated intermediates occurs faster, or as fast, as that of the dichlorinated parent compounds.

The number of putatively-halorespiring *Chloroflexi* was measured with qPCR to determine if the halorespiring *Chloroflexi* may grow on chlorinated xanthones. Interestingly, no growth in *Chloroflexi* was detected in the reactors (FIG 5.5).

To determine which organisms might be responsible for the dechlorination of 2,7-dichloroxanthone and 1,3-dihydroxy-5,7-dichloroxanthone, PCR-DGGE was performed on the Tilden, PV, Maple, and Leech reactors (FIG. 5.6). DGGE bands that were present in these reactors but not in the control reactors, were excised and sequenced (Table 5.1; sequences provided in SI). These sequences are presumed to include the organisms enriched by the addition of the chloroxanthones and therefore potentially responsible for their dechlorination; nevertheless, it is possible that other organisms, below the detection limit of the DGGE method, were also involved in the dechlorination of these xanthones as well. A total of 12 sequences were excised and sequenced: four from the Tilden reactors, three from the PV reactors, four from the Maple reactors, and 1 from the Leech

reactors. The majority of these sequences are closely related to the *Clostridia* class of the *Firmicutes* phylum, though few of these sequences are closely related to any cultured bacteria. Interestingly, a similar organism was not enriched in all of the reactors, though two strains, Tilden-1 and Maple-2 were closely related to each other (98% similarity). Two of the sequences, Tilden-4 and PV-2 were closely related to cultured bacteria from dechlorinating cultures, though these strains have not been specifically identified as dechlorinating organisms (*Sedimentibacter* sp. JN18_V27_I is a strain from Bedard et al., 2007; *Actinobacterium* BVF2 has no published reference). Six additional sequences are close matches (>97% identity) to sequences obtained from different consortia dechlorinating polychlorinated biphenyls, chlorinated ethenes, dioxins or hexachlorobenzene (Gu et al., 2004; Yan et al, 2006a; Yan et al., 2006b; Ahn et al., 2007; Behrens et al., 2008; Tas et al., 2010; Wei and Finneran, 2011; Ho and Lui, 2011). Although it is unknown if any of these strains are dechlorinators, their common occurrence in dechlorinating cultures suggests that either they are indeed capable of dechlorination, or they have a symbiotic relationship with dechlorinators. Of the four sequences not matching any sequences from dechlorinating cultures, three (Tilden-2, Tilden-3, and PV-1) are not closely related to any cultured strains, and one strain (Maple-4) is related to a *Sedimentibacter*. Although more research is warranted to determine if any known dechlorinating microorganisms, such as *Dehalococcoides* or *Dehalobacter*, are present in these cultures, the results of the DGGE analysis suggests that yet to be isolated bacteria, predominantly of the phylum *Firmicutes*, may be involved in the dechlorination of the chlorinated xanthones in this study.

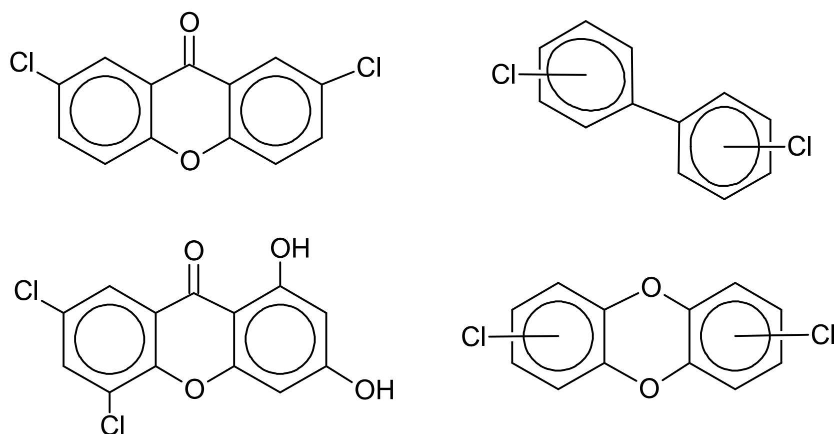

FIG 5.1. The structure of 2,7-dichloroxanthone (upper left), 1,3-dihydroxyl-5,7-dichloroxanthone (bottom left) compared to the structure of polychlorinated biphenyls (upper right) and dioxins (bottom right).

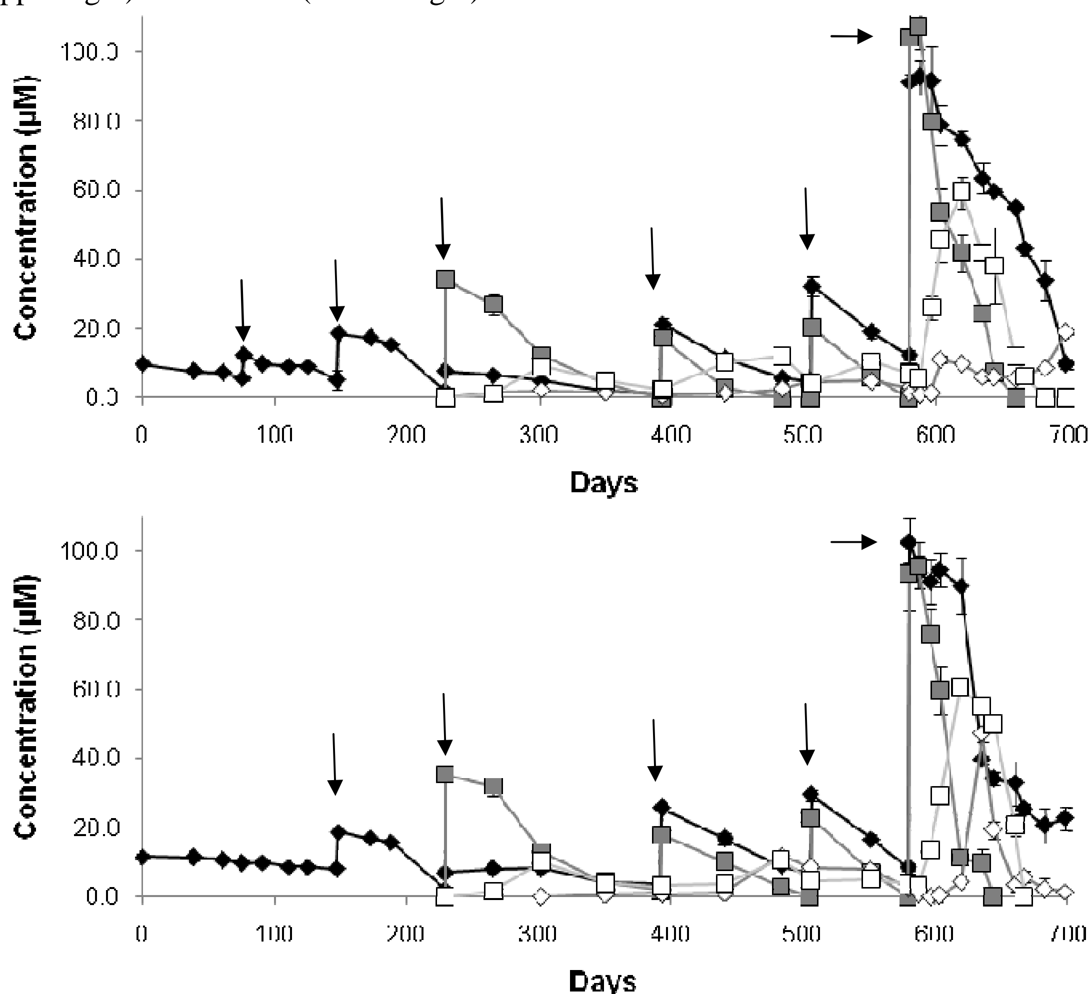

FIG 5.2. The concentrations of 2,7-dichloroxanthone (solid diamonds), 1,3-hydroxy1-5,7-dichloroxanthone (solid squares), xanthone (open diamonds), and 1,3-dihydroxylxanthone (open squares) over time for the Tilden reactors (above) and the PV

reactors (bottom). Errors bars represent standard error for triplicate reactors. Arrows point to times in which chlorinated xanthones were amended to the reactors.

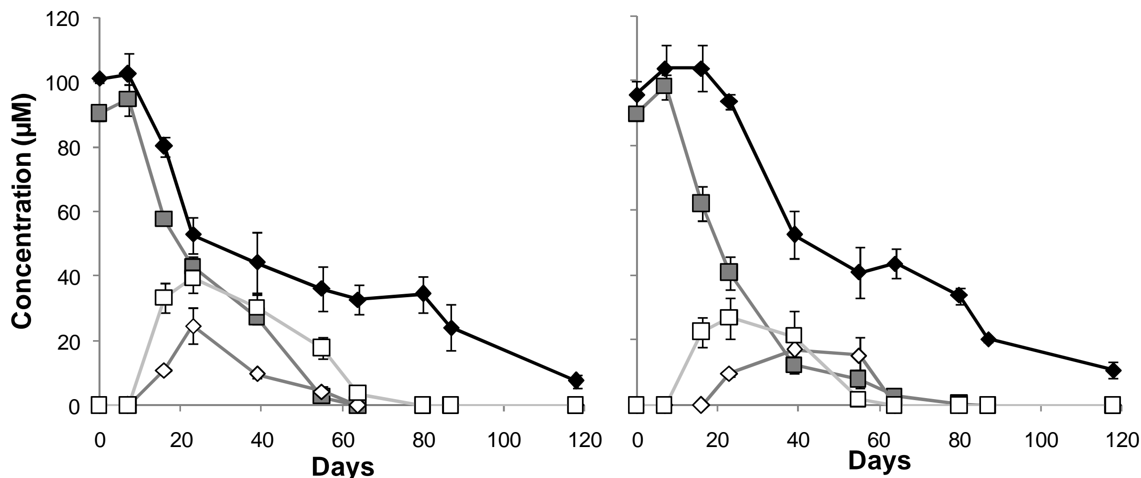

FIG 5.3. The concentrations of 2,7-dichloroxanthone (solid diamonds), 1,3-hydroxyl-5,7-dichloroxanthone (solid squares), xanthone (open diamonds), and 1,3-dihydroxylxanthone (open squares) over time for the Maple reactors (left) and the Leech reactors (right). Errors bars represent standard error for triplicate reactors.

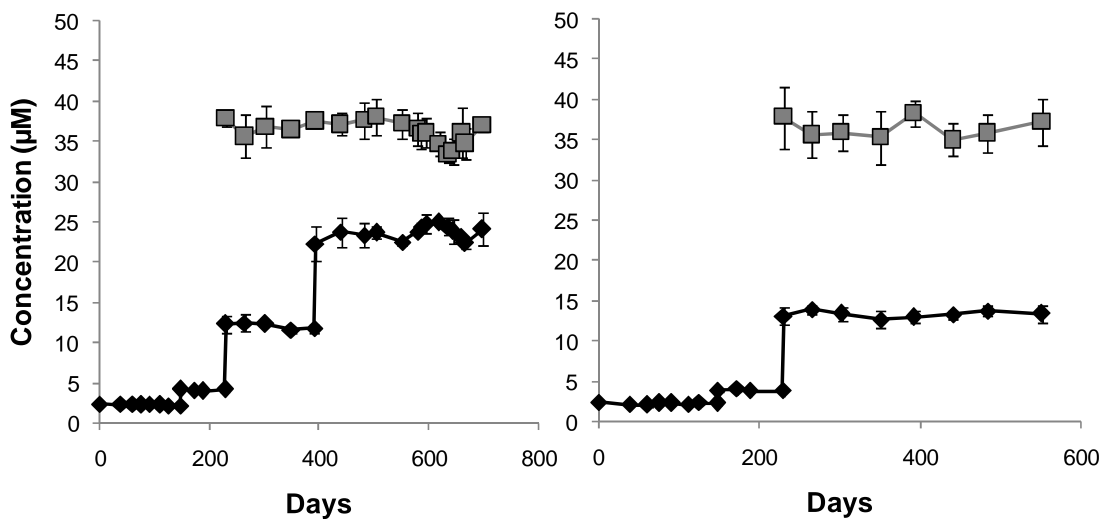

FIG 5.4. The concentrations of 2,7-dichloroxanthone (sold diamonds) and 1,3-hydroxyl-5,7-dichloroxanthone (solid squares) over time for the autoclaved reactors (left) and the Pine reactors (right).

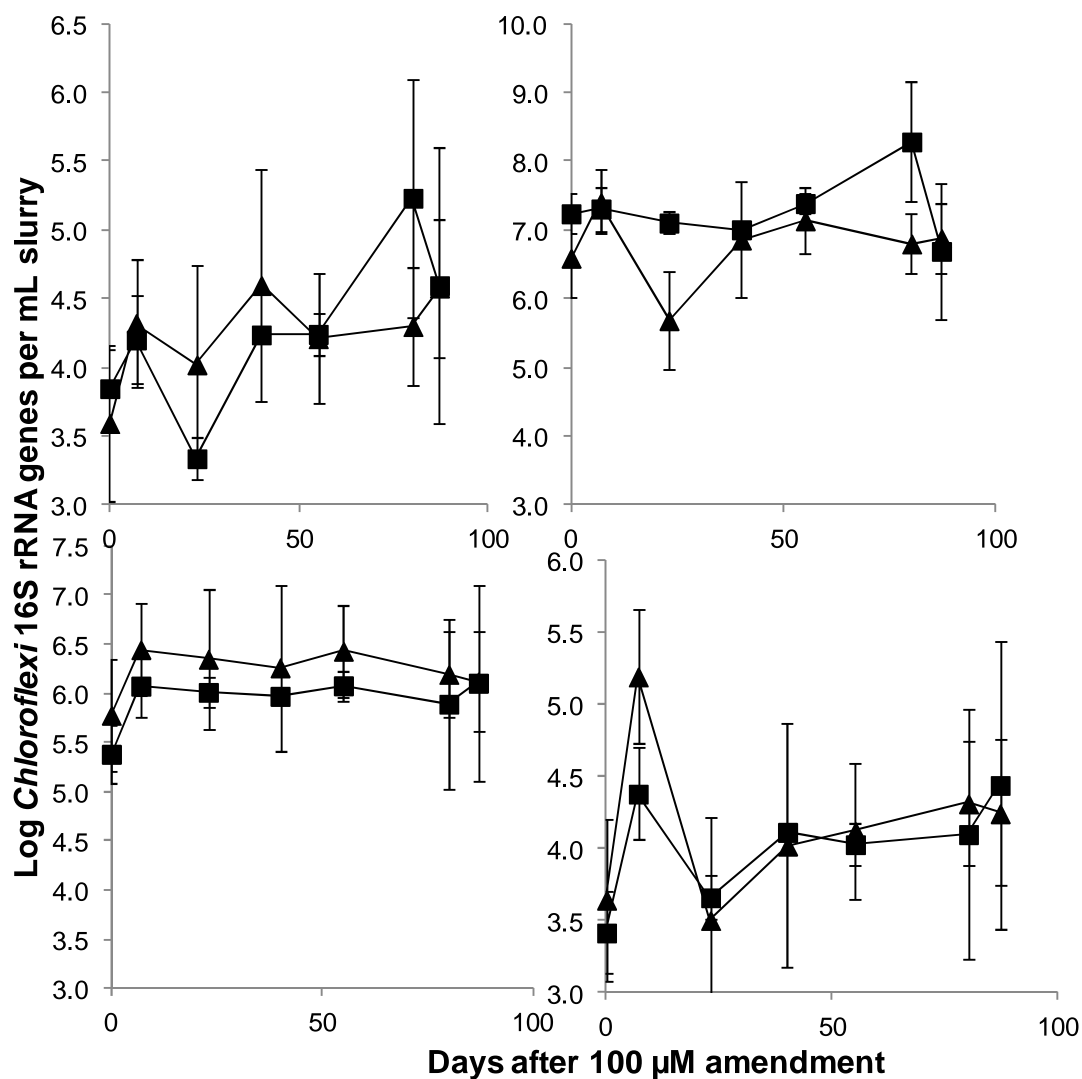

FIG 5.5 Abundance of putatively-halorespiring *Chloroflexi* in the Tilden (upper left), PV (upper right), Maple (lower left), and Leech (lower right) reactors following the amendment of 100 µM of each chloroxanthone. Triangle symbols indicate amended reactors and square symbols indicate unamended controls.

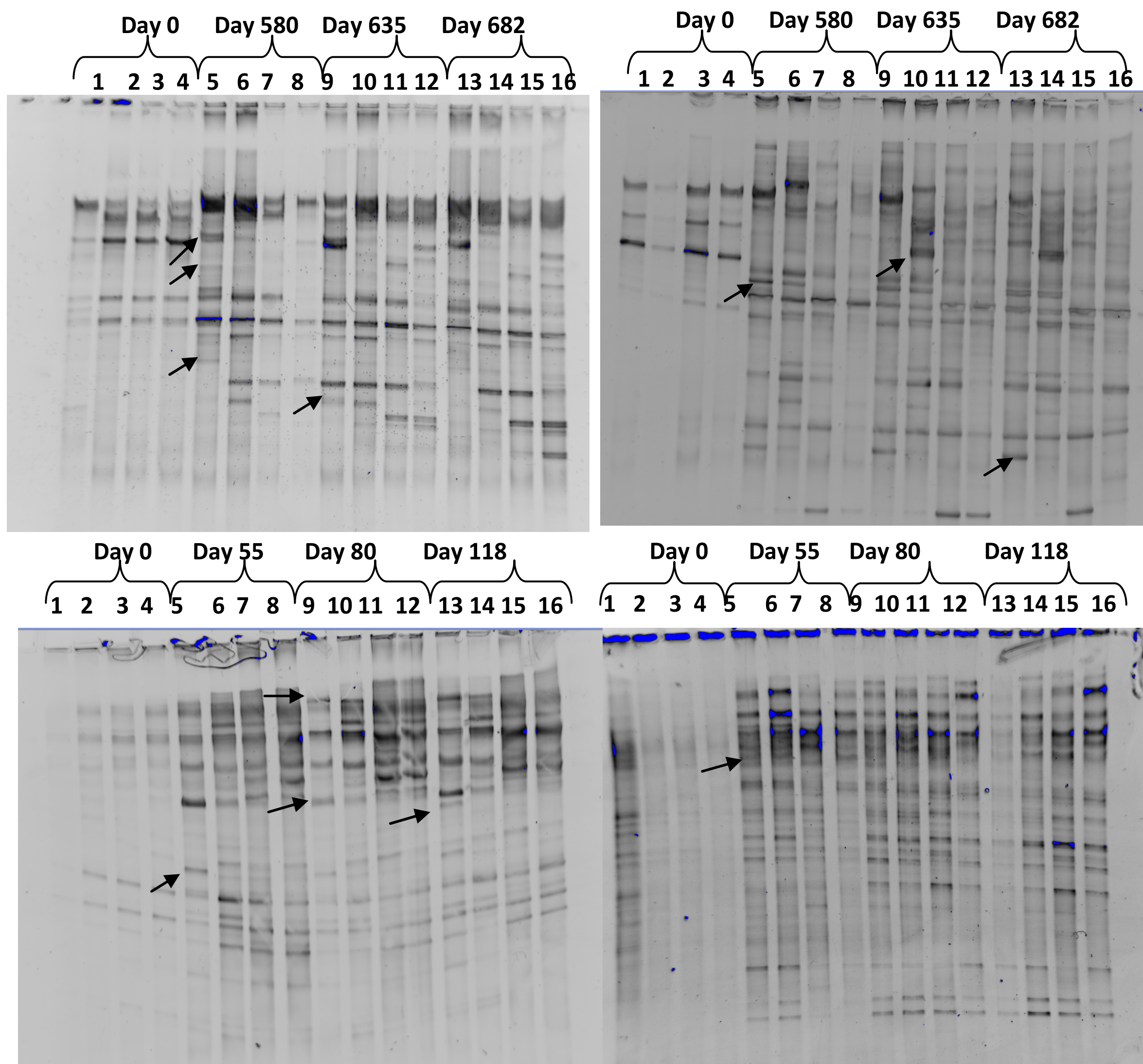

FIG 5.6. DGGE analysis of the Tilden (top left), PV (top right), Maple (bottom left) and Lake (bottom right). For each day indicated, the first two columns are from two batch reactors treated with chlorinated xanthones and the second two columns are from untreated controls. The arrows point to the bands excised and sequenced.

Table 1. The results of BLAST inquiries of the sequenced DGGE bands

DGGE Band	Closest Match (cultured), Phylum (Accession Number)	Similarity	Matches to uncultured bacteria from dechlorinating consortia (>97%)
Tilden-1	*Clostridium* -like species, clone 16SX-2, *Firmicutes* (U27711)	97%	PCE-dechlorinating consortium clone (FJ810668) 97%
			PCB-dechlorinating consortium clone (DQ080197) 97%
Tilden-2	*Clostridium* sp. 6-16, *Firmicutes* (FJ808609)	92%	None
Tilden-3	*Tindallia* sp. Mic8c12, *Firmicutes* (AB546246)	91%	None
Tilden-4	[1] *Actinobacterium* BVF2, *Actinobacteria* (DQ833388)	99%	Hexachlorobenzene-dechlorinating consortia clones (FJ810777 and FJ10762) 98%
PV-1	*Desulfotomaculum acetoxidans* DSM 771, *Firmicutes* (CP001720)	85%	None
PV-2	[2] *Sedimentibacter* sp. JN18_V27_I, *Firmicutes* (EF059533)	99%	PCE-dechlorinating consortium clone (FM178803) 98%
			TCE-dechlorinating consortium clone (AF349757) 98%
PV-3	Eubacterium sp. C2, *Firmicutes* (AF044945)	88%	Dioxin dechlorinating consortium clone (EF471886) 97%
Maple-1	*Dethiosulfovibrio* sp USBA 82, *Synergistetes* (EU719657)	91%	Hexachlorobenzene-dechlorinating consortia clones (FJ810770; FJ810689) 99%
			TCE-dechlorinating consortium clones (FJ810750; FJ810707; FJ810673) 99%
			PCB-dechlorinating consortium clones (DQ080193, AY754845) 98%
Maple-2	*Bacteroidales* bacterium 65bZ, *Bacteroidetes* (GU129100)	96%	TCE-dechlorinating consoritum clone (JF502584) 99%
			Chlorinated solvent dechlorinating consortium clone (FR821427) 99%
Maple-3	*Clostridium* -like species, clone 16SX-2, *Firmicutes* (U27711)	99%	PCB dechlorinating consortium clone (DQ080197) 100%
			PCE-dechlorinating consortium clone (FJ810668) 98%
Maple-4	*Sedimentibacter honkongensis* strain KI, *Firmicutes* (AY571338)	98%	None
Leech-1	Anaerobic bacterium sk.prop8, *Firmicutes* (AY538172)	97%	TCE dechlorinating consortium clone (HM749854) 100%
			PCB dechlorinating consortium clones (GQ859873, GU180170) 97-100%

[1] Cultured from VC dechlorinating enrichment culture, physiology uknown

[2] Cultured from Aroclor dechlorinating culture, physiology unknown

5.4 Discussion

The discovery of naturally occurring chlorinated electron acceptors that support the growth of halorespirers could lead to new technologies for bioremediation. The biostimulation of dechlorinators using alternative electron acceptors, often called priming, holds promise for the remediation of toxic chlorinated compounds. Dibromobiphenyls and several chlorinated aromatics have been shown to stimulate the dechlorination of PCBs (Bedard et al, 2006; Bedard et al, 1998; Deeward and Bedard, 1999; Krumins et al, 2009), and several chlorinated aromatic compounds were found to stimulate the dechlorination of dioxins (Ahn et al, 2007; Ahn et al, 2008). One barrier to the use of these types of compounds for biostimulation in the environment is that these halogenated compounds are anthropogenic and toxic themselves, and cannot therefore be administered *in situ*. Although the toxicity of the chlorinated xanthones used in this study is unknown, this paper provides evidence that xanthones are one source of natural compounds that are halorespired. Furthermore, because many chlorinated xanthones in nature contain methoxyl- and hydroxyl- groups (Tomasek and Crawford, 1986;Elix and Bennett, 1990; Elix and Crook, 1992), many natural chlorinated xanthones may be more soluble, and thus, more bioavailable, than the anthropogenic compounds previously used for halopriming. Additionally, because natural compounds, such as xanthones, often contain substituents such as methoxyl- and hydroxyl- groups, one could speculate that reductive dehalogenase genes may be better evolved to dechlorinate compounds containing these groups, though more study on the range and activities of reductive dehalogenases is needed.

Bioaugmentation, the addition of exogenous microorganisms with particular metabolic abilities into a contaminated system, has also been shown to be a potentially effective strategy for the enhancement of bioremediation, both for PCBs (Ahn et al., 2008; Winchell and Novak, 2008; Krumins et al., 2009) and for chlorinated ethenes (Adamson et al., 2003; Harkness et al., 1999; Major et al., 2002; Ritalahti et al., 2005). Unfortunately, concerns exist that augmented strains exhibit reduced fitness or the distribution of augmented cultures through a contaminated site is not optimal, which has hampered the application of this technology (Adamson et al., 2003; Lee et al., 2010). The development of robust halorespiring cultures derived from the halorespiration of natural organochlorines may help these cultures maintain their original fitness, and thus increase the applicability of bioaugmentation as well.

The DGGE results are inconclusive with respect to the population(s) that was/were responsible for the dechlorination of the two chloroxanthones. Several members within the *Firmicutes* phylum were identified in the dechlorinating cultures that were absent in the control cultures. In addition, organisms of a similar phylogeny were identified in cultures dechlorinating PCBs, dioxins, chlorobenzenes and chlorinated ethenes. Within the *Firmicutes*, several members of *Dehalobacter* and *Desulfitobacterium* have been isolated, as well as a halorespiring *Clostridium* strain (Dennie et al., 1998; Chang et al., 2000; Yoshida et al., 2009). Although the halorespiring *Chloroflexi* have proven important in bioremediation (Major et al., 2002), and have putatively been shown to be a part of natural environments (Chapters 3 and 4), more work needs to be done with respect to the halorespiring *Firmicutes* and their role in both contaminant remediation and the natural chlorine cycle. Additionally, the ability of

Chloroflexi, especially the *Dehalococcoides*, to use natural chlorinated substrates such as chlorinated xanthones needs further study.

5.5 References

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Chapter 6. Conclusions, Recommendations, and Future Work.

6.1 Conclusions

This dissertation focuses on naturally occurring halorespirers, their role in the natural chlorine cycle, the geochemical parameters that influence a common group of these bacteria, and the ability of these organisms to dechlorinate and grow on natural organochlorines. The general and specific conclusions of this research are as follows:

- Putatively-halorespiring *Chloroflexi* are present in uncontaminated environments. In soil samples, the amount of putatively-halorespiring *Chloroflexi* ranged from below the detection limit ($<5 \times 10^2$) to 4.2×10^7 gene copies/g soil and averaged 0.04% of the total *Bacteria* 16S rRNA genes. In lake sediments, the number of putatively halorespiring *Chloroflexi*16S rRNA genes ranged from $3.5 \times 10^4$to 8.5×10^{10} copies 16S rRNA genes/g dry sediment, and averaged 4.1% of the total *Bacteria* 16S rRNA genes.

- The amount of organochlorine content in soils impacts the abundance of putatively halorespiring *Chloroflexi*. The number of putatively halorespiring *Chloroflexi* 16S rRNA genes/g soil in soil samples correlated to [organochlorine]/TOC (ρ=0.31, P=0.012), but did not correlate with depth, TOC, or [chloride] (ρ=0.10, P=0.40,ρ=-0.16, P=0.18, and ρ=-0.03, P=0.82, respectively).

- Naturally produced organochlorines support the growth of putatively halorespiring *Chloroflexi*. The number of putatively halorespiring *Chloroflexi*

16S rRNA increased in batch reactors when amended with an enzymatically-produced mixture of organochlorines. For each amendment of enzymatically-produced organochlorines the number of *Chloroflexi* 16S rRNA genes increased by 2.9±0.3 to 4.0±0.2 orders of magnitude. Extracted organic matter alone increased the number of *Chloroflexi* 16S rRNA genes, but by a significantly lower amount (0.89±0.2 and 1.7±0.3 orders of magnitude). The number of *Bacteria* 16S rRNA genes was also measured with qPCR and did not increase significantly during these experiments. The accumulation of chloride increased by 2.4±0.5 mM and 2.7±0.4 mM concomitant with the increase of putatively halorespiring *Chloroflexi*.

- In contrast, when fed a specific natural chlorinated organic, 2,7-dichloroxanthone and 1,3-dihydroxyl-5,7-dichloroxanthone, putatively halorespiring *Chloroflexi* did not appear to be selectively enriched. Rather, organisms closely related to those from consortia dechlorinating anthropogenic compounds, such as PCBs, appeared to thrive as these chloroxanthones were dechlorinated. Most of the sequences enriched during the dechlorination of 2,7-dichloroxanthone and 1,3-dihydroxyl-5,7-dichloroxanthone were deeply branching members of the *Clostridia* class of the *Firmicutes* phylum. Although it is unknown if any of these strains are dechlorinators, their common occurrence in dechlorination cultures suggest that either they are indeed capable of dechlorination, or they have a symbiotic relationship with dechlorinators.

- As suggested above, chlorinated xanthones are one class of natural organochlorines that are actively dechlorinated in uncontaminated soils and sediments and are presumed to serve as a terminal electron acceptor for halorespiring bacteria. Both 2,7-dichloroxanthone and 1,3-dihydroxyl-5,7-dichloroxanthone were dechlorinated in anaerobic batch cultures inoculated with different soils and sediments, and the dechlorination products xanthone and 1,3-dihydroxylxanthone were produced.

- In lake sediments, putatively halorespiring *Chloroflexi* were correlated to the number of *Bacteria* and negatively correlated to the amount of dissolved sulfur present (Spearman's ρ=-0.46, P<0.001). Dissolved sulfur actually appeared to act as a switch at about 1 mM in the porewater or 0.1 mM in the lake water column, where the number of putatively halorespiring *Chloroflexi*, as well as the ratio of *Chloroflexi* to *Bacteria*, was negatively impacted by higher sulfur concentrations.

- In low sulfur lakes, the quantity of both *Bacteria* (16S rRNA genes) and the putatively-halorespiring *Chloroflexi* (16S rRNA genes) correlated strongly to the organic matter content of the samples (measured as mass loss on ignition at 550°C) (Spearman's ρ=0.77 $P<0.001$, ρ<0.74 $P<0.001$, Figure 2). This correlation did not exist in the samples containing high (>1 mM) sulfur. Thus, sulfur content impacts the correlation of putatively halorespiring *Chloroflexi* to organic matter in sediments.

- The diversity of the class *Dehalococcoidetes* was statistically higher (Student t-test $P<0.001$) in low sulfur sediments than higher sulfur sediments, signifying that sulfur impacts the diversity of halorespirers as well.

6.2 Recommendations.

A better understanding of the natural niche of halorespiring organisms may lead to better strategies and new technologies for bioremediation. The quantity, and type, of organic chlorine, as well as the amount of sulfur present in an environment, may impact the ease with which *in situ* bioremediation is applied. Additionally, the discovery of natural organochlorines, which have the potential to be non-toxic, may lead to new technologies in which halopriming may stimulate the *in situ* dechlorination of contaminants. The greatest need for this sort of stimulation may be at PCB-contaminated sites where dechlorination is often incomplete and slow. Of interest would be non-toxic natural organochlorines that can stimulate the same reductive dehalogenase genes used for the dechlorination of PCBs and dioxins. Future research is needed to determine which reductive dehalogenase genes can dechlorinate specific anthropogenic contaminants such as PCBs, and whether any natural organohalogens (and which ones) stimulate these genes. The similar structure of xanthones to PCBs suggests that they may be promising haloprimers.

On a more global scale, these organisms also impact chlorine, and possibly carbon cycling. Others have found that natural organochlorines are widely produced and present at high concentrations in the environment. Organochlorines have also been found to be recalcitrant in the environment. The results of the fifth chapter of this thesis, in which the

degradation of *non-chlorinated* xanthones occurred upon the dechlorination of their parent compounds, suggest that the process of dechlorination may serve as a modulator of the entrance of organic carbon into the anaerobic carbon cycle. While the pool of organochlorine is generally not explicitly included in carbon cycling models, the research contained herein suggests that this pool may be relevant, especially in environments where natural halorespirers may be inhibited in some way (such as via the addition of sulfur from agriculture). In models of the chlorine cycle, the pool of organochlorines have been shown to be significant, with the fluxes out of this pool presumed to be leaching and chemical/biological dechlorination. This dissertation provides, for the first time, evidence of the dechlorination of natural organochlorines via halorespiring organisms. Research on the role of halorespirers in the carbon cycle and further research into what controls their function in the chlorine cycle are needed.

6.3 Future Work.

This dissertation presents the opportunity for further data analysis and continuing work. First, data on the abundance and diversity of reductive dehalogenase genes may be gathered on the DNA extracts gathered from the soils in Chapters 3 and 4. Because of the geochemical data gathered for these papers, the geochemical pressures on these genes can be analyzed statistically as well. In fact, research has begun to identify the reductive dehalogenase genes from the DNA extracts from Chapter 3 (see Appendix D). Additionally, with DNA extracts, as well as RNA extracts, gathered from the reactors in Chapter 5, the presence and expression of reductive dehalogenase genes during the dechlorination of chlorinated xanthones will be studied. With these DNA extracts, further

analysis on the community structure of the chlorinated xanthone dechlorinating cultures may be performed as well. Specifically, these extracts may be probed to determine which, if any known halorespirers (*i.e.*, *Dehalobacter* or *Dehalococcoides*) are present. Finally, with clone library analysis, the phylogenetic relationship of the sequences identified by the DGGE method may be better studied. Sequences from putatively dechlorinating bacteria may then be quantified with qPCR analysis, and tracked over the life of those reactors to specifically tie growth to dechlorination, as was accomplished in Chapter 3.

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Appendix A. Supporting Information for Chapter 3.

Analysis of Specificity of qPCR Primers. The specificity of the primers used for the quantification of putatively-halorespiring *Chloroflexi* was verified with cloning and sequencing of the amplified qPCR products. Cloning was performed with the PGEM-T Easy Vector System II (Promega). Sequencing was performed at the University of Minnesota Biogenomics Medical Center using an ABI PRISM™ 3730xl DNA Analyzer. Plasmids were sequenced in both directions with M13 primers. The forward and reverse directions for each sequence were aligned and the quality of sequence data was checked with Sequence Scanner v1.0 software (Applied Biosystems). Reported sequences are the consensus of bi-directional sequence information. Thirteen unique clone sequences were obtained out of 42 clones analyzed, and these were aligned with sequences obtained from BLAST along the 86 bp between the *Chloroflexi* primers, using the ClustalW alignment with the software Mega4 *(4)*. The evolutionary history was inferred using the Neighbor-Joining method *(3)*. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches *(1)*. Values of less than 50 percent agreement are not shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method *(5)* and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). The tree was not manually rooted or structurally manipulated and the final topology is the result of the consensus

tree. Phylogenetic analyses were conducted in MEGA 4 *(4)*. The subphyla *Chloroflexi* and *Thermomicrobia*, as defined previously *(2)*, are shown with respect to the known halorespiring and putatively-halorespiring *Chloroflexi*.

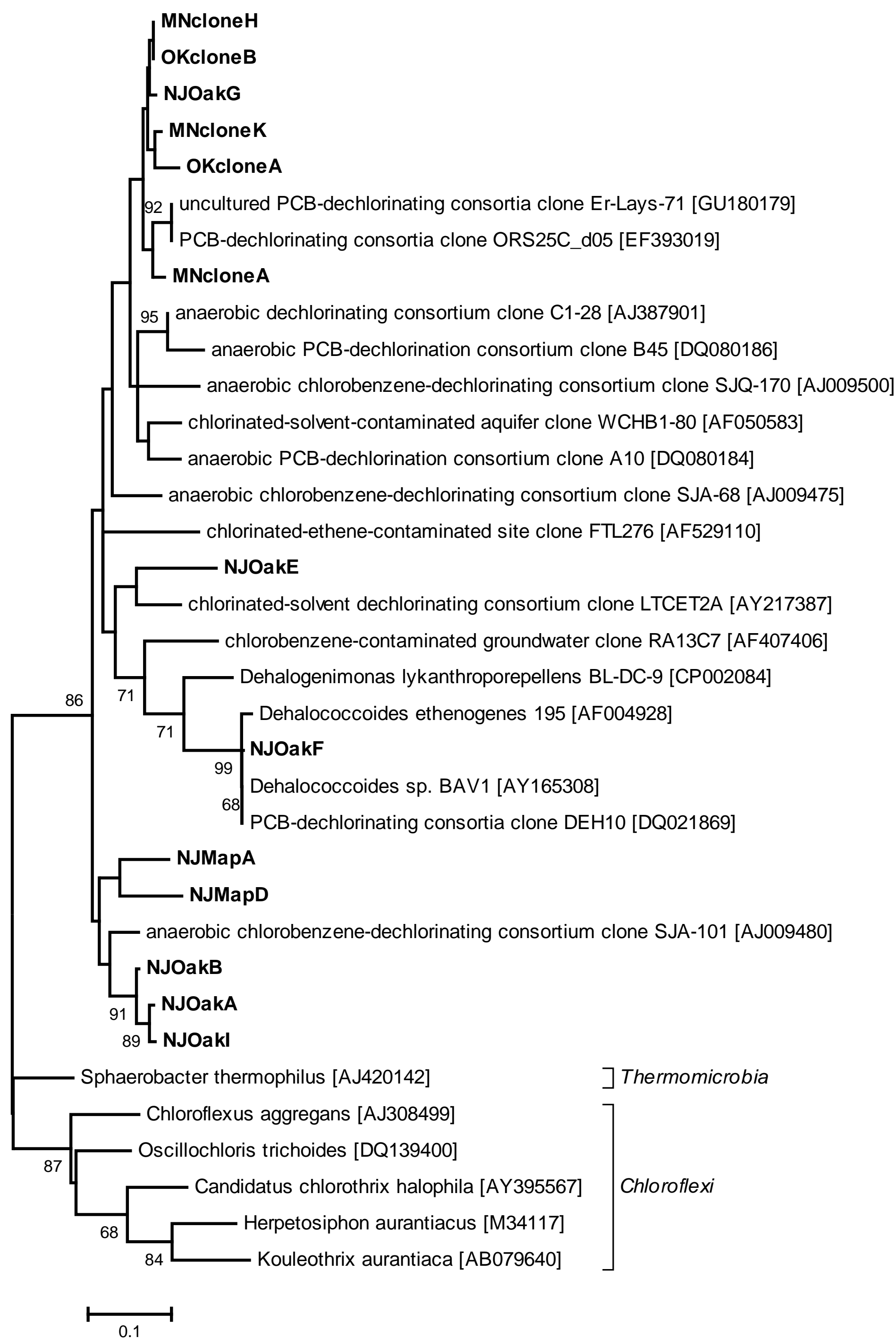

FIG. A.1. Phylogenetic tree of sequences amplified with the qPCR method targeting the putatively-halorespiring *Chloroflexi*, performed to assure the specificity of the primers used in this study. The subphyla *Thermomicrobia* and *Chloroflexi* are shown as defined previously *(Hugenholtz and Stackebrandt, 2004)* with the putatively-halorespiring branch of the phylum *Chloroflexi*. Clone sequences from this study are in bold.

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Appendix B. Supporting Information for Chapter 4.

Table B.1. Longitude and latitude of sampling location for the lakes sampled in this study.

Lake, County, State	Latitude	Longitude
Salt Lake, Lac Qui Parle County, MN	44° 57' 51.3606"	-96° 26' 7.0254"
Dry Lake, Codington County, SD	45° 2' 3.6636"	-97° 18' 43.2072"
Waubay Lake, Day County, SD	45° 28' 18.4038"	-97° 23' 11.2554"
Lake Parmley, Edmunds County, SD	45° 26' 30.573"	-98° 43' 56.3802"
Richmond Lake, Brown County, SD	45° 31' 56.3772"	-98° 35' 36.4338"
Sand Lake, Brown County, SD*	45° 41' 28.5108"	-98° 18' 23.1732"
Devils Lake, Ramsey County, ND	48° 4' 3.5076"	-98° 53' 18.744"
Free People's Lake, Benson County, ND	47° 54' 12.024"	-98° 44' 1.017"
Elbow Lake, Benson County, ND	47° 54' 4.9788"	-98° 44' 22.6464"
East Stump Lake, Nelson County, ND	47° 50' 38.958"	-98° 21' 35.0526"
Long Lake, Clearwater County, MN*	47° 16' 58.4286"	-95° 18' 36.6228"
Lake Itasca, Clearwater County, MN*	47° 12' 14.403"	-95° 10' 37.686"
Leech Lake, Cass County, MN	47° 11' 11.8356"	-94° 37' 36.4404"
Lake Winnebigoshish, Cass County, MN	47° 21' 5.1948"	-94° 12' 24.2418"
Moose Lake, Itasca County, MN	47° 23' 35.9628"	-93° 40' 31.281"
Ball Club Lake, Itasca County, MN	47° 22' 54.9624"	-93° 58' 45.7242"
Pelican Lake, Crow Wing County, MN	46° 36' 41.9616"	-94° 9' 29.1996"
Gladstone Lake, Crow Wing County, MN*	46° 28' 22.569"	-94° 13' 56.4744"
Nokay Lake, Crow Wing County, MN	46° 22' 16.9026"	-93° 58' 40.3176"
Mille Lacs Lake, Crow Wing County, MN	46° 15' 9.6186"	-93° 49' 1.887"

*Sediment cores in addition to a grab sample were collected at these sites.

Table B.2. Data from sediment samples.

		Measured with ICP-AES							
		Al	B	Ca	Co	Cr	Cu	Fe	K
		(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)
Lake Itasca Core	0-2 cm	NA	NA	NA	NA	NA	NA	NA	NA
	2-4 cm	NA	NA	NA	NA	NA	NA	NA	NA
	4-6 cm	NA	NA	NA	NA	NA	NA	NA	NA
	6-8 cm	NA	NA	NA	NA	NA	NA	NA	NA
	8-10 cm	NA	NA	NA	NA	NA	NA	NA	NA
	10-12 cm	NA	NA	NA	NA	NA	NA	NA	NA
	12-14 cm	NA	NA	NA	NA	NA	NA	NA	NA
Sand Lake Core	0-2 cm	7737	40	37927	7.96	15.56	20.52	15214	2593
	2-4 cm	NA	NA	NA	NA	NA	NA	NA	NA
	4-6 cm	7892	40	35513	8.25	15.65	21.36	15520	2673
	6-8 cm	NA	NA	NA	NA	NA	NA	NA	NA
	8-10 cm	9516	43	34929	7.91	18.66	21.08	16577	2936
	10-12 cm	NA	NA	NA	NA	NA	NA	NA	NA
	12-14 cm	6796	37	38585	7.46	13.30	21.48	14525	2404
	14-16 cm	9353	44	37397	8.26	17.61	20.62	16276	2819
	16-18 cm	7916	41	39231	8.23	15.41	21.70	16442	2650
	18-20 cm	11382	51	38707	10.29	21.06	21.21	19067	3211
	20-22 cm	NA	NA	NA	NA	NA	NA	NA	NA
	22-24 cm	NA	NA	NA	NA	NA	NA	NA	NA
	24-26 cm	6744	32	30385	6.55	14.51	18.21	13716	2361
	26-28 cm	NA	NA	NA	NA	NA	NA	NA	NA
	28-30 cm	8389	34	30977	6.13	17.33	18.41	13455	2780
Gladstone Lake Core	0-2 cm	NA	NA	NA	NA	NA	NA	NA	NA
	2-4 cm	733	1	478	<3.12	2.10	<1.36	871	45
	4-6 cm	NA	NA	NA	NA	NA	NA	NA	NA
	6-8 cm	1182	2	800	<3.12	2.38	<1.36	1219	67
	8-10 cm	NA	NA	NA	NA	NA	NA	NA	NA
	10-12 cm	3095	3	2231	<3.12	8.40	<1.36	2511	156
	12-14 cm	NA	NA	NA	NA	NA	NA	NA	NA
	14-16 cm	2094	2	1911	<3.12	5.17	<1.36	1785	107
	16-18 cm	NA	NA	NA	NA	NA	NA	NA	NA
	18-20 cm	NA	NA	NA	NA	NA	NA	NA	NA
	20-22 cm	8540	7	6562	<3.12	17.44	3.24	5689	345
	22-24 cm	NA	NA	NA	NA	NA	NA	NA	NA
	24-26 cm	NA	NA	NA	NA	NA	NA	NA	NA
	26-28 cm	923	2	803	<3.12	1.97	<1.36	1001	52
	28-30 cm	NA	NA	NA	NA	NA	NA	NA	NA
Long Lake Core	0-2 cm	NA	NA	NA	NA	NA	NA	NA	NA
	2-4 cm	NA	NA	NA	NA	NA	NA	NA	NA
	4-6 cm	NA	NA	NA	NA	NA	NA	NA	NA
	6-8 cm	NA	NA	NA	NA	NA	NA	NA	NA
	8-10 cm	NA	NA	NA	NA	NA	NA	NA	NA
	10-12 cm	NA	NA	NA	NA	NA	NA	NA	NA
	12-14 cm	NA	NA	NA	NA	NA	NA	NA	NA
	14-16 cm	NA	NA	NA	NA	NA	NA	NA	NA
	16-18 cm	NA	NA	NA	NA	NA	NA	NA	NA
	18-20 cm	NA	NA	NA	NA	NA	NA	NA	NA
	20-22 cm	NA	NA	NA	NA	NA	NA	NA	NA

		Measured with ICP-AES							
		Mg	**Mn**	**Na**	**Ni**	**P**	**Pb**	**S**	**Zn**
		(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)
Lake Itasca Core	0-2 cm	NA	NA	NA	NA	NA	NA	NA	NA
	2-4 cm	NA	NA	NA	NA	NA	NA	NA	NA
	4-6 cm	NA	NA	NA	NA	NA	NA	NA	NA
	6-8 cm	NA	NA	NA	NA	NA	NA	NA	NA
	8-10 cm	NA	NA	NA	NA	NA	NA	NA	NA
	10-12 cm	NA	NA	NA	NA	NA	NA	NA	NA
	12-14 cm	NA	NA	NA	NA	NA	NA	NA	NA
Sand Lake Core	0-2 cm	9788	1026	831	23.72	980	15.64	7408	63
	2-4 cm	NA	NA	NA	NA	NA	NA	NA	NA
	4-6 cm	10021	957	878	24.77	892	16.80	8220	67
	6-8 cm	NA	NA	NA	NA	NA	NA	NA	NA
	8-10 cm	10386	831	866	27.58	798	14.83	9329	70
	10-12 cm	NA	NA	NA	NA	NA	NA	NA	NA
	12-14 cm	9776	1000	781	26.14	811	14.66	9044	64
	14-16 cm	10356	1056	835	26.47	823	15.16	8741	67
	16-18 cm	9839	1029	858	26.19	840	18.51	9786	68
	18-20 cm	10253	1035	1024	29.75	871	15.71	9769	74
	20-22 cm	NA	NA	NA	NA	NA	NA	NA	NA
	22-24 cm	NA	NA	NA	NA	NA	NA	NA	NA
	24-26 cm	10144	705	803	23.67	748	<14.08	4491	61
	26-28 cm	NA	NA	NA	NA	NA	NA	NA	NA
	28-30 cm	10754	619	845	23.63	721	<14.08	1917	64
Gladstone Lake Core	0-2 cm	NA	NA	NA	NA	NA	NA	NA	NA
	2-4 cm	283	8	78	<2.56	<29.6	<14.08	28	<1.12
	4-6 cm	NA	NA	NA	NA	NA	NA	NA	NA
	6-8 cm	332	11	203	<2.56	41	<14.08	36	2
	8-10 cm	NA	NA	NA	NA	NA	NA	NA	NA
	10-12 cm	797	21	86	3.68	127	<14.08	158	6
	12-14 cm	NA	NA	NA	NA	NA	NA	NA	NA
	14-16 cm	607	15	78	<2.56	112	<14.08	194	4
	16-18 cm	NA	NA	NA	NA	NA	NA	NA	NA
	18-20 cm	NA	NA	NA	NA	NA	NA	NA	NA
	20-22 cm	1941	34	319	7.67	241	<14.08	663	13
	22-24 cm	NA	NA	NA	NA	NA	NA	NA	NA
	24-26 cm	NA	NA	NA	NA	NA	NA	NA	NA
	26-28 cm	328	9	220	<2.56	43	<14.08	57	2
	28-30 cm	NA	NA	NA	NA	NA	NA	NA	NA
Long Lake Core	0-2 cm	NA	NA	NA	NA	NA	NA	NA	NA
	2-4 cm	NA	NA	NA	NA	NA	NA	NA	NA
	4-6 cm	NA	NA	NA	NA	NA	NA	NA	NA
	6-8 cm	NA	NA	NA	NA	NA	NA	NA	NA
	8-10 cm	NA	NA	NA	NA	NA	NA	NA	NA
	10-12 cm	NA	NA	NA	NA	NA	NA	NA	NA
	12-14 cm	NA	NA	NA	NA	NA	NA	NA	NA
	14-16 cm	NA	NA	NA	NA	NA	NA	NA	NA
	16-18 cm	NA	NA	NA	NA	NA	NA	NA	NA
	18-20 cm	NA	NA	NA	NA	NA	NA	NA	NA
	20-22 cm	NA	NA	NA	NA	NA	NA	NA	NA

		TOC - LOI	Measured with QPCR		Pore Water Concentrations determined by IC					
			Log *Bacteria*	Log *Chloroflexi*	F^-	Cl^-	Br^-	NO_3^-	PO_4^{-2}	SO_4^{-2}
		Percent	(per dry g)	(per dry g)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)
Lake Itasca Core	0-2 cm	3.07	11.24	9.98	BDL	0.20	0.018	0.064	BDL	0.36
	2-4 cm	2.92	11.00	9.82	BDL	0.23	0.030	0.165	0.057	0.35
	4-6 cm	1.18	10.97	9.81	0.033	0.15	0.017	0.064	BDL	0.45
	6-8 cm	1.74	10.79	9.55	0.082	0.25	0.026	0.077	BDL	0.53
	8-10 cm	0.90	10.93	9.62	BDL	0.03	0.003	0.012	0.008	0.01
	10-12 cm	0.33	10.24	9.05	BDL	0.15	0.016	0.046	BDL	0.13
	12-14 cm	0.33	10.06	8.62	0.035	0.25	0.010	0.038	0.025	0.44
Sand Lake Core	0-2 cm	13.03	10.86	9.70	0.002	1.58	0.009	0.031	0.011	15.24
	2-4 cm	11.41	10.39	9.12	BDL	1.81	0.039	0.053	0.022	3.02
	4-6 cm	14.08	10.67	9.44	0.008	2.21	0.025	0.030	0.016	10.41
	6-8 cm	12.85	9.80	8.24	0.006	2.20	0.023	0.036	0.028	19.27
	8-10 cm	12.50	9.18	7.11	0.005	1.92	0.003	0.029	0.014	15.55
	10-12 cm	12.43	8.74	BDL	0.013	1.58	0.024	0.034	0.014	8.30
	12-14 cm	12.27	8.75	BDL	0.006	1.56	0.007	0.033	0.011	6.79
	14-16 cm	12.79	11.11	9.85	0.007	1.75	0.009	0.030	0.015	5.68
	16-18 cm	12.88	9.42	BDL	0.009	1.76	0.007	0.030	0.011	7.92
	18-20 cm	13.72	10.03	8.45	0.008	1.88	0.030	0.037	0.020	3.56
	20-22 cm	12.59	9.03	6.99	0.003	2.15	0.007	0.035	0.012	6.13
	22-24 cm	12.31	8.49	BDL	0.002	2.63	0.035	0.041	0.019	10.00
	24-26 cm	11.09	8.23	BDL	0.005	0.22	0.005	0.016	0.010	2.11
	26-28 cm	11.76	8.50	BDL	0.020	0.78	0.011	0.052	0.021	4.75
	28-30 cm	7.68	9.07	7.23	0.020	0.47	0.011	0.017	0.008	2.60
Gladstone Lake Core	0-2 cm	0.21	9.83	7.84	0.034	0.14	BDL	0.071	0.028	0.10
	2-4 cm	0.23	9.99	8.18	0.045	0.11	0.014	0.061	0.000	0.06
	4-6 cm	0.39	9.99	8.10	0.035	0.10	0.005	0.033	0.004	0.04
	6-8 cm	0.79	10.81	9.24	0.011	0.11	BDL	0.079	BDL	0.09
	8-10 cm	2.06	11.36	9.84	0.022	0.15	BDL	0.066	BDL	0.08
	10-12 cm	3.23	11.08	9.59	0.057	0.17	0.040	0.354	BDL	0.28
	12-14 cm	3.05	11.07	9.72	0.026	0.12	0.009	0.132	BDL	0.10
	14-16 cm	4.65	11.15	9.82	0.034	0.30	BDL	0.203	BDL	0.20
	16-18 cm	11.85	11.41	10.01	0.058	0.17	0.007	0.189	0.047	0.15
	18-20 cm	25.81	11.92	10.17	0.012	0.12	0.000	0.061	BDL	0.11
	20-22 cm	8.83	10.14	8.14	0.072	0.17	0.015	0.088	0.025	0.10
	22-24 cm	3.67	11.03	9.52	0.003	0.10	BDL	0.008	BDL	0.02
	24-26 cm	0.83	10.49	9.02	0.007	0.10	0.006	0.011	BDL	0.01
	26-28 cm	3.59	10.10	8.95	0.009	0.08	0.003	0.016	0.013	0.02
	28-30 cm	0.62	10.29	8.78	0.006	0.12	0.006	0.013	0.006	0.03
Long Lake Core	0-2 cm	0.55	11.19	9.38	BDL	0.51	0.016	0.059	0.068	0.65
	2-4 cm	0.41	10.50	9.07	0.037	0.45	0.017	0.046	0.036	0.55
	4-6 cm	0.39	10.17	8.75	BDL	0.69	0.032	0.109	0.098	0.21
	6-8 cm	0.38	9.82	8.37	BDL	0.68	0.015	0.039	0.014	0.08
	8-10 cm	0.37	9.85	8.38	0.047	0.58	0.028	0.089	0.048	0.08
	10-12 cm	0.43	9.58	8.09	0.020	0.57	0.014	0.035	0.012	0.06
	12-14 cm	0.37	9.70	8.45	0.036	0.65	0.023	0.064	BDL	0.08
	14-16 cm	0.39	9.90	8.46	0.000	0.59	0.020	0.041	0.220	0.26
	16-18 cm	0.32	9.64	8.26	0.035	0.58	0.012	0.035	0.020	0.16
	18-20 cm	0.58	9.85	8.33	0.018	0.50	0.014	0.025	0.021	0.14
	20-22 cm	0.52	9.56	8.15	0.059	1.37	0.043	0.081	BDL	0.39

		Measured with ICP-AES							
		Al	B	Ca	Co	Cr	Cu	Fe	K
		(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)
Grab Samples	Salt	995	11	36102	<3.12	3.61	3.82	2311	295
	Waubay	2704	17	52281	6.22	8.52	8.09	8285	747
	Dry	800	3	9325	<3.12	2.29	<1.36	2089	183
	Parmley	8439	32	16424	29.34	11.21	33.95	8965	2869
	Richmond	4354	24	41402	6.89	10.23	11.42	11939	1624
	Sand	6134	34	35679	9.64	12.71	19.77	13155	2254
	Devils	4588	29	20775	5.57	10.40	9.11	8904	1667
	Free People's	1942	17	21348	4.68	5.21	2.13	5593	850
	Elbow	2407	13	16581	4.64	5.64	3.67	6198	841
	Stump	4333	27	41242	7.14	8.32	11.81	11596	1559
	Long	1236	3	4986	3.46	4.08	<1.36	2173	174
	Itasca	1773	6	4276	<3.12	4.71	<1.36	2942	256
	Leech	2715	15	52188	<3.12	5.32	11.71	4879	477
	Winnebigoshish	961	3	1565	<3.12	2.09	<1.36	2054	88
	Moose	657	2	6019	<3.12	1.95	<1.36	1326	74
	Ball Club	1105	4	8157	<3.12	3.83	<1.36	2531	135
	Pelican	3465	36	166230	<3.12	8.33	11.22	18592	718
	Gladstone	2402	3	1677	<3.12	6.75	<1.36	2049	147
	Nokay	2716	11	10859	4.92	10.36	6.53	8598	225
	Mille Lacs	4930	14	4669	8.18	17.08	6.57	10737	566
		Mg	Mn	Na	Ni	P	Pb	S	Zn
		(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)	(ppm)
Grab Samples	Salt	5681	137	1059	5.93	353	<14.08	780	8
	Waubay	8982	794	336	16.16	377	<14.08	120	22
	Dry	2542	179	468	3.06	173	<14.08	149	5
	Parmley	4530	6967	1316	102.87	217	24.07	127	76
	Richmond	12466	4103	670	20.75	460	<14.08	532	38
	Sand	9805	889	958	22.67	905	14.80	7612	61
	Devils	6121	481	865	14.41	556	<14.08	2121	35
	Free People's	5757	436	1103	6.84	414	<14.08	715	16
	Elbow	4705	554	464	9.22	428	<14.08	159	18
	Stump	8513	1159	1004	22.41	439	<14.08	394	36
	Long	1336	25	204	4.23	135	<14.08	56	8
	Itasca	1183	44	164	2.82	161	<14.08	96	8
	Leech	2380	82	231	5.17	624	15.61	4498	28
	Winnebigoshish	575	20	184	<2.56	172	<14.08	94	4
	Moose	1632	36	153	<2.56	210	<14.08	199	4
	Ball Club	2869	36	511	3.33	283	<14.08	49	6
	Pelican	4142	717	401	8.47	748	14.43	2276	33
	Gladstone	618	18	239	<2.56	110	<14.08	86	5
	Nokay	2287	631	361	8.49	215	<14.08	507	14
	Mille Lacs	3195	142	335	12.70	359	<14.08	352	27

		Measured with QPCR		Pore Water Concentrations determined by IC					
		Log *Bacteria*	Log *Chloroflexi*	F^-	Cl^-	Br^-	NO_3^-	PO_4^{-2}	SO_4^{-2}
		(per dry g)	(per dry g)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)
Grab Samples	Salt	10.63	8.69	0.016	13.00	0.068	0.116	BDL	46.84
	Waubay	9.33	5.84	0.002	0.46	0.004	0.044	0.012	5.74
	Dry	11.73	8.56	0.001	0.44	0.001	0.054	0.029	8.91
	Parmley	8.38	5.10	0.003	2.01	0.022	0.048	0.011	3.21
	Richmond	8.05	4.55	0.004	1.79	0.024	0.136	0.060	3.25
	Sand	9.90	7.93	0.001	1.78	0.009	0.038	0.016	5.35
	Devils	9.24	6.48	0.009	4.23	0.054	0.118	0.023	8.34
	Free People's	11.76	9.39	0.021	5.73	0.068	0.105	BDL	15.29
	Elbow	11.92	9.30	BDL	1.12	BDL	0.240	BDL	0.39
	Stump	9.80	5.70	0.013	8.51	0.018	0.166	0.048	18.39
	Long	10.04	8.59	0.009	1.28	0.000	0.086	0.048	0.73
	Itasca	10.84	9.81	0.021	0.08	0.012	0.030	BDL	0.03
	Leech	11.73	10.93	0.003	0.09	0.012	0.046	0.025	0.05
	Winnebigoshish	10.48	9.41	0.017	0.14	0.000	0.063	BDL	0.24
	Moose	9.73	9.01	0.037	0.33	0.018	0.093	0.294	0.87
	Ball Club	10.03	8.87	0.037	0.17	0.014	0.070	0.022	0.11
	Pelican	11.79	10.83	0.004	0.20	0.000	0.061	BDL	0.09
	Gladstone	10.63	9.24	0.001	0.24	0.008	0.011	0.008	0.03
	Nokay	10.50	9.56	0.001	0.10	0.007	0.019	0.008	0.04
	Mille Lacs	11.02	9.83	0.011	0.10	0.005	0.010	0.003	0.33

		Lake Water Concentrations determined by IC						Specific		
		F^-	Cl^-	Br^-	NO_3^-	PO_4^{-2}	SO_4^{-2}	Conductivity	pH	TOC - LOI
		(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(μS/cm)		Percent
Grab Samples	Salt	BDL	23.69	0.403	0.114	BDL	79.34	77000	8.35	0.74
	Waubay	BDL	0.57	0.015	0.116	BDL	11.36	1377	8.26	1.00
	Dry	0.003	0.50	BDL	0.020	0.019	10.01	1743	8.4	16.10
	Parmley	0.006	1.72	0.012	0.028	0.050	2.95	924	8.76	2.23
	Richmond	0.006	1.61	0.041	0.002	0.020	3.41	987	9.2	1.79
	Sand	0.007	1.64	0.077	0.100	0.003	5.69	1557	7.79	11.77
	Devils	0.008	3.91	0.091	0.029	BDL	9.05	16700	8.93	9.67
	Free People's	BDL	12.15	BDL	0.050	BDL	20.03	40100	9.34	1.60
	Elbow	0.010	1.10	0.051	0.004	BDL	0.48	1146	9.04	1.21
	Stump	BDL	10.21	0.228	0.044	BDL	35.57	37700	8.96	3.38
	Long	0.003	0.27	0.016	0.002	BDL	0.02	156.3	8.44	0.56
	Itasca	0.004	0.02	0.007	0.010	0.002	0.01	144	7.98	1.04
	Leech	0.004	0.10	0.011	0.002	0.003	0.02	145.2	8.17	25.96
	Winnebigoshish	0.003	0.13	0.011	0.003	0.001	0.03	138.8	7.95	0.41
	Moose	0.004	0.05	0.008	BDL	BDL	0.02	118.5	8.47	0.65
	Ball Club	0.003	0.07	0.007	0.001	BDL	0.02	126.4	7.93	0.34
	Pelican	0.005	0.12	0.009	0.001	0.001	0.01	87.7	7.46	29.91
	Gladstone	0.004	0.10	0.007	0.001	0.003	0.00	129.5	7.66	2.22
	Nokay	0.005	0.05	0.008	0.003	BDL	0.03	113	8.36	1.21
	Mille Lacs	0.005	0.23	0.012	0.070	0.003	0.06	126.4	7.52	3.34

TRFLP fragments by clone library analysis. To match the TRFs from the TRFLP analysis to the *Dehalococcoidetes* class or other *Chloroflexi* classes, clone libraries were constructed from the amplification products of Dry Lake, Sand Lake, and Leech Lake. From these three clone libraries, a total of 11 TRFs were identified out of 29 total TRFs from the TRFLP analysis. Partial clone libraries from an additional four samples (grab samples from Salt Lake, Long Lake, Devils Lake, and Ball Club Lake) produced a classification of a total of 4 additional TRFs (60: *Dehalococcoidetes*, and 185, 187, 197: other). A total of 8 TRFs grouped within the *Dehalococcoidetes* class while 7 grouped outside of this class (FIG. B.2). These sequences were aligned *Chloroflexi* sequences obtained from NCBI's BLAST database. Sequences were aligned using the CLUSTAL W alignment tool in MEGA 4.1 software. Tree topology and branch lengths were obtained from a bootstrap analysis (1000 replicates) using the Neighbor-Joining method and Maximum Likelihood Composite method.

Table B.3. Summary of results from clone library analysis TRFLP primers between *Dehalococcoidetes* (*Ddts*) class and other *Chloroflexi* classes (Other)

	Number of Sequences		Number of unique sequences		Fragments Identified	
	Ddts	Other	*Ddts*	Other	*Ddts*	Other
Dry Lake	5	47	2	2	269	266
Sand Lake	3	55	1	29	269	133, 266, 296
Leech Lake	60	40	54	18	154, 178, 193, 268, 269, 270, 283	84, 266

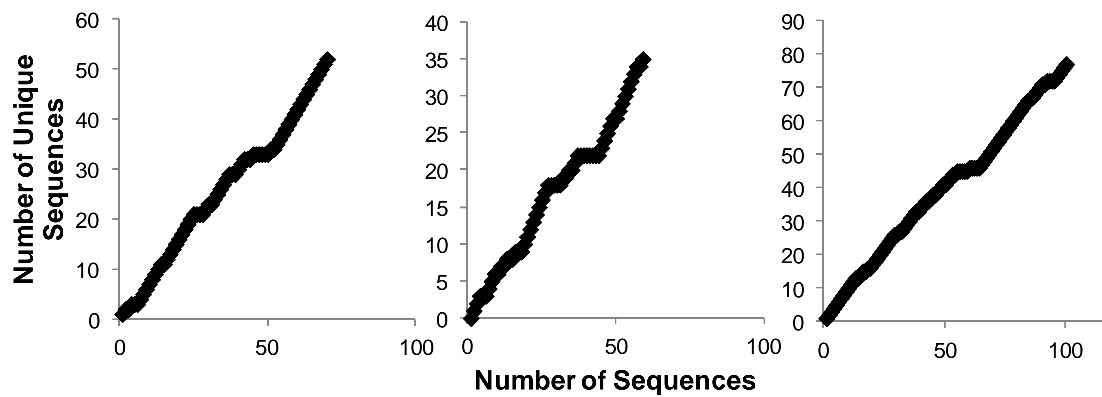


FIG. B.1. Collector's curves showing number of sequences analyzed versus number of unique sequences for Dry Lake sediment, Sand Lake sediment, and Leech Lake sediment.

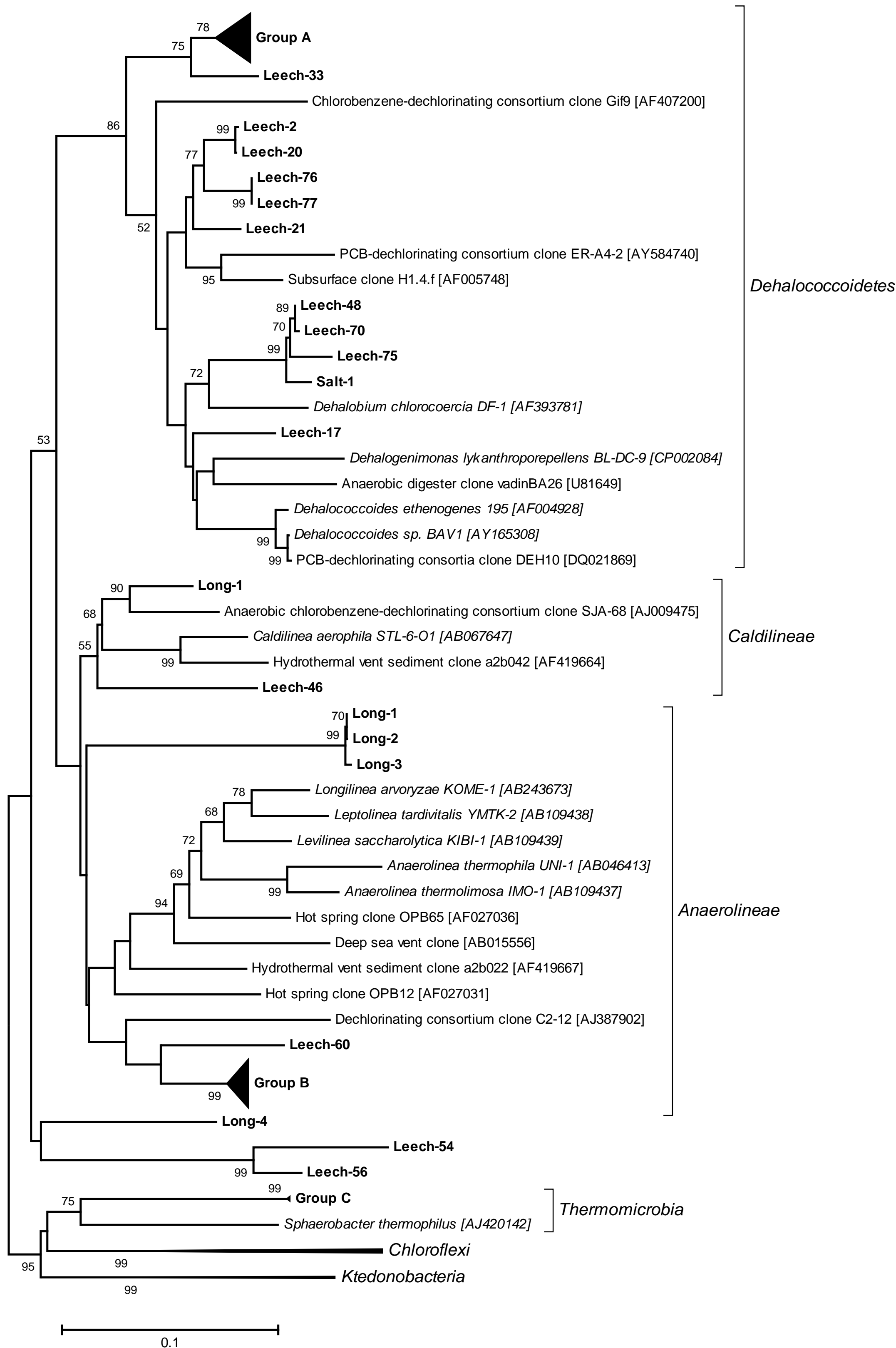
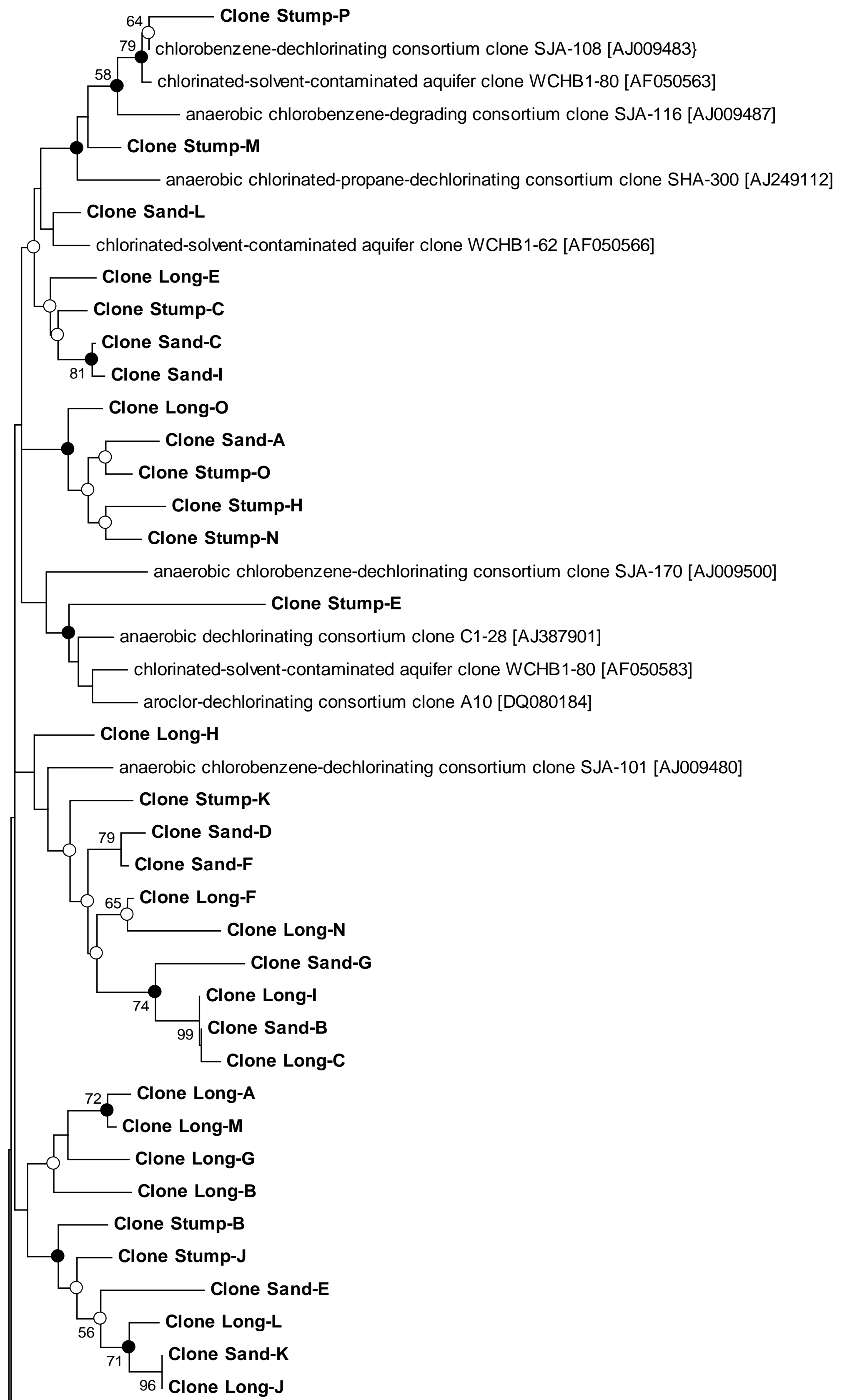


FIG. B.2. Phylogenetic tree of sequences from clone libraries show the relationship of

the sequences analyzed in the TRFLP method within the *Chloroflexi*. Group A, B, and C contain 47, 42 and 5 unique sequences, respectively. The evolutionary history was inferred using the Neighbor-Joining method *(*Santou and Nei, 1987*)* and the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). Values at the nodes indicate percentage agreement from bootstrap analysis (1000 replicates) when above 50 percent *(*Dopazo, 1994; Rzhetsky and Nei, 1992*)*. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). Phylogenetic analyses were conducted in MEGA4 (Tumara et al, 2004).

Verification of qPCR amplification. To test the specificity of the qPCR amplification, clone libraries were constructed from the amplification products of three samples: the 2-4 cm depth section of the sediment core from Sand Lake, SD, the 2-4 cm depth section of the sediment core from Long Lake, MN, and the grab sediment section from East Stump Lake, ND. Out of 48 clones sequenced, 45 unique sequences were obtained. These sequences were aligned with known halorespiring *Chloroflexi* and with putative halorespiring clones obtained from NCBI's BLAST database, as well as three non-halorespiring *Chloroflexi*. Sequences were aligned using the CLUSTAL W alignment tool in MEGA 4.1 software. Tree topology and branch lengths were obtained from a bootstrap analysis (1000 replicates) using the Neighbor-Joining method and Maximum Likelihood Composite method. The tree topology was not rooted or manually manipulated. Because of the small number of bp in the analysis (~90 bp), bootstrap values were generally weak. Thus, the Interior Branch test (1000 replicates) was performed to obtain a second statistical analysis on the tree topology. Both statistics are shown on the tree. All 45 sequences grouped among the 16S rRNA sequences from the halorespiring and putatively-halorespiring *Chloroflexi*.


Clone Stump-P
64
79
chlorobenzene-dechlorinating consortium clone SJA-108 [AJ009483}
58
chlorinated-solvent-contaminated aquifer clone WCHB1-80 [AF050563]
anaerobic chlorobenzene-degrading consortium clone SJA-116 [AJ009487]
Clone Stump-M
anaerobic chlorinated-propane-dechlorinating consortium clone SHA-300 [AJ249112]
Clone Sand-L
chlorinated-solvent-contaminated aquifer clone WCHB1-62 [AF050566]
Clone Long-E
Clone Stump-C
Clone Sand-C
81
Clone Sand-I
Clone Long-O
Clone Sand-A
Clone Stump-O
Clone Stump-H
Clone Stump-N
anaerobic chlorobenzene-dechlorinating consortium clone SJA-170 [AJ009500]
Clone Stump-E
anaerobic dechlorinating consortium clone C1-28 [AJ387901]
chlorinated-solvent-contaminated aquifer clone WCHB1-80 [AF050583]
aroclor-dechlorinating consortium clone A10 [DQ080184]
Clone Long-H
anaerobic chlorobenzene-dechlorinating consortium clone SJA-101 [AJ009480]
Clone Stump-K
79
Clone Sand-D
Clone Sand-F
65
Clone Long-F
Clone Long-N
Clone Sand-G
Clone Long-I
74
Clone Sand-B
99
Clone Long-C
72
Clone Long-A
Clone Long-M
Clone Long-G
Clone Long-B
Clone Stump-B
Clone Stump-J
Clone Sand-E
56
Clone Long-L
Clone Sand-K
71
96
Clone Long-J

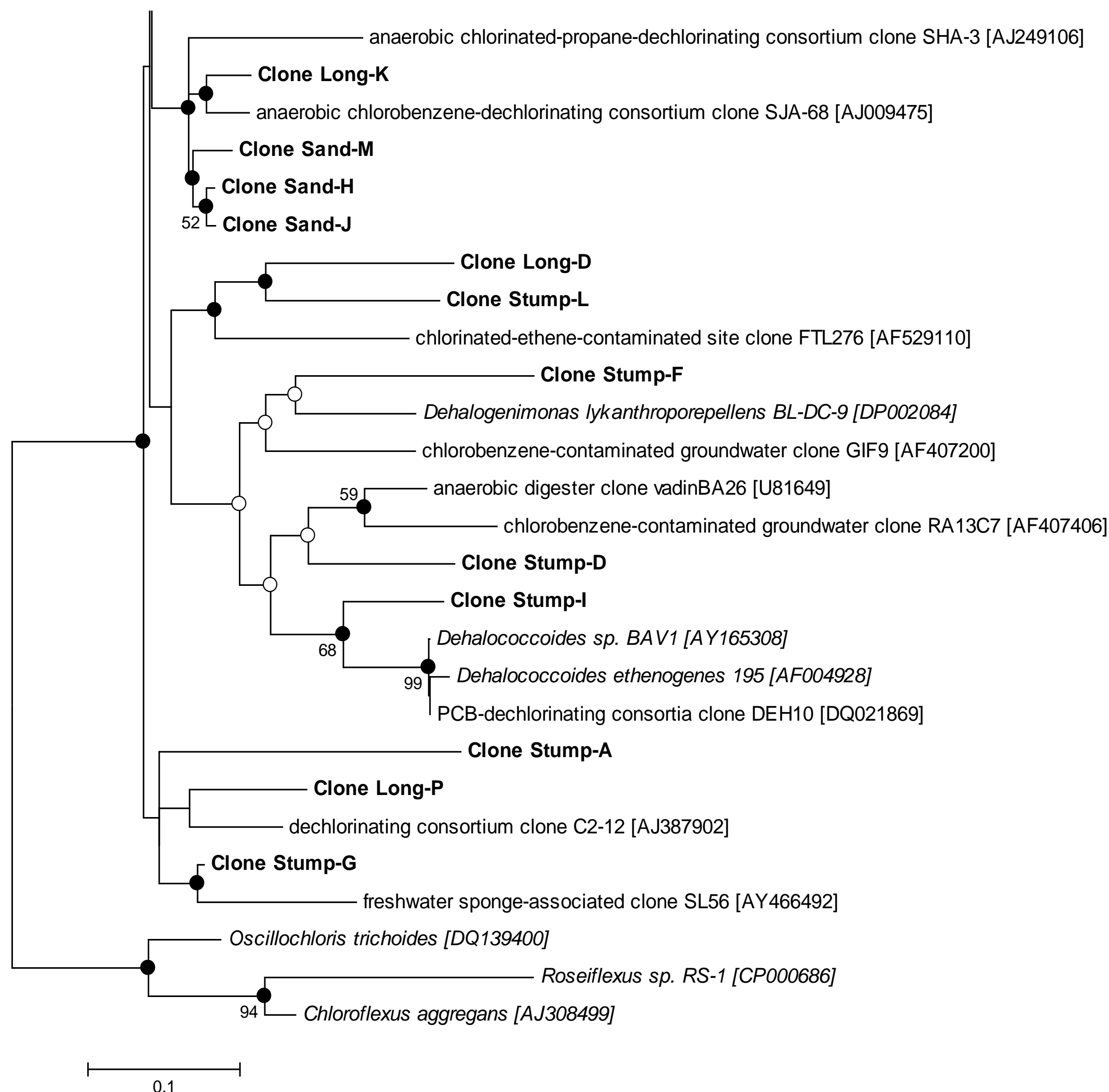


FIG. B.3. Relationship of clones obtained from qPCR amplification using the *Chloroflexi* primers 1150F and 1286R in this study. The evolutionary history was inferred using the Neighbor-Joining method (Santou and Nei, 1987) and the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). Values at the nodes indicate percentage agreement from bootstrap analysis (1000 replicates) when above 50 percent (Felsenstein, 1985). Solid circles at nodes indicate between 75-100 percent agreement using the interior branch test (1000 replicates), open circles at nodes indicate between 50-100 percent agreement, and empty nodes indicate less than 50 percent agreement (Dopazo, 1994; Rzhetsky and Nei, 1992). All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 94 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tumara et al, 2004).

Dopazo, J. (1994). Estimating errors and confidence intervals for branch lengths in phylogenetic trees by a bootstrap approach. *J. Mol. Evol.* 38:300-304.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evol.* 39:783-791.

Rzhetsky, A.; Nei, M. (1992). A simple method for estimating and testing minimum evolution trees. *Mol. Biol. Evol.* 9:945-967.

Saitou, N.; Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406-425.

Tamura, K.; Nei, M.; Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *PNAS* (*USA*) 101:11030-11035.

Tamura, K.; Dudley, J.; Nei, M.; Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24:1596-1599.

Appendix C. Supporting Information for Chapter 5.

Sequences obtained from DGGE Analysis.

Tilden-1
TGGGGAATATTGCACAATGGAGGGAACTCTGATGCAGCAACGCCGCGTGGAG
GACGAAGGTTTTCGGATTGTAAACTCCTGTCTTTTGGGACGATAATGACGGTA
CCCAAAGAGGAAGCCCCGGCTAACTACCTG

Tilden-2
TGGGGAATATTGCGCAATGGGGGAAACCCTGACGCAGCGACGCCGCGTGAA
GGAAGAAGGTTTTCGGATTGTAAACTTCTTTGGCCGGGGACGAAAAAAATGA
CGGTACCCGGTTAACAAGCCACGGCTAACTACGTG

Tilden-3
TGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGC
GAAGAAGGCCTTCGGGTTGTAAAGCTCTGTCTGGAGGGACGAACAAGATGAC
GGTACCTCCGGAGGAAGCCACGGCTAACTACGTG

Tilden-4
TGGGGGATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGG
GAAGACGGTCTTCGGATTGTAAACCTCTGTCTTCGGGGACGATAATGACGGT
ACCCGAGGAGGAAGCTCCGCAAGTCGTACGGT

PV-1
CTCCTACGGGTGGGGAATCTTGCGCAATGGGGGAAAGCCTGACGCAGCAACG
CCGCGTGAGTGATGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTAGGGGACGA
AAGTACCCGAGTAAACAGCTTGGGAGATGACGGTACCCTAAGAGGAAGCCC
CGGCTAACTACGTG

PV-2
TGGGGAATATTGCACAATGGAGGAAACTCTGATGCAGCGACGCCGCGTGAGT
GAAGAAGGTCTTCGGATCGTAAAACTCTGTCCTTGGTGAAGAAAAATAATGA
CGGTAGCCAAGGAGGAAGCCCCGGCTAACTACGTG

PV-3
TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGAG
GATGAAGGCCTTCGGGTCGTAAACTCCTGTCTGGGGGGATGAATACTGACAG
TACCCCCGGAGGAAGCCCCGGCTAACTACCTGCCCGCCGCCGCGGTAATAGC
CA

Maple-1
TGGGGAATATTGGGCAATGGGCGAAAGCCTGACCCAGCAACGCCGCGTGAG
GGAAGAAGGTTTTCGGATTGTAAACCTCTGTCGCAGATGACGAAGAAAGTGA
CGGTAATCTGTGAGGAAGCCCCGGCTAACTACGTGCCCG

Maple-2
TGAGGAATATTGGTCAATGGGCGCAAGCCTGAACCAGCCACGTCGCGTGAAG
GAAGACGGCCCTACGGGTTGTAAACTTCTTTTGTAAGGGAATAAAGTGTAGT
ACGTGTACTATTTTGCATGTACCTTACCAATAAGGATCGGCTAACTCCGTG

Maple-3
TGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCAACGCCGCGTGGA
GGACGAAGGTTTTCGGATTGTAAACTCCTGTCTTTTGGGACGATAATGACGGT
ACCAAAAGAGGAAGCCACGGCTAACTACGTG

Maple-4
TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGC
GAAGAAGGTTTTCGGATCGTAAAGCTCCGTCCTTTGGGAAGAAAAAAATGAC
GGTACCAAAGGAGGAAGCCCCGGCTAACTACGTG

Leech-1
TGGGGAATTTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGGGT
GAAGAAGGCCTTCGGGTTGTAAAGCCCTGTCTTATGGGAAGAAGAAAGTGAC
GGTACCATAGGAGGAAGCCACGGCTAACTACGTG

Appendix D. Unfinished Work.

D.1 Reductive Dehalogenases In Uncontaminated Environments.

Brief Description of Work. The purpose of this work was to measure the diversity of putative reductive dehalogenases in the environment with the data gathered for Chapter 3. Briefly, putative reductive dehalogenases were amplified with a previously developed method (Krajmalnik-Brown et al., 2004) for all of the samples collected for the soil cores in that chapter. This amplification produced a lot of non-specific amplification, detected by gel electrophoresis, but amplified products were optimized to remove all bands of non-specific size. DNA extract was subjected to two rounds of PCR, with the second round of PCR containing a fluorescently-labeled primer for TRFLP analysis. PCR products were digested and submitted for fragment analysis. The TRFLP was successfully performed and many TRFs matched the lengths of several reductive dehalogenases used as standards (these standards were developed by Kevin Drees). The work, however, was left unfinished as attempts at verifying the TRFLP method with clone libraries of the putative reductive dehalogenase genes failed to successfully produce verifiable reductive dehalogenase genes. The results of clone library attempts were a diversity of sequences in which the primer, B1R served as both the forward and reverse primer. Furthermore, because of the biasing towards smaller fragments during the cloning procedure, the dominant fraction of these sequences was shorter than the 1500-1700 bp fragments that were optimized by the PCR method. Still, because the primer, B1R was not fluorescently labeled, these non-specific sequences were not analyzed by the TRFLP method. Nevertheless, their dominance of the PCR amplification precluded

the ability to easily obtain the sequences containing the fluorescently labeled primer RRF2. It is thus still unknown if the TRFLP method truly measured the diversity of putative-reductive dehalogenases or not. A method to work around the non-specific amplification and cloning is being discussed currently with Kevin Drees and this work may still lead to a future publication. The methodology and results of the TRFLP technique follows.

TRFLP of putative rdhs. TRFLP was used to analyze the putative rdh gene fingerprint of all samples. A nested PCR reaction was used to increase the amount of amplified PCR product. The PCR reaction was adopted from (Krajmalnik-Brown et al., 2004) but was further optimized to minimize nonspecific amplification as determined by gel electrophoresis. The PCR reaction totaling 30 μL contained 5X Promega PCR Buffer, 1.0 mM $MgCl_2$, 20 μg BSA, 0.2 mM dNTP mix, 500 nM RRF2, 300 nM B1R, 0.4 U GoTaq DNA Polymerase (Promega), and 1 μL template DNA. The PCR temperature protocol was used as previously described (Krajmalnik-Brown et al., 2004) The PCR products from this first reaction were cleaned (GENECLEAN II Kit, Qbiogene) and diluted by 100 fold. A second PCR reaction was performed on these amplicons in the same manner as the first reaction, but with the RRF2 primer labeled with 6-Carboxyfluorescein. The PCR products from the second reaction were cleaned and 6 μL was digested with *RsaI* (Promega). The digested PCR products were sent to the University of Minnesota Biogenomics Medical Center and analyzed on an ABI 3730xl capillary instrument with MapMarker-1000 standard reference dye. Results were analyzed using Peak Scanner Software v1.0 and Microsoft Excel. The areas, heights, and length of all peaks with a height of 5 FU between 50 and 1700 bp were transferred to a

spreadsheet. To separate the true peaks from the background noise, the method of Abdo et al (2006) was adapted. Briefly, real peaks (as opposed to background peaks) were considered to be those with a peak area greater than the average plus three standard deviations of the areas of the remaining (background) peaks. The relative areas for each peak were calculated and used for further analysis. Peaks greater than 1000 bp were discarded, so that all peaks were within the standards of the internal reference dye (MapMarker1000) of the analysis. The trfs of similar lengths were binned manually. The difference between the longest and shortest trf in each bin did not exceed 1.2 nucleotides.

Canoco v4.5 was used to analyze the TRFLP fingerprints. Correspondence Analysis (CA) was used to determine the distribution of data without constraining the axis by environmental variables. Partial Canonical Correspondence Analysis (pCCA) was used to analyze the association of the TRFLP fingerprint to the following environmental variables: the log (*Dehalococcoides*), the log (Bacteria), aliphatic organochlorine (aliphatic-Cl), aromatic organochlorine (aromatic-Cl), inorganic chloride (inorganic-Cl), TOC, cover type (oak, pine, and maple), depth, and sampling event (September 2006 or January 2008). Each variable was tested for significance ($p<0.05$) using Monte Carlo permutation tests (9999 permutations) with all other variables included as covariables. Thus, the significance of each variable was tested after the variation in the data explained by all other variables was removed. Canonical correspondence analysis (CCA) was used with all significant environmental variables to assess the relative strength of each variable to the other variables. The total amount of variance explained by the measured variables was calculated by dividing the total eigenvalues of the canonical relationship with the environmental variables to the total eigenvalues of the unconstrained TRFLP data.

Between the 6 soil cores, a total of 82 samples were used for the CA analysis and the pCCA analysis of the environmental variables, with the exception of the organochlorine variables, for which there were 67 samples. ‘Biplot scaling’ and ‘downweighting of rare species’ were chosen options for all analyses.

Diversity of putative rdhs in uncontaminated soil cores. Canonical analysis was used to analyze the ability of the environmental variables to explain the variation in the TRFLP fingerprints of putative rdh genes. The first step in this analysis was an unconstrained CA of the TRFLP fingerprints. With CA, the variability between samples is determined with only the TRFLP data considered, and thus, the raw TRFLP data can be analyzed. After CA, there is a minor grouping observed according to cover type (FIG D.1), indicating that this particular variable (cover type) may be important. In general, however, there was a lack of strong sample grouping according to any of the environmental variables that were investigated.

To determine which environmental variables were significant ($p<0.05$) in explaining the TRFLP profiles, pCCA was used. This allowed for testing how well a single variable explained the variability of the TRFLP data, and was tested after the variations explained by all other variables was partialed-out of the analysis. Monte Carlo simulation tests indicated that the log(*Bacteria*), the amount of organochlorine, the sampling event, the cover types (oak, pine and maple) and the depth were all significant in their ability to explain the variability in the TRFLP data. Because these variables were tested independently after the variation explained by all other measured variables was factored out, each of these variables explain unique components of the TRFLP fingerprints. The variables of inorganic chloride, TOC and log(*Dehalococcoides*) did not

explain the TRFLP data, according to pCCA. Finally, CCA using all statistically significant variables was performed to analyze the relative strength of the significant variables in explaining the TRFLP data (FIG D2). From the length of the arrows, it is apparent that maple cover, oak cover, organochlorine content, depth, and log(*Bacteria*), all have equally large influences on the variation of the TRFLP profile, while the sampling date and pine coverage have relatively smaller influences.

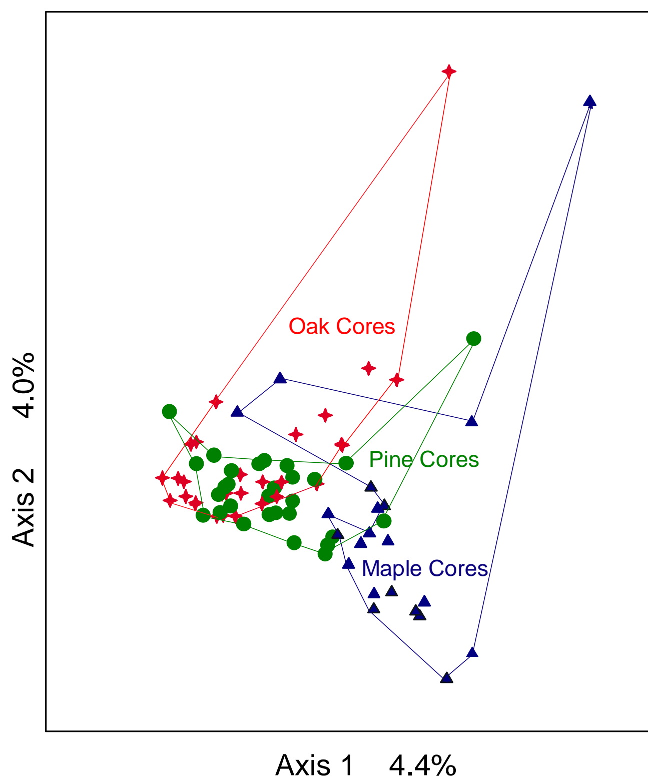


FIG. D.1. An ordination plot of unconstrained CA results for the TRFLP fingerprints of putative rdh genes. The plot shows weak groupings according to cover type. The percentages on the axis correspond to the percentage of the variance of the TRFLP fingerprints explained by the axis; thus only 8.4% of the variation between samples can be represented by this plot.

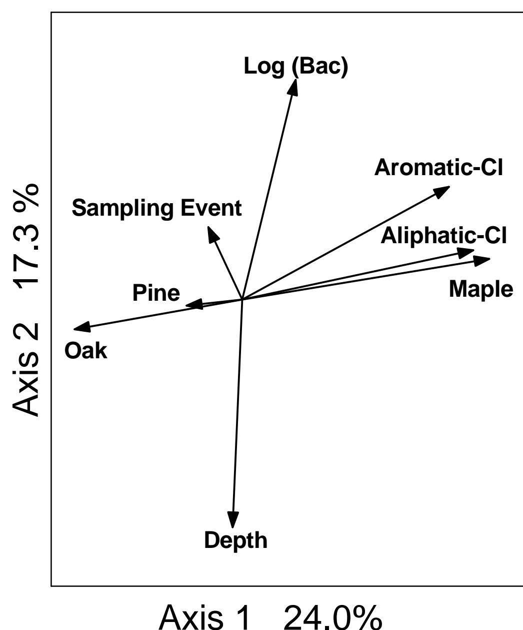


FIG. D.2. Ordination plot of the CCA results. The percentages on the axis correspond to the percentage variance of the environmental-TRFLP fingerprint relationship explained by the axis. Thus, the plot displays 41.3% of the variability in the TRFLP data that can be explained by the environmental variables shown. The length of a given arrow corresponds to the relative strength of that variable in explaining the TRFLP fingerprint variability and the direction indicates the steepest increase of the variable.

Both the CA and CCA of the data show that no single measured variable dominates with respect to its ability to explain the variability in the TRFLP data. It is likely that additional variables not measured in this study, such as pH, trace nutrients, or specific organochlorine moieties, play an important role in selecting for particular rdhs. Indeed, only 26.2 percent of the variance in the TRFLP data can be explained by these measured factors. Further controlled studies would help elucidate the variables that impact rdhs in uncontaminated environments.

D.2 Dechlorination of the anthropogenic chemical trichloroethene.

Brief Description. Many experiments were set-up and maintained to study the dechlorination of trichloroethene (TCE) by fresh, uncontaminated sediment. In short, these reactors were set-up and maintained as the batch reactors in Chapter 5, with the exception of trichloroethene used as an amendment instead of chlorinated xanthones. The amount of TCE was measured with analysis of the headspace with gas chromatography equipped with a photoionization detector (GC-PID), and the amount of TCE was quantified with external standards. This experiment was tried using several different soils as innocula. The results of these experiments, however, were not convincing enough to continue. In short, the decrease of TCE either did not occur, or occurred intermittently. Figure D.3 below shows one set of these experiments. In this set up, the initial spike of 20 mg/L of TCE was initially dechlorinated by the reactor seeded with an uncontaminated soil ("Natural Soil"), but then stalled, was again dechlorinated, and then either stopped dechlorinating or dechlorinated slowly. In comparison, the reactors seeded with TCE-contaminated aquifer contaminated dechlorinated three aliquots of 20 mg/L TCE. The products of dechlorination, cis-dichloroethene, trans-dichloroethene, and vinyl chloride were also all monitored by GC-PID analysis. Although the 'TCE aquifer' reactors produced nearly stoichiometric amounts of *trans*-dichloroethene, and then vinyl chloride, the 'natural soil' reactors had intermittent amounts of dechlorination products. Other attempts at this experiment with uncontaminated soils produced either no dechlorination or similarly intermittent dechlorination.

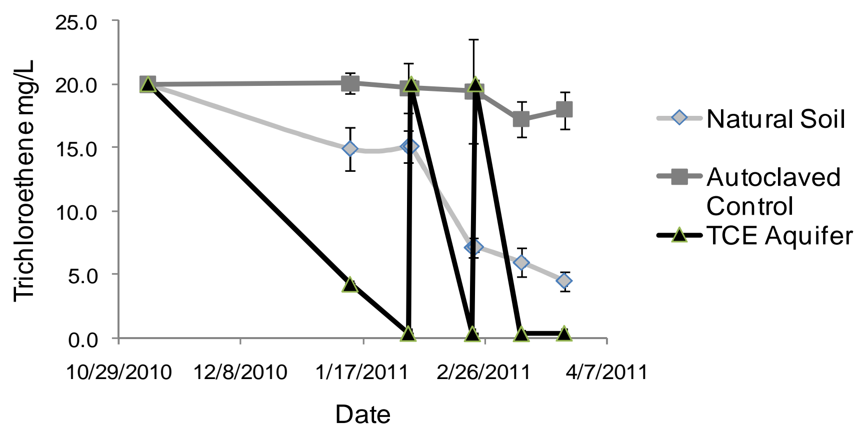


FIG. D.3. The amount of trichloroethene in batch reactors. Error bars indicate measurements from triplicate reactors.

Similarly, experiments with fill-and-draw reactors containing soil from uncontaminated soils were exposed to TCE. A 1-L reactor capped with a Teflon septum was started with the addition of 35 g of uncontaminated soil, 600 mL of anaerobic mineral media, 20 mM each of acetate, butyrate, and propionate, and 0.6 mM of TCE. A control reactor was also set up and treated the same except without TCE added. Every two days, 40 mL of the settled reactor contents is drawn from the reactor and replaced with 40 mL of fresh media, for a hydraulic residence time of 30 days. Approximately 2 μL of neat TCE was added immediately after the media exchange through a Teflon septum so that TCE is supplied at a concentration of 0.6 mM. The reactor is stored and maintained in an anaerobic glovebag with a three percent hydrogen headspace. Every 4th draw and fill, approximately 2 mL of the slurry was withdrawn from the reactor after it has been vigorously mixed, transferred to 2 microcentrifuge tubes, and immediately stored at -70 ^{o}C for later analysis. Samples of the reactor headspace will be taken approximately every two weeks to determine if TCE dechlorination is detectable and if

dechlorination products are present. TCE and its dechlorinated products will be analyzed on a GC equipped with a photoionization detector (PID).

After approximately 12 months of this experiment, only small, intermittent amounts of TCE dechlorination products were detectable and analysis of the community by DNA extraction and qPCR (same qPCR method as Chapter 3) showed no increase in the amount of putatively-dechlorinating *Chloroflexi*. Since the purpose of this experiment was to track the changes in community structure as a previously-uncontaminated soil was pressured to dechlorinate TCE, this experiment was discontinued.