REDUCTIVE DEHALOGENATION OF CHLORINATED BENZENES: A ROLE FOR *DEHALOBACTER* SPP.

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Chlorinated benzenes have been extensively used in industry and agriculture and are common groundwater pollutants. Chlorobenzenes readily migrate to anaerobic zones in sediment and groundwater, thus understanding the fate of these compounds and anaerobic remediation are important goals. In previous studies in this laboratory, sediment microcosms were developed that reductively dehalogenated dichlorobenzene (DCB) isomers to monochlorobenzene (MCB) and MCB to benzene at high rates. Enrichment cultures that dehalogenated either 1,2-DCB, 1,3-DCB, or 1,4-DCB to MCB were derived from these microcosms, and *Dehalobacter* spp. were identified in an enrichment using 1,2-DCB. In this study, quantitative PCR (qPCR) of 16S rRNA genes indicated *Dehalobacter* spp. were responsible for dehalogenation in all DCB cultures, and 16S rRNA clone library analysis indicated 1,2- and 1,4-DCB dehalogenating *Dehalobacter* spp. were closely related while the *Dehalobacter* sp. in 1,3-DCB cultures was more divergent. In the course of purification of these cultures, methanogens and acetogens were eliminated, and culture conditions for growth of the dehalogenators were optimized. Extracts from a *Sedimentibacter* sp. isolated from a 1,2-DCB enrichment culture were added to 10^{-8} dilution cultures, allowing the isolation of 1,2-DCB and 1,3-DCB dehalogenating *Dehalobacter* sp. strains 12DCB1 and 13DCB1, although the extracts were not required for growth in subsequent transfers. *Dehalobacter* sp. strain 14DCB1 grew

more slowly and was characterized in a highly enriched 1,4-DCB dehalogenating culture. Dehalogenation capabilities of each strain were tested with all chlorobenzene isomers, dichlorotoluenes, and tetrachloroethene (PCE), and each strain dehalogenated different combinations of these compounds. Strain 12DCB1 dehalogenated predominantly singly flanked chlorines on aromatic compounds and PCE, and did not dehalogenate compounds with only doubly flanked chlorines or those without any flanked chlorines. Strain 13DCB1 had the widest dehalogenation range of compounds tested, utilizing singly flanked chlorines, doubly flanked chlorines, *meta* substituted unflanked chlorines, and PCE, while strain 14DCB1 had the narrowest substrate range, dehalogenating *para* substituted chlorines and slowly dehalogenating some singly flanked chlorines. MCB dehalogenation to benzene was investigated in microcosms constructed with sediment from two different contaminated areas within the Chambers Works site. Attempts to transfer dehalogenation activity to sediment-free enrichment culture were not successful, though transfers into medium containing a commercial potting mix were achieved, which reduced the reliance upon limited supplies of sediment for perpetuation of MCB dehalogenation activity. A threshold below which MCB was not dehalogenated was investigated, and results suggested MCB was not utilized at concentrations below ca. 15 μM in microcosms despite the thermodynamic favorability of the reaction. Lastly, qPCR confirmed *Dehalobacter* spp. played a role in MCB dehalogenation, and different *Dehalobacter* spp. were detected in 16S rRNA clone libraries from the two sediment types which, together with DCB culture sequences, suggested 16S rRNA gene sequence is not a good predictor of *Dehalobacter* spp. dehalogenation spectra. These studies have established a role for *Dehalobacter* spp. in the dehalogenation of chlorobenzenes, and this genus should be considered when determining the fate of diverse halogenated organic compounds.

BIOGRAPHICAL SKETCH

Jennifer Lynn Nelson was born on March 6th, 1985, in Helena, MT, to Sam and Arlene Nelson. At age nine months, Jenny learned to walk due to an intense desire to follow her big brother wherever he went. Jenny spent countless days with her brother and cousins at their grandparents' house in the Montana mountains which instilled a love of flowers, animals, and the outdoors. While attending Helena High School, Jenny lettered in academics, choir, and softball, and worked part time as a delivery driver for Papa John's Pizza. She graduated as valedictorian in 2003 and enrolled at Morningside College in Sioux City, IA, where she received an athletic scholarship to play fastpitch softball. As a college sophomore in 2005, Jenny was the Morningside Mustangs' starting right fielder and received a Research Experience for Undergraduates at the University of Nebraska-Lincoln in the lab of Dr. Greg Somerville, where she continued doing research each summer through 2007. After graduating from Morningside with a B.S. in biology, Jenny began graduate school in the Department of Microbiology at Cornell University in the fall of 2007. She joined Dr. Stephen Zinder's laboratory in March of 2008 and enjoyed four years of graduate research. During her time at Cornell, Jenny continued to play softball, survived a bout of thyroid cancer, learned to play ice hockey, and could be found enjoying Zinder Lab Wine Hour with good friends almost every late Friday afternoon.

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CHAPTER ONE

REDUCTIVE DEHALOGENATION OF CHLOROBENZENES: AN INTRODUCTION TO *DEHALOBACTER* AND *DEHALOCOCCOIDES SPP*.

Chlorinated organic compounds have been used for decades in the production of herbicides, pesticides, dyes, insulators, and as industrial solvents (9). Since many chlorinated compounds are known to cause disease or are suspected of causing disease in humans, they are numerous and high ranking on the 2011 CERCLA Priority List of Hazardous Substances (7) which ranks substances based on a combination of their toxicity, abundance, and potential for human exposure. The first organic compound on this Substance Priority List (SPL) (ranked fourth) is the known carcinogen vinyl chloride. Benzene is ranked sixth, and eight of the twelve chlorinated benzene isomers are scattered throughout the SPL as shown in Table 1.1.

The three dichlorobenzene (DCB) isomers are among the chlorobenzenes on the SPL. Of more than 1700 national priority sites in the U.S. in 2011, 1,2-DCB, 1,3-DCB, and 1,4-DCB were found in at least 277, 153, and 321 sites, respectively (6). Monochlorobenzene (MCB) was found in at least 491 of these sites, while benzene was detected in at least 968 sites (7).

Table 1.1. Benzene and chlorinated benzenes in order of ranking on the 2011 CERCLA Priority List of Hazardous Substances (atsdr.cdc.gov/SPL) (7).

Rank	Substance	Current/former applications
6	Benzene	Gasoline or petroleum fuel processing (9, 13)
77	Pentachlorobenzene (QCB)	Herbicide intermediate, fire retardant (18)
93	Hexachlorobenzene (HCB)	Fungicide, manufacturing fireworks and ammunition (15)
117	Monochlorobenzene (MCB)	Pesticide (precursor to DDT); production of phenol and aniline; degreasing solvent (14)
139	1,2,3-Trichlorobenzene (TCB)	Chemical solvent, heat transfer medium (18)
159	1,4-Dichlorobenzene (DCB)	Main ingredient in mothballs, restroom deodorizers, insecticide component targeting fruit borers and ants (17)
175	1,2-DCB	Herbicide intermediate, insecticide targeting termites and borers, industrial solvent, wastewater deodorizer (16)
199	1,2,4-TCB	Herbicide, degreaser, lubricant (12)
204	1,3-DCB	Intermediate in production of dyes, pesticides, herbicides, and medicines (6)

1,2-DCB is primarily used as a chemical intermediate in the production of herbicides and pesticides, though 1,2-DCB may also be added to waste water as a deodorizer or used in dye production (16). 1,2-DCB typically enters the environment through industrial waste disposal and waste water effluent (6), and between 1987-1993 at least 240,000 pounds of 1,2-DCB were released to the environment in the U.S. (the largest releases came from New Jersey). 1,2-DCB has been shown to bioaccumulate in fish from the Great Lakes (16), and human contact with high levels of 1,2-DCB has the potential to damage the nervous system, kidneys, liver, and blood cells, though 1,2-DCB is not listed as a carcinogen due to insufficient studies on the impact of 1,2-DCB on human health (16).

1,3-DCB is the least abundant of the DCB isomers, and is primarily used as an intermediate in the production of herbicides, insecticides, and medicines. Little is known about potential health effects associated with human exposure, but disposal into aquatic environments has been shown to negatively affect fish (6).

1,4-DCB is the highest ranking and most abundant DCB isomer in priority sites. Most of the 1,4-DCB in the U.S. is used to manufacture mothballs or toilet deodorizers (17) which are almost entirely composed of 1,4-DCB, though some mothballs are manufactured using naphthalene. Chronic human exposure and consumption of 1,4-DCB have shown liver, skin, and central nervous system effects, and tests with rats and mice have been shown to damage the liver, kidneys, and respiratory system, however these studies stopped short of labeling 1,4-DCB as a carcinogen (17). Currently 1,4-DCB is listed as a "probable carcinogen".

MCB is more abundant than DCBs in contaminated sites and is ranked higher on the SPL. In 1990, over 370 million pounds of MCB were produced in the U.S., mainly used to manufacture industrial solvents and pesticides (19), and other minor uses include production of adhesives, rubber, and paints (14). Animal tests with MCB showed adverse effects to the male reproductive system in rats, mice, and dogs, and moderate liver and kidney effects were observed in both sexes of rats and mice. Similar to 1,4-DCB, MCB has not been classified as a carcinogen due to a lack of human data and inadequate animal data (19).

Benzene is a known carcinogen and is the third highest ranking organic compound on the priority list (5). Benzene is a well-known component of petroleum fuels and may be naturally spread to the environment through volcanic eruptions, cigarette smoke, and forest fires. Industrial uses of benzene include the synthesis of resins, plastics, nylon and as a precursor for other chemicals (13), and it has been has been found in more than half of SPL sites, extensively

deposited in the environment as industrial waste and as a component of oil spills. Chronic human exposure to benzene has resulted in cancers in blood-forming organs, and benzene has been shown to impact developing fetuses via maternal blood (5).

Under aerobic conditions, trichlorobenzene (TCB) isomers (1, 32), DCB isomers (10, 43, 45), MCB (35), and benzene (27) can be broken down by *Pseudomonas* spp. and other organisms by well-known oxygenase pathways, though higher chlorinated benzenes including hexachlorobenzene (HCB), pentachlorobenzene (QCB), and 1,2,3,5-tetrachlorobenzene (TeCB) have rarely been degraded aerobically (47). Under anaerobic conditions, every chlorobenzene isomer has been shown to be reductively dehalogenated (3, 23, 40), which is important as these compounds readily form dense non-aqueous phase liquids (DNAPLs) and migrate to anaerobic zones such as groundwater and sediments. A major concern that complete chlorobenzene dehalogenation results in the formation of benzene can be stemmed by the fact that many studies have shown anaerobic benzene degradation under methanogenic conditions as well as nitrate, sulfate, and Fe(III) reducing conditions (33, 48).

This introduction will largely focus on anaerobic chlorobenzene-dehalogenating mixed cultures and dehalogenating specialists *Dehalococcoides* and *Dehalobacter* spp., which almost exclusively require molecular hydrogen as electron donor and a chlorinated electron acceptor for growth (4, 29, 37). Several other dehalogenating organisms with more versatile capabilities at using electron donors have been investigated including members of the *Desulfitobacterium*, *Desulfovibrio*, *Geobacter*, *Desulfomonile*, *Anaeromyxobacter*, and *Sulfurospirillum* genera (44); however, these organisms have not been shown to dehalogenate chlorobenzenes and are beyond the scope of this introduction.

Chlorobenzene dehalogenation in microcosms and mixed cultures

Mixed community experiments using sources including digester sludge and contaminated river or lake sediments have seen chlorobenzene dehalogenation to varying degrees. Ramanand et al. (41) provided a mixture of HCB, QCB, and 1,2,4-TCB to soil slurry microcosms and observed MCB as the predominant end product. Enrichment cultures derived from these microcosms given individual TeCB or TCB isomers produced DCBs instead of MCB as end products. Masunaga et al. (36) constructed microcosms from contaminated river sediment that dehalogenated all chlorobenzenes with two or more chlorines to MCB, and different dehalogenation patterns were observed with different isomers. Bosma et al. (8) showed dehalogenation of all TCB isomers to MCB via DCB intermediates in sediment microcosms constructed using Rhine River sediment. In these studies, 1,2,3-TCB and 1,3,5-TCB were dehalogenated to 1,3-DCB while 1,2,4-TCB was dehalogenated to 1,4-DCB. Only trace amounts of 1,2-DCB were observed. Fathepure et al. (21) showed dehalogenation of HCB and 1,2,3,5-TeCB to mainly 1,3,5-TCB in anaerobic sewage sludge. Low amounts of 1,2,4-TCB appeared transiently in these studies and was likely dehalogenated to DCBs which were detected at low concentrations. Middeldorp et al. (38) showed that an enrichment culture from Rhine River sediment maintained on 1,2,4-TCB could dehalogenate higher chlorinated benzenes to 1,3,5-TCB, 1,2-DCB, 1,3-DCB and MCB as main end-products. This enrichment dehalogenated doubly flanked, singly flanked, or unflanked chlorines (Figure 1.1) from specific isomers which the authors hypothesized was due to the presence of more than one dehalogenating organism. Nowak et al. (40) provided TCBs or DCBs as electron acceptors to mixed cultures derived from contaminated Saale River sediment and observed accumulation of MCB with trace amounts of

benzene produced during TCB and DCB dehalogenation. This was the first example of chlorobenzene dehalogenation to benzene, though benzene production was likely a cometabolic process, only occurring in small amounts concomitantly with DCB dehalogenation to MCB. Hölscher et al. (30) developed mixed cultures from German sewage sludge and showed HCB and QCB were dehalogenated to DCBs and MCB, and cultures given only 1,3,5-TCB produced 1,3-DCB followed by slow accumulation of MCB. In all of these studies, organisms responsible for chlorobenzene dehalogenation were not identified.

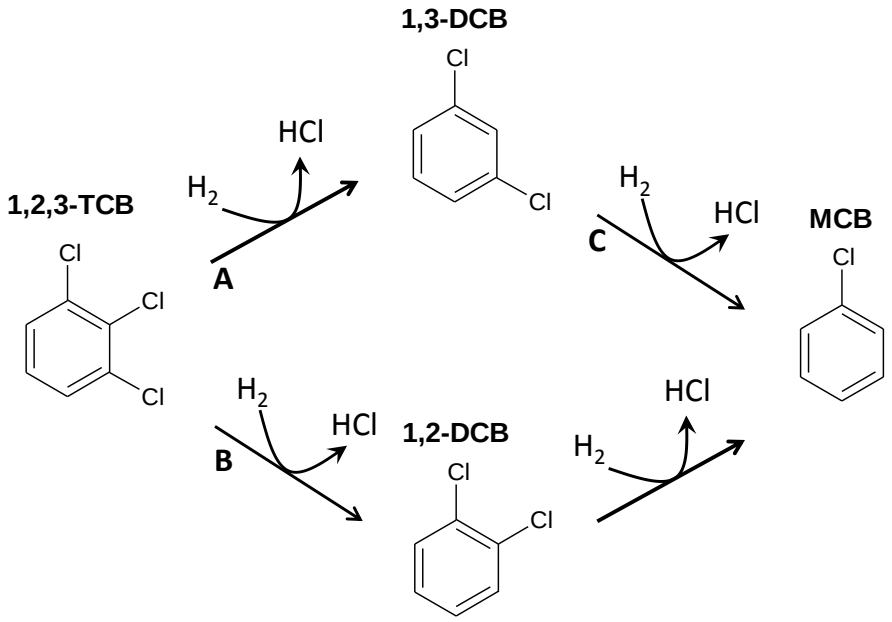

Figure 1.1. Reductive dehalogenation of chlorinated benzenes proceeds via removal of a doubly flanked chlorine (pathway A), a singly flanked chlorine (pathway B), or an unflanked chlorine (pathway C). Not shown is removal on an unflanked *para*-substituted chlorine, such as dehalogenation of 1,4-DCB to MCB.

Recently, sediment microcosms from a contaminated site in Salem County, New Jersey, were shown by our laboratory to dehalogenate all DCB isomers to MCB, with further dehalogenation of MCB to benzene at unprecedented rates and amounts (23). Additional MCB

amendments were dehalogenated more rapidly in these microcosms and benzene often accumulated to > 5 mmol/L. Much of the work in this thesis was aimed at identifying and further characterizing organisms responsible for chlorobenzene dehalogenation in these sediments. *Dehalococcoides* spp. were originally thought to be good candidates for MCB dehalogenation as they were the only organisms known at the time to dehalogenate chlorobenzenes and had been shown to completely dehalogenate chloroethenes (11, 37). However, as described in Chapter 2, no *Dehalococcoides* spp. sequences were detected in 16S rRNA gene clone libraries of DCB and MCB dehalogenating microcosms and enrichment cultures, though approximately one third of sequences had high sequence identity to known dehalogenating organisms *Dehalobacter* spp. (39).

Dehalococcoides spp.

Dehalococcoides spp. and other closely related members of the *Chloroflexi* reductively dehalogenate a wide variety of chlorinated organic compounds in pure culture including chlorinated ethenes, ethanes, dibenzodioxins, naphthalenes, phenols, propanes and biphenyls (2, 22, 37, 53). Additionally, chlorobenzenes are dehalogenated by *Dehalococcoides* spp. (4, 22, 52). *Dehalococcoides* sp. strain CBDB1 was the first isolate shown to dehalogenate chlorinated benzenes, able to dehalogenate isomers with three or more chlorines to a mixture of 1,3,5-TCB, 1,3-DCB and 1,4-DCB (4). Specifically, 1,2,3,4-TeCB and 1,2,4,5-TeCB were dehalogenated via 1,2,4-TCB to 1,4-DCB and 1,3-DCB; 1,2,3-TCB was dehalogenated to 1,3-DCB; and 1,2,3,5-TeCB dehalogenation accounted for accumulation of 1,3,5-TCB (3, 4). Additionally, *Dehalococcoides ethenogenes* strain 195 dehalogenated HCB by removing doubly flanked chlorines to 1,2,4,5-TeCB and 1,3,5-TCB (22). A close relative of *Dehalococcoides*, strain DF1,

dehalogenated HCB and QCB to 1,2,3,5-TeCB and 1,3,5-TCB (52). Patterns of chlorine removal (Figure 1.1) differ between the *Dehalococcoides* spp. in that strain CBDB1 removed both doubly flanked and singly flanked chlorines on chlorobenzenes while strains 195 and DF1 removed only doubly flanked chlorines. No *Dehalococcoides* spp. has been shown to remove unflanked chlorines on chlorobenzenes.

Dehalobacter spp.

Dehalobacter spp., like *Dehalococcoides* spp., are obligate organochloride respiring organisms that use only molecular H_2 as electron donor, although formate was also used in one case (31, 46). While there are fewer published pure culture representatives in comparison to *Dehalococcoides* spp., *Dehalobacter* have been cultivated in mixed cultures from diverse environments and shown to dehalogenate a wide variety of chlorinated organic compounds (Table 1.2). *Dehalobacter restrictus* strain PER-K23, the best characterized isolate, has been shown to dehalogenate tetrachloroethene (PCE) and trichloroethene (TCE) to *cis*-1,2-dichloroethene (*cis*-DCE) (29). Other *Dehalobacter* sp. strains can also dehalogenate PCE to *cis*-DCE, though further dehalogenation to vinyl chloride or ethene has not been observed (51) (Chapter 3). *Dehalobacter* sp. strain WL in mixed cultures dehalogenated 1,1,2-trichloroethane (TCA) or 1,2-dichloroethane (DCA) to vinyl chloride or ethene, respectively, via a dihaloelimination reaction in which two chlorines are removed simultaneously (25, 26). Biochemical studies investigating the PCE reductive dehalogenase in *D. restrictus* concluded the enzyme contained a cobalamin-type corrinoid, associate with the required addition of vitamin B_{12} to culture medium for growth.

Table 1.2. Overview of the dehalogenation spectra of *Dehalobacter* spp. from pure or mixed culture studies. Abbreviations are as follows: tetrachloroethene (PCE); trichloroethene (TCE); dichloroethene (DCE); vinyl chloride (VC); trichloroethene (TCA); dichloroethane (DCA); monochloroethane (CA); monochlorobenzene (MCB). Not included in table is a *Dehalobacter* sp. that ferments (rather than dehalogenates) dichloromethane to acetate (31).

***Dehalobacter* spp.**	**Dehalogenates**	**Dehalogenation Products**	**Reference**
D. restrictus strain PER-K23	PCE, TCE	*cis*-1,2-DCE	(29)
strain TEA	PCE, TCE	*cis*-1,2-DCE	(51)
strain TCA1	1,1,1-TCA	1,1-DCA, CA	(46)
strain FTH1/2	Tetrachlorophthalate	Monochlorophthalate	(54)
Dehalobacter sp.	Dichloropropane	Propene	(42)
Dehalobacter sp.	Tetrachlorobiphenyl	Monochlorobiphenyl	(55)
Dehalobacter sp.	Trichlorodibenzo-*p*-dioxin	Dichlorodibenzo-*p*-dioxin	(55)
Dehalobacter sp.	β-Hexachlorocyclohexane	MCB, benzene	(49)
strain WL	1,2-DCA	Ethene	(25)
strain WL	1,1,2-TCA	VC	(26)
strain CF	Trichloromethane	Dichloromethane	(24)

Real-World Applications: Bioremediation of contaminated sites

Due to widespread release of chemicals into the environment as a result of current or previous manufacturing processes, thousands of contaminated sites exist in the United States alone. Remediation is crucial to prevent contamination of water supplies and human exposure to known and potential carcinogens. Reductively dehalogenating microorganisms can play a significant role in remediation as many of these sites contain halogenated compounds (7).

Different strategies of bioremediation exist, and can be accomplished either *ex situ* or *in situ*. *ex situ* strategies such as pump-and-treat and air stripping are effective, but expensive remediation strategies. Pump-and-treat methods involve pumping contaminated groundwater to the surface and applying appropriate treatment above ground, and air stripping strategies pump air through groundwater to remove volatile contaminants. Introducing oxygen into anaerobic zones is another treatment option, though certain compounds have not been shown to degrade under aerobic conditions and oxygen may not be distributed efficiently enough to allow for cost-

effective treatment. Two treatment strategies relevant to this work are *in situ* biostimulation and bioaugmentation. Biostimulation involves adding a limiting nutrient, electron donor, or electron acceptor to an environment to stimulate naturally occurring microorganisms to degrade contaminants present in that environment (50), and bioaugmentation involves the addition of microorganisms competent for a desired degradation activity along with nutrients when necessary (20).

Both biostimulation and bioaugmentation have been applied to environments contaminated with chlorinated compounds (20, 28, 34) and were shown to be a cost-effective strategies to detoxify contaminants from anaerobic zones. The chlorobenzene-dehalogenating *Dehalobacter* spp. described in this thesis could be potential candidates for use in bioremediation applications, though important considerations including optimal and minimum growth temperatures, pH sensitivities, redox conditions, and nutritional requirements should aid in determining the best candidate organisms and sites for bioremediation.

Conclusions

Reductive dehalogenation of chlorinated compounds has been studied extensively with *Dehalococcoides* spp., though fewer studies have focused on *Dehalobacter* spp. The goals of my research were to enrich, isolate, and characterize organisms from microcosms constructed using chlorobenzene contaminated sediment that dehalogenated all DCB isomers to MCB, and MCB further to benzene. Initial unpublished studies by Fung and Cadillo-Quiroz in this laboratory identified *Dehalobacter* sp. as a major constituent of a dehalogenating enrichment fed yeast extract and 1,2-DCB and 1,3-DCB, and further studies implicated *Dehalobacter* in other DCB-dehalogenating cultures. Chapter 2 of this work focuses on three enrichment cultures that

each contained different *Dehalobacter* spp. and dehalogenated a specific DCB isomer (1,2-DCB, 1,3-DCB, or 1,4-DCB) to MCB, and was published in Environmental Science and Technology (39). Isolation of *Dehalobacter* sp. strains 12DCB1 and 13DCB1 (that dehalogenated 1,2-DCB and 1,3-DCB, respectively), and description of highly enriched 1,4-DCB-dehalogenating *Dehalobacter* sp. strain 14DCB1 are covered in Chapter 3. Potential electron donors, DCBs and non-chlorinated electron acceptors, growth temperatures, and growth rates were also examined in Chapter 3. In Chapter 4, all chlorobenzene isomers, tetrachloroethene, and dichlorotoluenes were tested as electron acceptors with each of the *Dehalobacter* strains described in Chapter 3. Lastly, Chapter 5 discusses MCB dehalogenation to benzene and the *Dehalobacter* spp. involved in this process from two different sediment samples as well as our attempts to transfer this activity away from sediment. Evidence is accruing that *Dehalobacter* spp., like *Dehalococcoides* spp., have broad dehalogenation spectra that cannot be predicted by 16S rRNA gene sequence alone. The work presented here shows *Dehalobacter* spp. should be considered when examining remediation strategies for contaminated sites.

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CHAPTER TWO

A ROLE FOR *DEHALOBACTER* SPP. IN THE REDUCTIVE DEHALOGENATION OF DICHLOROBENZENES AND MONOCHLOROBENZENE[1]

Abstract

Previously, we demonstrated the reductive dehalogenation of dichlorobenzene (DCB) isomers to monochlorobenzene (MCB), and MCB to benzene in sediment microcosms derived from a chlorobenzene-contaminated site. In this study, enrichment cultures were established for each DCB isomer and each produced MCB and trace amounts of benzene as end products. MCB dehalogenation activity could only be transferred in sediment microcosms. The 1,2-DCB-dehalogenating culture was studied the most intensively. Whereas *Dehalococcoides* sp. were not detected in any of the microcosms or cultures, *Dehalobacter* spp. were detected in 16S rRNA gene clone libraries from 1,2-DCB enrichment cultures, and by PCR using *Dehalobacter*-specific primers in 1,3-DCB and 1,4-DCB enrichments and MCB-dehalogenating microcosms. Quantitative PCR showed *Dehalobacter* 16S rRNA gene copies increased up to three orders of magnitude upon dehalogenation of DCBs or MCB, and that nearly all of bacterial 16S rRNA genes in a 1,2-DCB-dehalogenating culture belonged to *Dehalobacter* spp. *Dehalobacter* 16S rRNA genes from DCB enrichment cultures and MCB-dehalogenating microcosms showed considerable diversity, implying that 16S rRNA sequences do not predict dehalogenation-spectra of *Dehalobacter* spp. These studies support a role for *Dehalobacter* spp. in the reductive

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dehalogenation of DCBs and MCB, and this genus should be considered for its potential impact on chlorobenzene fate at contaminated sites.

Introduction

The three dichlorobenzene isomers (DCBs) and monochlorobenzene (MCB) are used as industrial solvents and in the manufacturing of pesticides and dyes(8), and have been extensively released into the environment. As DCBs and MCB are important groundwater pollutants and probable human carcinogens, understanding their fate in the environment and their potential for remediation is critical. DCBs and MCB can be metabolized to CO_2 under aerobic conditions by well characterized pathways(16). However, DCBs and MCB readily form dense non-aqueous phase liquids (DNAPLs) and migrate to anaerobic environments such as sediments and groundwater. Anaerobically, halogenated aromatic compounds can be degraded via reductive dehalogenation, in which the halogenated compound serves as the terminal electron acceptor for organisms such as *Dehalococcoides* spp., *Desulfitobacterium* spp., and *Dehalobacter* spp.(19, 34).

Previous studies have demonstrated dehalogenation of chlorobenzenes with three or more chlorines in culture to trichlorobenzenes (TCBs) and DCBs by *Dehalococcoides* strain CBDB1(3, 21) *Dehalococcoides ethenogenes* strain 195(11), and by strain DF-1, a member of the *Chloroflexi* related to *Dehalococcoides*(39). Additionally, *Dehalococcoides* spp. can dehalogenate a range of halogenated aromatics including polychlorinated biphenyls, chlorophenols, and polychlorinated dibenzofurans and dioxins(1, 4, 7, 11). A 16S rRNA gene clone library from a microbial consortium dehalogenating TCBs to MCB contained several members of the *Chloroflexi* other than *Dehalococcoides* spp.(38). That survey also identified five 16S rRNA genes with between 98.8-99.4% nucleotide identity to that of *Dehalobacter restrictus*,

although no further work was done to confirm whether these organisms played a role in chlorobenzene dehalogenation(38). Other *Dehalobacter* spp. can dehalogenate chloroaromatic compounds, such as a 4,5,6,7-tetrachlorophthalide(40).

Prior to our recent work(12), dehalogenation of the less chlorinated DCBs and MCB was observed mainly in sediment microcosms (23, 28-30). These studies did not address the organisms responsible for the reductive dehalogenation, and MCB was dehalogenated to benzene in only trace amounts, if at all., In a recent study, we used sediments from a contaminated site in Salem County, New Jersey, and monitored microcosms given DCBs or MCB. All DCB isomers were dehalogenated to MCB, and MCB was further dehalogenated to unprecedented amounts of benzene(12). Of the three DCB isomers, 1,2-DCB was the most rapidly dehalogenated, consistent with other studies.

In this study, sediment microcosms were used to establish enrichment cultures that reductively dehalogenate individual isomers of DCB to MCB, as well as sediment microcosms that dehalogenate MCB to benzene. 16S rRNA gene clone libraries and quantitative PCR were used to investigate the organisms involved in the degradation of these compounds. Our results demonstrate that *Dehalobacter* spp. were mainly responsible for the reductive dehalogenation of DCBs and MCB in enrichment cultures and sediment microcosms.

Materials and Methods

Chemicals.

All dichlorobenzene isomers, chlorobenzene, and benzene of the highest available purity were purchased from Sigma-Aldrich (1,3-DCB: 98%, 1,2- and 1,4-DCB: 99%, and MCB:

HPLC grade 99.9%). 1,4-DCB was dissolved in acetone to make a 5M solution or dissolved in hexadecane to make a 4 M solution. Gases were purchased from Airgas East (Elmira, NY).

Sediment Microcosms.

Sediment samples were obtained from a water-saturated drainage ditch in at the DuPont Chambers Works site, Salem County, New Jersey. One liter sediment samples were stored in non-sterile one liter plastic bottles at 4°C in the dark with no precautions against oxygen. Samples used for microcosm studies were taken from below the surface and away from the sides of the plastic bottles , and assumed to be anaerobic due to the production of methane. Three batches of sediment ca. 4 L each from this site were used in this study, obtained in March 2006, June 2007, and December 2008.

Microcosm preparation for MCB dehalogenation studies was described previously(12). To each sediment microcosm, 200 mg/L yeast extract was supplied as an electron donor and organic nutrient source and 1 g/L $NaHCO_3$ was supplied as a buffer. Microcosms were amended with 1 mmol/L MCB (nominal concentration) via microsyringe and incubated at 30°C in the dark, shaking at 300 RPM.

DCB Enrichment Cultures.

A mineral salts medium designed for *Dehalococcoides* strain CBDB1(2) containing 12 mM $NaHCO_3$ and 0.05 mg/L vitamin B_{12} from a vitamin solution(24) was used as the growth medium for DCB enrichment cultures. CBDB1 medium (50 mL) was dispensed inside an anaerobic glovebox into 160 mL serum vials that were sealed using Teflon-coated butyl rubber stoppers and aluminum crimps. All vial headspaces were flushed with N_2/CO_2 (70%/30%) to remove H_2 present in the glovebox atmosphere. Sediment slurries (sediment collected June,

2007 or December 2008) from actively reductively dehalogenating microcosms described above were used to inoculate DCB enrichment cultures on the benchtop using N_2-flushed syringes with 18 gauge needles (2% vol/vol corresponding to ca. 0.4 g sediment).

Preliminary enrichment culture optimization tested a variety of reducing agents including 0.8 mM Na_2S, 0.8 mM Ti(III) citrate, and 0.8 mM Ti(III) NTA. 1,2-DCB dehalogenation proceeded most rapidly when reduced with Na_2S, however 1,3- and 1,4-DCB enrichment cultures were inhibited by Na_2S and were instead reduced with 1 mM amorphous FeS(6). 2 mM sodium butyrate as electron donor was routinely added to early transfers of all DCB enrichment cultures. 200 mg/L yeast extract was added to first transfers of each enrichment culture, but was replaced with 20 mg/L Casamino Acids by the second, seventh, and fourth transfer of 1,2-, 1,3-, and 1,4-DCB enrichment cultures, respectively. Uninoculated culture medium containing DCBs did not show more than trace amounts of MCB when incubated for over 30 days, indicating the reducing agents used were not responsible for the reductive dehalogenation observed in enrichment cultures.

After several successive transfers using butyrate as electron donor, dilution-to-extinction series were performed into medium in which butyrate was replaced with 5 mL H_2/CO_2 (80%/20%-nominal 3.3 mmol/L H_2) as electron donor and 2 mM sodium acetate as a carbon source. Dehalogenation of DCBs generally occurred as far as the 10^{-6} dilution, and these 10^{-6} dilution cultures were used as inocula for subsequent culture transfers. Methanogenesis was inhibited with either 5 mM 2-bromoethanesulfonate (BES) (1,2-DCB and 1,4-DCB enrichments) or 10 µM mevinolin (1,3-DCB enrichments), an inhibitor of archaeal lipid biosynthesis(26). Tables 2.1 and 2.2 provide a more detailed description of the enrichment process for the 1,2-

DCB and 1,3-DCB cultures. As the 1,3-DCB and 1,4-DCB cultures followed a similar path of enrichment, we only present a detailed table for 1,3-DCB.

Early transfers of the 1,2- and 1,3-DCB enrichment cultures received initial doses of approximately 50 µmol/L 1,2- or 1,3-DCB. 1,4-DCB was initially dissolved in acetone (5 M) and added to enrichment cultures at 40 µmol/L. These are nominal concentrations which facilitate mass balance and volumetric productivity calculations. Using Henry's law constants from EPA factsheets(10) we estimated that the aqueous concentrations of 1,2-DCB, 1,3-DCB, 1,4-DCB, MCB, and benzene to be 86%, 80%, 82%, 73%, and 67% respectively of the total nominal concentrations. In later cultures studies, a two-liquid phase system was used in which these DCB isomers were dissolved in 0.5 ml sterile hexadecane at a total nominal concentration in the vials of 1 mmol/L(18). Because significant fractions of the chlorobenzenes were dissolved in the hexadecane phase, new calibrations for the gas chromatograph were needed. For example a vial receiving nominal concentrations of 1 mmol/L 1,2-DCB or MCB in hexadecane gave readings equivalent to nominal concentrations of 0.04 mmol/L and 0.3 mmol/L respectively in cultures lacking hexadecane, representing the lowered concentrations perceived by the organisms.. Subsequent doses of DCBs were added directly to the hexadecane phase already present in the culture vial. 1,4-DCB was added as a 4 M solution in hexadecane. Serial tenfold dilutions were carried out with at least three vials representing the higher dilutions.

Table 2.1. Conditions used to establish a highly enriched 1,2-DCB dehalogenating culture.

Transfer Number	Reducing Agent	Electron Donor	Casamino Acids	1,2-DCB dissolved in hexadecane	Methanogen Inhibitor	Comments
1	0.8 mM Na_2S	200 mg/L Yeast extract	None	No	BES	1% inoculum of a 1,2-DCB utilizing sediment microcosm slurry derived from June 2007 Chambers Works ditch sediment
2	Na_2S	2 mM Butyrate	200 mg/L	No	BES	Dehalogenation continued without an appreciable lag when butyrate replaced yeast extract as the electron donor.
3-5	Na_2S	2 mM Butyrate	20 mg/L	No	BES	Methane not detected in transfer 3. H_2 added to vials in the transfer 5 series rapidly consumed H_2, indicating presence of acetogens.
6-7	Na_2S	2 mM Butyrate	20 mg/L	Yes	BES	
8	Na_2S	H_2	20 mg/L	Yes	None	A dilution-to-extinction series was performed into medium in which H_2 and acetate replaced butyrate, dehalogenation occurred as far as the 10^{-6} dilution, which was used as an inoculum for generation 9 cultures. 2 mM acetate served as a potential carbon source.
9-17	Na_2S	H_2	20 mg/L	Yes	None	qPCR studies of 16S rRNA concentrations and dehalogenation over time were performed on 13^{th} transfer cultures (Fig. 2). The clone library presented was performed on a 17^{th} transfer culture (Table 2.3)
Post 17	1 mM FeS	H_2	20 mg/L	Yes	None	Culture grew more rapidly with FeS but these cultures are not included in this study.

Table 2.2. Conditions used to establish a 1,3-DCB dehalogenating culture. Reducing agent: 1 mM amorphous FeS precipitate

Transfer Number	Reducing Agent	Electron Donor	Casamino Acids	1,3-DCB dissolved in hexadecane	Methanogen Inhibitor	Comments
1	FeS	200 mg/L Yeast extract (YE)	None	No	None	1% inoculum of sediment microcosm slurry derived from December 2008 Chambers Works ditch sediment
2-3	FeS	2 mM Butyrate, 200 mg/L YE	None	No	None	
4-5	FeS	Butyrate, 200 mg/L YE	None	Yes	None	
6	FeS	Butyrate	200 mg/L	Yes	BES	BES inhibits 1,3-DCB dehalogenation
7-15	FeS	Butyrate	20 mg/L	Yes	Mevinolin	PCR product sequenced from Transfer 8 (1,3-DCB_Gen8 in Fig. 1)

Analytical Procedures.

Headspace analysis was used to detect chlorobenzenes, benzene, and methane, by injecting a 0.1 mL-headspace sample with a pressure locked syringe into a Perkin-Elmer 8500 gas chromatograph outfitted with a macrocapillary column and a flame ionization detector as previously described by Fung et al.(12). H_2 in the headspace was determined using a Carle 110 thermal conductivity gas chromatograph (detection limit 0.0001 atm) and aqueous acetate and other organic acids by HPLC as described previously(5).

DNA Extraction, 16S rRNA Gene Clone Libraries, and Phylogenetic Analyses.

The FastDNA SPIN Kit for Soil (MP Biomedicals, LLC, Solon, OH) was used to extract genomic DNA according to the manufacturer's protocol. DNA was eluted in 55 μL sterile, deionized water. DNA was extracted from 2 mL of sediment microcosm material or 1.6 mL of enrichment culture.

16S rRNA gene clone libraries were constructed using DNA extracted from fourth generation and 17th generation 1,2-DCB enrichment cultures, first generation 1,3-DCB enrichment cultures, and using DNA extracted from a sediment microcosm dehalogenating MCB to benzene. PCR products of primers 27f and 1492r (1,2-DCB enrichment cultures) or *Dehalobacter*-specific primers 110f and 1273r (sediment microcosm) (Table 2.3) were cloned using the Topo TA Cloning Kit into pCR2.1 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Inserts of the expected size were PCR amplified using primers M13f and M13r and digested with the restriction enzymes HhaI and HaeIII (New England Biolabs). Representatives from each restriction fragment length polymorphism (RFLP) type were

sequenced at the Life Sciences Core Laboratory Center at Cornell University. We determined one of our sequences was chimeric using the Chimera Check feature of greengenes.lbl.gov and removed it from subsequent analysis. In the case of some 1,3-DCB and 1,4-DCB cultures, the sequence of the PCR product using *Dehalobacter*-specific primers 110f and 1273r was determined directly using the PCR primers.

Table 2.3. Primers used in this study. *Dehalobacter* (Dhb), eubacteria (Eub), *Dehalococcoides* (Dhc)

Primer Name	Target	Purpose	Annealing Temperature	Sequence
Dhb477f	16S rDNA	rDNA qPCR	62	GATTGACGGTACCTAACGAGG
Dhb647r	16S rDNA	rDNA qPCR	62	TACAGTTTCCAATGCTTTACGG
Eub331f	16S rDNA	rDNA qPCR	62	TCCTACGGGAGGCAGCAGT
Eub797r	16S rDNA	rDNA qPCR	62	GGACTACCAGGGTATCTAATCCTGTT
Dhc385f	16S rDNA	rDNA qPCR	62	GGGTTGTAAACCTCTTTTCAC
Dhc692r	16S rDNA	rDNA qPCR	62	TCAGTGACAACCTAGAAAAC
Dhb110f	16S rDNA	Clone Library	50	AGTAACGCGTGGGTAACCTG
Dhb1273r	16S rDNA	Clone Library	50	CTTCCGTCTGTACCGTCCAT
27f	16S rDNA	Clone Library	50	AGAGTTTGATCCTGGCTCAG
1492r	16S rDNA	Clone Library	50	ACGGYTACCTTGTTACGACTT
M13f	pCR2.1 Vector	Clone Library	50	GTAAAACGACGGCCAG
M13r	pCR2.1 Vector	Clone Library	50	CAGGAAACAGCTATGAC

16S rRNA gene sequences were aligned using ClustalX 2.1, and DNA distances using 837 bases shared by the sequences were analyzed using DNADist as implemented in the PHYLIP 3.69 package. A nearly identical tree topology was obtained using DNAML maximum likelihood analysis.

Quantitative PCR.

Quantitative PCR (qPCR) was used to estimate the concentrations of *Dehalobacter*, *Dehalococcoides*, and eubacterial populations in enrichment cultures and sediment microcosms using specific primers (15, 27) (Table 2.3). *Dehalobacter* primers 110f and 1273r were designed in this study (Table 2.3). Reaction mixtures (final volume 25 µL) contained 12.5 µL iQ SYBR Green Super Mix (BioRad, Hercules, CA), forward and reverse primers (200 nM each) and 1 µL template DNA. Cycling conditions were as follows: 10 min. at 95 °C, 35 cycles of 15 s at 95 °C and 1 min. at 62 °C, followed by melting curve analysis from 60 to 95 °C to screen for primer dimers using a MyiQ Single Color Real Time PCR Detection System (Bio-Rad).

Quantification of *Dehalobacter* and *Dehalococcoides*16S rRNA genes were achieved by analyzing dilution series of a known quantity of a plasmid containing a partial 16S rRNA gene from *Dehalobacter* obtained from a clone library of the 1,2-DCB enrichment culture (described above), or a full length 16S rRNA gene from *Dehalococcoides* strain 195, respectively. Bacteria were quantified using a plasmid containing the 16S rRNA gene from *Bacillus subtilis* strain 168. DNA concentrations were estimated spectrophotometrically using a ND-1000 spectrophotometer (Nanodrop, Wilmington, DE). Values presented and their standard deviations are the average of triplicate samples from individual enrichment cultures or microcosms, though each experiment was performed on at least two different cultures or microcosms with similar results.

Nucleotide sequence accession numbers

The nucleotide sequence data presented has been deposited into Genbank,, accession numbers JN051262 to JN051274.

Results

Development of 1,2-DCB Dehalogenating Enrichment Cultures

Sediment slurries from microcosms dehalogenating all DCB isomers to MCB and benzene (described in Fung et al. 2009(12)) were transferred into a growth medium based on that used to culture *Dehalococcoides* strain CBDB1(3). Preliminary optimization experiments with enrichment cultures showed that 1,2-DCB was most rapidly dehalogenated of the DCB isomers, that 0.8 mM Na_2S was superior as a reducing agent to Ti (III) citrate or Ti (III) NTA, that yeast extract could be replaced by butyrate or H_2 as an electron donor, that 20 mg/L Casamino Acids could support growth, and that methanogenesis could be inhibited with 5 mM BES (data not presented). However these studies reached an impasse when H_2-utilizing acetogens outgrew the dehalogenators in cultures provided with H_2 as the electron donor, as evidenced by cessation of dehalogenation (Figure 2.1) accompanied by H_2 depletion and appearance of 2.5 mM acetate. Numerous attempts to remove the acetogens failed, including using the antibiotic vancomycin, which inhibited dehalogenation, in contrast to cultures containing *Dehalococcoides*, which is vancomycin resistant(24).

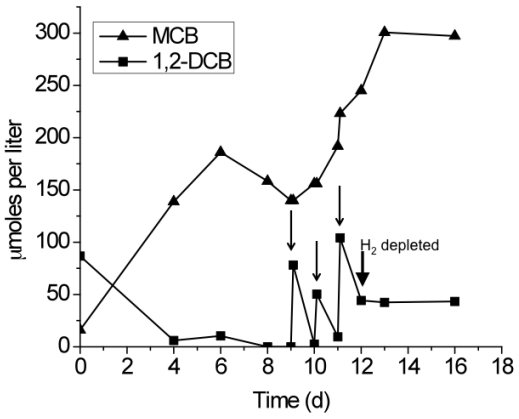

Figure 2.1. Reductive dehalogenation of 1,2-dichlorobenzene (1,2-DCB) (added near) to monochlorobenzene (MCB) in enrichment cultures stalled on Day 12 concomitant with the depletion of exogenously-added H_2 gas in the culture vial headspace. H_2 was added in stoichiometric excess to DCB, and the acetate concentration increased from the initial value of 2.0 mM to 4.5 mM on Day 12. The consumption of excess H_2 and production of excess acetate are indicative of the presence of hydrogenotrophic acetogens (5). Small arrows indicate additional doses of 1,2-DCB and the large arrow indicates when hydrogen was depleted from vial headspace. Data presented is from an individual enrichment culture but is representative of duplicate cultures. The cultures were incubated statically in an attempt to minimize H_2 transfer to acetogens, so MCB appearance sometimes lags behind DCB utilization.

Using this information, a new enrichment series on 1,2-DCB was initiated (Table 2.1). By the sixth transfer, the culture utilized butyrate as the electron donor, methanogenesis had been inhibited with BES, and a nominal concentration of 1 mmol/L 1,2-DCB was provided in a hexadecane phase (see Materials and Methods). Utilizing butyrate as the electron donor takes advantage of the ability of organisms catalyzing butyrate oxidation to poise hydrogen at a concentration thermodynamically unfavorable for acetogenesis(31), whereas reductive dehalogenation remains favorable under such conditions(33). Transferring the enrichment cultures six successive times with butyrate as the electron donor apparently lowered then number of H_2-utilizing acetogens to the point that on transfer 8, in which a serial tenfold dilution series was performed, 1,2-DCB dehalogenation was detected in a 10^{-6} dilution, whereas no extra H_2

consumption occurred at this dilution or transfers from this dilution, indicating the absence of H_2-utilizing methanogens and acetogens.

The enrichment culture tolerated up to 100 μmol/L 1,2-DCB added neat, however when 1,2-DCB was dissolved in hexadecane(18), the enrichment culture could be routinely amended with an initial dose equivalent to 1 mmol/L 1,2-DCB nominal concentration (see Materials and Methods), allowing for more rapid and extensive growth of the dehalogenating organisms. After the enrichment culture consumed the initial dose of 1,2-DCB, subsequent additions of electron acceptor were routinely increased to up to 3 mmol/L. In subsequent studies, we found that this culture was unable to dehalogenate 1,3-DCB or 1,4-DCB when provided with these substrates alone or together with 1,2-DCB (data not presented).

Community analysis of the 1,2-DCB utilizing enrichment cultures

We considered *Dehalococcoides* spp. to be likely candidates for carrying out DCB and MCB dehalogenation. However, attempts to detect *Dehalococcoides* spp. in cultures or microcosms using specific 16S rRNA PCR primers (see Materials and Methods and Table 2.3) uniformly failed (data not presented). To examine whether other organisms were involved in dehalogenation, a 16S rRNA gene clone library using universal bacterial primers was constructed from an enrichment culture during the preliminary optimization studies that was fed butyrate,1,2-DCB, and 1,3-DCB. This library was compared to one from a culture given all amendments except DCBs. In the culture fed DCBs, a restriction type representing 44% (30 /71) of the clones contained sequences nearly identical (99.7%) with *Dehalobacter restrictus* strain PER-K23 (1,2/1,3-DCB in Figure 2.2), whereas no *Dehalobacter* sequences were found in the cultures not amended with DCBs. *Dehalococcoides* sequences were not detected in these

libraries, and other sequences were related to anaerobic fermentative *Firmicutes* and *Bacteroidetes* (data not presented). Thus, *Dehalobacter* spp. were good candidates for being responsible for dehalogenation.

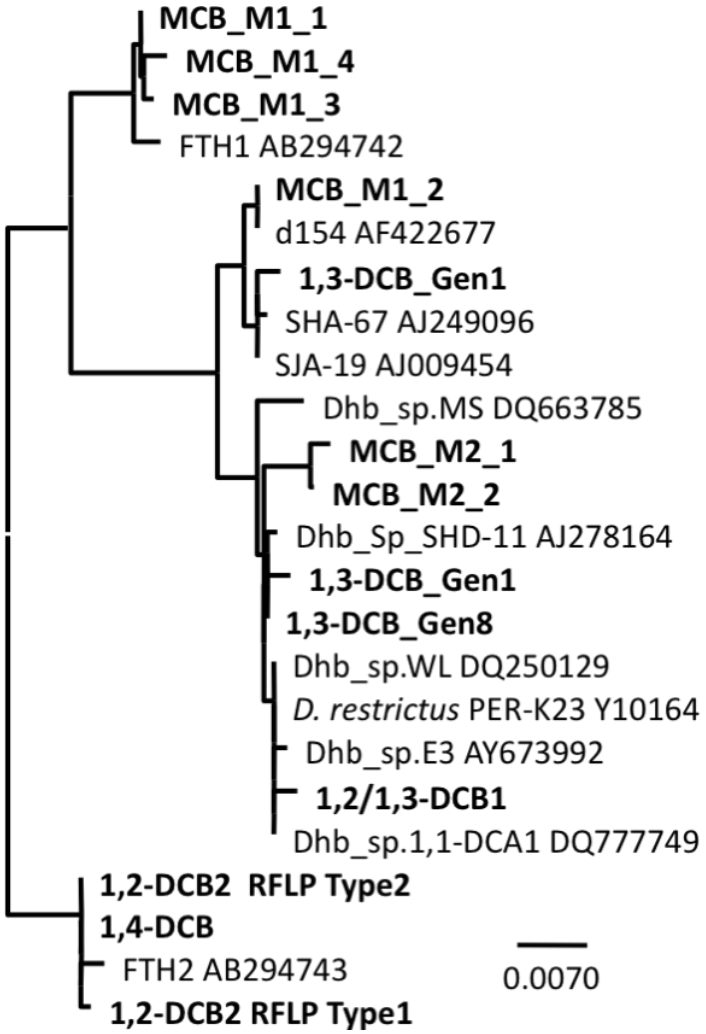

Figure 2.2. Phylogenetic tree for *Dehalobacter* 16S rRNA genes from cultures reductively dehalogenating 1,2-, 1,3-, or 1,4-dichlorobenzene (DCB) to monochlorobenzene (MCB) and trace amounts of benzene, and microcosms reductively dehalogenating MCB to benzene. A sequence from a clone library of an 8th generation culture fed 1,2-DCB and 1,3-DCB in preliminary experiments is named 1,2/1,3-DCB1, while sequences of two restriction types found in a subsequently derived 17th generation culture fed only 1,2-DCB are called 1,2-DCB2. Sequences from a first generation 1,3-DCB enrichment culture are called 1,3-DCB_Gen1 whereas the only sequence from an 8th generation culture is called 1,3-DCB_Gen8. MCB-dehalogenating microcosms from two different sediment samples are denoted MCB_M1 and MCB_M2. The 16S rRNA gene from *Desulfitobacterium hafniense* Y51 was used as an outgroup (not shown).

We also examined the composition of the bacterial community in an independently-derived 17th-transfer culture that was fed a total of 5 mmol/L 1,2-DCB, H_2, and 20 mg/L Casamino Acids (Table 2.1). A 16S rRNA gene clone library was constructed using primers 27f and 1492r, which contained 82 non-chimeric clones. Two RFLP types (RFLP Types 1&2) accounted for 80 of the 82 clones. Representatives from both RFLP types had high sequence identity (>99%) with *Dehalobacter* strain FTH2 from a 4,5,6,7-tetrachlorophthalide dehalogenating culture(40) (Figure 2.2, Table 2.4); however the sequence of RFLP Type 2, comprising approximately 39% (31/80) of *Dehalobacter* spp. sequences, contained a ~100 bp insertion near the 5' end, a situation similar to the insertions observed in 16S rRNA genes of *Desulfitobacterium* spp.(37). The remaining two clones in this library were related to *Sedimentibacter* spp. and *Desulfovibrio* spp. This community analysis was consistent with microscopic observations of the culture, which showed mainly thin rods resembling *Dehalobacter* PER-K23(17) with small numbers of thicker rods and spirals (Figure 2.3).

To examine the role of *Dehalobacter* spp. in 1,2-DCB dehalogenation, we used qPCR with primers specific for *Dehalobacter* spp.(15) or universal bacterial primers(27). As shown in Figure 2.4, numbers of *Dehalobacter* spp. 16S rRNA gene copies increased three orders of magnitude in cultures that converted several doses of 1,2-DCB to MCB, with numbers essentially identical with total bacterial rRNA gene copies. In cultures not provided with 1,2-DCB, bacterial numbers increased about one order of magnitude, presumably due to growth of heterotrophs on the small amount of amino acids added to the culture, whereas those for *Dehalobacter* spp. did not increase.

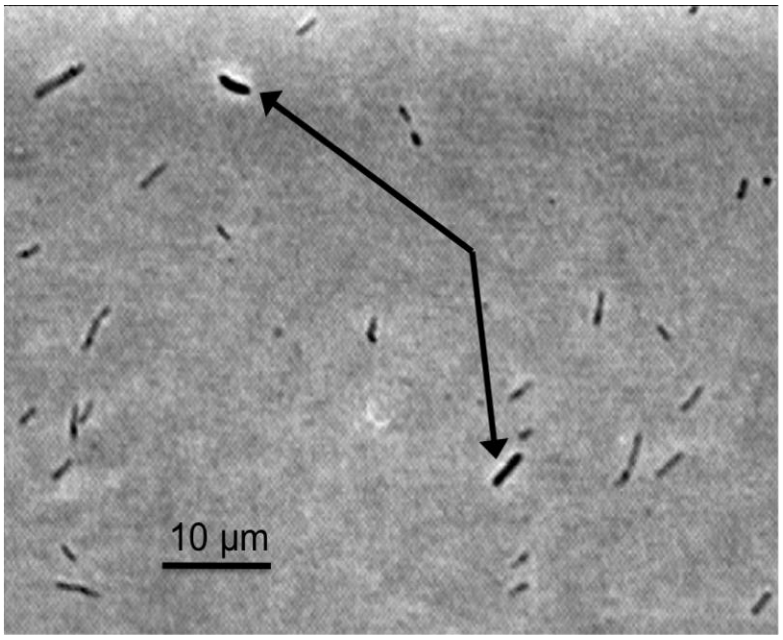

Figure 2.3. Phase contrast micrograph of a 17th generation 1,2-dichlorobenzene (1,2-DCB) enrichment culture grown on H_2/CO_2, Casamino Acids, and 1,2-DCB. The smaller rod-shaped organisms are assumed to be *Dehalobacter* as this organism is numerically dominant in the culture. Arrows indicate larger, more phase-dark rods assumed to be *Sedimentibacter* or *Desulfovibrio*.

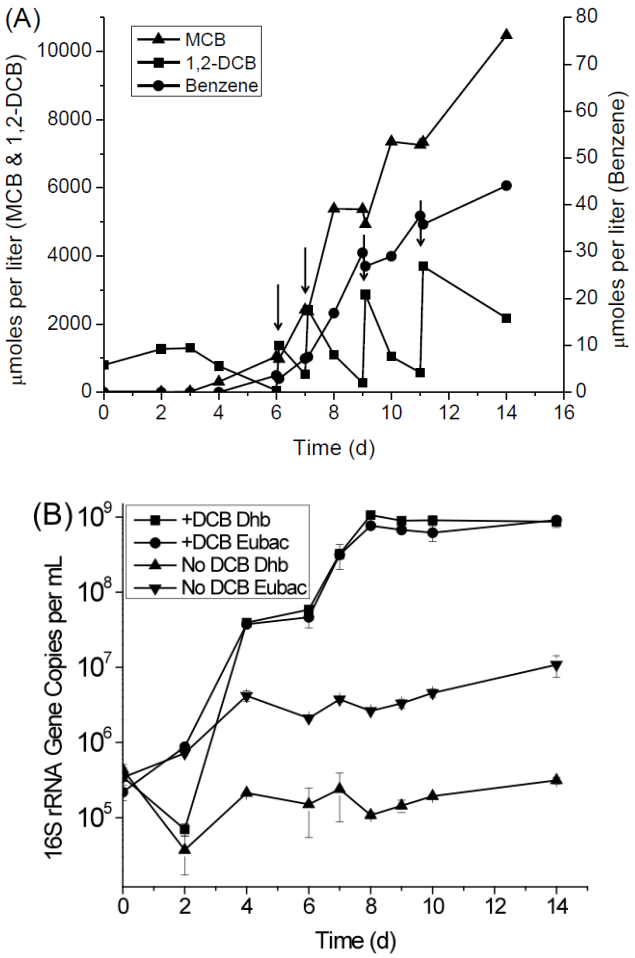

Figure 2.4. (A) Reductive dehalogenation of 1,2-dichlorobenzene (1,2-DCB) to monochlorobenzene (MCB) by an enrichment culture and (B) changes in *Dehalobacter* spp. (Dhb) and total bacterial (Eubac) 16S rRNA gene copy numbers in that culture (+DCB) compared to a control culture not receiving DCB, as measured by quantitative PCR (qPCR). Arrows in (A) indicate additional amendments of 1,2-DCB. Error bars denote the standard deviation of the mean of triplicate DNA samples. Data presented is from an individual enrichment culture, but is representative of triplicate cultures.

1,3- and 1,4-DCB utilizing Enrichment Cultures

1,3-DCB and 1,4-DCB utilizing enrichment cultures were more difficult to establish than those for 1,2-DCB, and cultures often lost the ability to be transferred more than a few times. We were able to obtain stable enrichment cultures using these substrates when we used amorphous FeS as the reducing agent rather than Ti (III) or Na_2S. These cultures dehalogenated their respective isomers to MCB with trace amounts of benzene produced, similar to the 1,2-DCB enrichment culture (Figure 2.5). While these enrichments were established similarly to the 1,2-DCB enrichment cultures, minor differences in the enrichment process are as follows and highlighted in Table 2.2. 5 mM BES was used to inhibit methanogens in 1,4-DCB cultures, but BES inhibited 1,3-DCB dehalogenation, so 10 µM mevinolin, which inhibits archaeal lipid synthesis(26), was used to inhibit methanogens in 1,3-DCB enrichment cultures. Repeated transfer with butyrate followed by dilution likely cured both cultures of acetogens. DNA was extracted from representatives of each of these cultures with or without their respective DCB isomer and were analyzed with *Dehalobacter*-specific qPCR primers as above. Representative cultures containing DCBs were highly enriched for *Dehalobacter* 16S rRNA genes compared to the total bacterial pool, while cultures without DCB show no appreciable increase in *Dehalobacter* 16S rRNA gene copy number (Figure 2.6A&B).

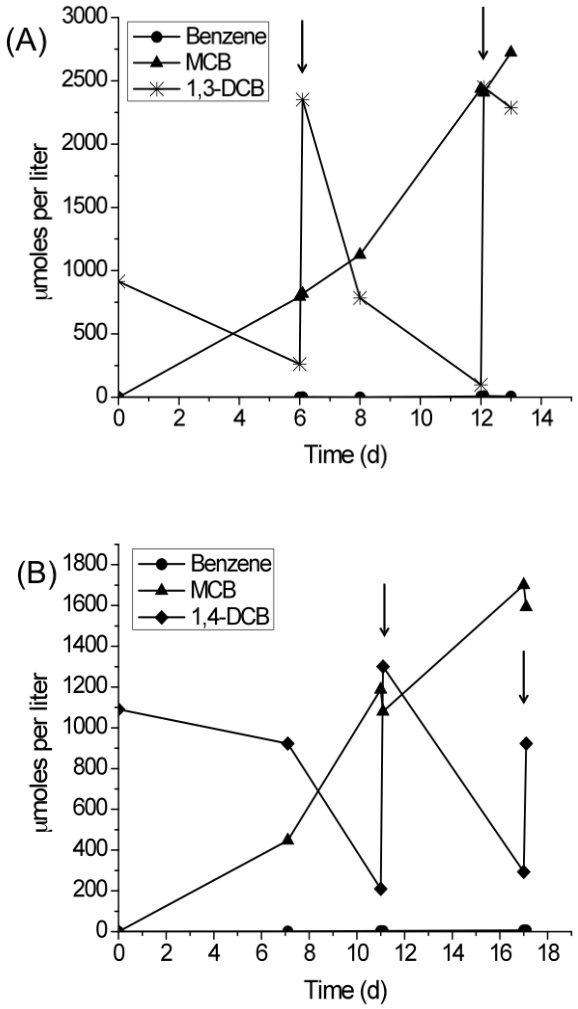

Figure 2.5. Reductive dehalogenation of 1,3-dichlorobenzene (1,3-DCB) (A) and 1,4-dichlorobenzne (1,4-DCB) (B) to monochlorobenzene (MCB) in enrichment cultures. Arrows indicate additional feedings of the respective DCB isomer. Data presented are from individual enrichment cultures, but are representative of duplicate cultures.

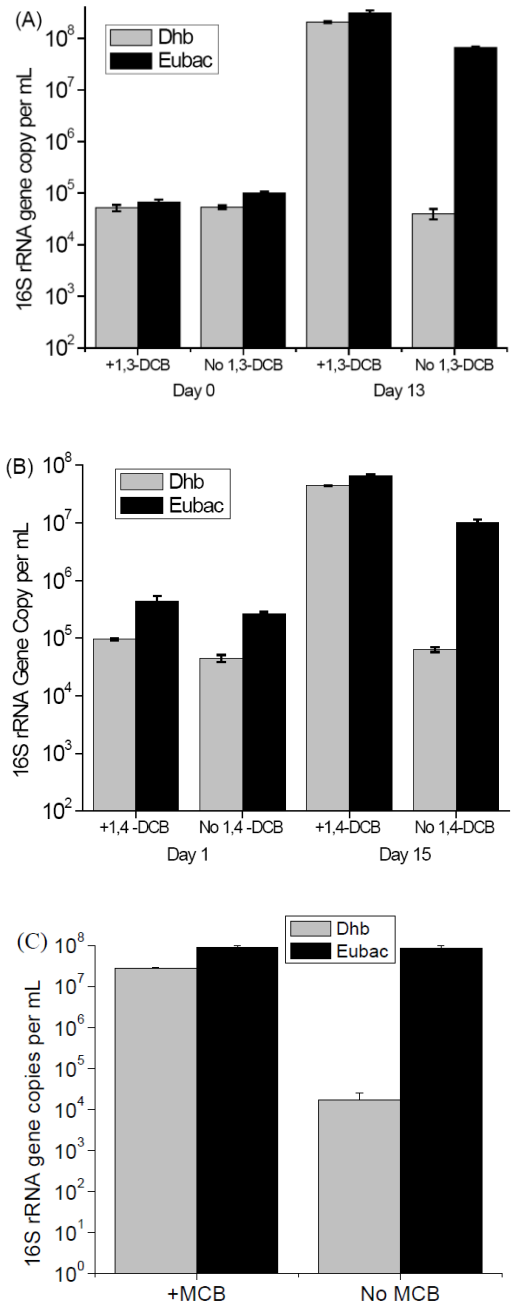

Figure 2.6. Effect of dehalogenation of 1,3-dichlorobenzene (DCB) (A) and 1,4-DCB (B) to monochlorobenzene (MCB), or dehalogenation of MCB to benzene (C) on *Dehalobacter* spp. (Dhb) and total bacterial (Eubac) 16S rRNA gene copy numbers in enrichment cultures as measured by quantitative PCR (qPCR). The 1,3-DCB enrichment culture produced 2.15 mmol/L MCB when DNA samples were taken on Day 13, and the 1,4-DCB enrichment culture produced 1.04 mmol/L MCB on Day 14. The MCB-containing microcosm produced ~3.5 mmol/L benzene when DNA samples were taken after 50 days of incubation. Error bars represent the standard deviation of the mean of triplicate DNA samples.

PCR products from DNA extracted from the 1,3- and 1,4-DCB enrichment cultures using *Dehalobacter*-specific primers (110f & 1273r) were directly sequenced. Sequences from the 1,2-DCB2 enrichment culture and the 1,4-DCB enrichment cultures were 99.7% identical, while the 1,3-DCB sequences were considerably different from cultures grown with the other two isomers (Figure 2.2). Using *Dehalobacter*-specific primers, we sequenced clones from early (first culture generation) and PCR products from late (eighth culture generation) iterations of the 1,3-DCB enrichment culture. One of the early iteration 1,3-DCB sequences grouped closely with clone SJA-19, which was derived from a consortium dehalogenating TCBs to MCB(38), whereas the other 1,3-DCB sequences grouped close to *D. restrictus* PER-K23.

Reductive Dehalogenation of MCB in microcosms

In previous studies we found that MCB dehalogenation to benzene in sediment microcosms was much slower than DCB dehalogenation, but either incubating the sediments with DCBs first or adding an inoculum of MCB-adapted microcosm sediments hastened MCB dehalogenation in naïve (uninoculated) microcosms(12). Although MCB-dehalogenating activity could be transferred to naïve microcosms, we have not succeeded in transferring MCB-dehalogenating activity from these microcosms into various growth media despite numerous attempts.

Since we were interested in the potential role of *Dehalobacter* spp. in MCB-dehalogenating microcosms, we used qPCR to examine whether their growth correlated with MCB dehalogenation. In a naïve microcosm given MCB, benzene was detected after approximately 18 days of incubation and the microcosm consumed two more doses of 1 mmol/L

neat MCB by 50 days. In a DNA sample taken on Day 50, numbers of *Dehalobacter* spp. 16S rRNA genes were nearly equal to total bacterial numbers, whereas in a microcosm not given MCB, *Dehalobacter* spp. 16S rRNA gene copy numbers were about three orders of magnitude lower (Figure 2.6C and Figure 2.7). Following the finding that *Dehalobacter* spp. were abundant in sediment microcosms (called MCB_M1), a clone library using *Dehalobacter*-specific 16S rRNA primers was constructed and several *Dehalobacter* sequences were obtained which were most identical with sequence FTH1 from a 4,5,6,7-tetrachlorophtalide dehalogenating enrichment culture (Figure 2.2), although two of the eight clones sequenced were closer to SJA-19. Sequences from an MCB-dehalogenating microcosm derived from a different batch of sediment (MCB_M2) grouped in the clade containing *D. restrictus* PER-K23.

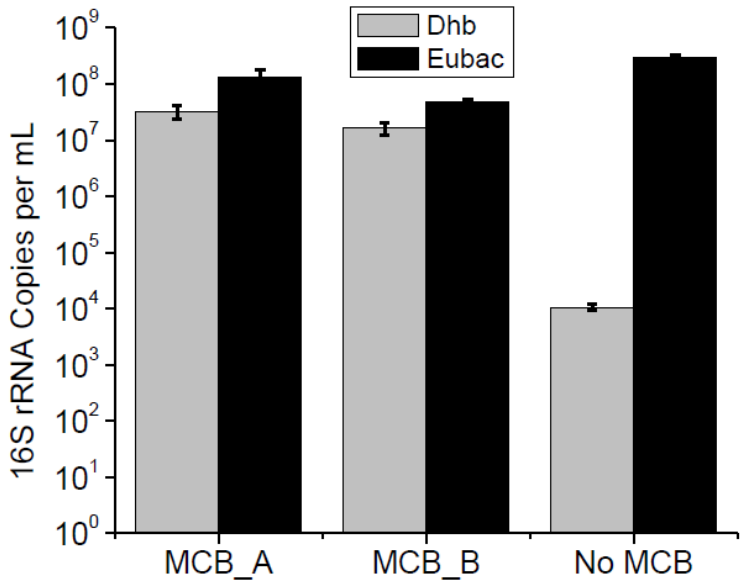

Figure 2.7. Effect of dehalogenation of MCB to benzene on *Dehalobacter* spp. (Dhb) and total bacterial (Eubac) 16S rRNA gene copy numbers in enrichment cultures as measured by quantitative PCR (qPCR). The MCB-containing microcosms produced ~4.4 mmol/L (MCB_A) and ~4.7 mmol/L (MCB_B) benzene when DNA samples were taken after 50 days of incubation. Error bars represent the standard deviation of the mean of triplicate reactions.

Discussion

Dehalogenation of DCBs to MCB has been previously detected in microcosms and culture medium, but here we establish a clear role for *Dehalobacter* spp. in this process. We have succeeded in culturing organisms that dehalogenate each of the three DCB isomers to MCB, and have a highly enriched culture using 1,2-DCB. We examined the use of various reducing agents in transferring activity, and found that while low concentrations of Na_2S were suitable for the enrichment of 1,2-DCB dehalogenators, use of amorphous FeS allowed fastest and most consistent growth of those using 1,3-DCB and 1,4-DCB.

A problem in purification was eliminating other hydrogenotrophic organisms capable of faster growth than the DCB dehalogenators. We eliminated hydrogenotrophic methanogens using the inhibitor BES, or in the case of 1,3-DCB cultures, mevinolin. Hydrogenotrophic acetogens were more problematical, and we could not eliminate them by using vancomycin, a strategy that works for the purification of *Dehalococcoides* spp.(24). To lower the number of acetogens that were present in early transfers of the enrichment cultures, we used butyrate as the electron donor in successive transfers, which does not support the syntrophic growth of acetogens(31), followed by serial dilution. Eliminating these two groups allowed us to use H_2 as the electron donor, thereby eliminating butyrate-oxidizing syntrophs, which did not grow on H_2. Finally, through minimizing the concentration of organic nutrients that can support growth of fermentative contaminants to 20 mg/L Casamino Acids and maximizing the amount of DCB added by dissolving it in a hexadecane phase(18) we were able to obtain a highly enriched culture of 1,2-DCB dehalogenators. A similar strategy is being used to purify 1,3- and 1,4-DCB dehalogenators.

Our expectation was that the organisms responsible for dehalogenation of DCBs would be *Dehalococcoides* spp, since they have been shown to use more highly chlorinated benzenes(3) and are known to dehalogenate more lightly chlorinated organic compounds like vinyl chloride(42). Instead, various lines of evidence indicated that *Dehalobacter* spp. were associated with dehalogenation of all three DCB isomers in cultures and of MCB in microcosms. Numbers of *Dehalobacter* spp. 16S rRNA genes increased only when chlorobenzene substrates were added to cultures, where they increased several orders of magnitude to become a significant fraction of the predicted total bacterial numbers. Indeed, in the optimized 1,2-DCB culture, clone library and qPCR analyses indicate that *Dehalobacter* was the predominant organism in those cultures. In recent studies, Hölscher et al.(20) were unable to detect *Dehalococcoides* in cultures removing singly flanked chlorines from trichlorobenzenes, but the identity of the responsible organisms was not determined(20).

Our analyses of *Dehalobacter* spp. sequences indicate that a surprisingly large diversity of strains is present in these samples, and that 16S rRNA gene sequence does not necessarily correlate with dehalogenation phenotype, a situation resembling that in *Dehalococcoides* spp. where there is strong evidence for horizontal transfer of reductive dehalogenation genes(25). The 16S rRNA gene sequence obtained from a clone library from a preliminary enrichment using 1,2- and 1,3-DCBs was considerably different from one from an independently derived and highly purified culture on 1,2-DCB taken later, as were sequences from different 1,3-DCB dehalogenating cultures and sequences from MCB dehalogenating microcosms derived from different batches of sediment (Figure 2.2). The inability of the purified 1,2-DCB dehalogenating culture to use other DCB isomers also supports the contention that diverse *Dehalobacter* strains are involved in chlorobenzene dehalogenation in these sediments.

Nearly full length 16S rRNA gene sequences from a clone library from the highly purified 1,2-DCB culture showed an interesting phenomenon in which about one third of the *Dehalobacter* sequences had an insert near the 5' end not present in the other sequences. This resembles the situation described for genomes of pure cultures of *Desulfitobacterium* spp.(37), members of the *Firmicutes* fairly closely related to *Dehalobacter* spp. that are also capable of reductive dehalogenation. These genomes contain three 16S rRNA gene copies, one of which has a ~100 bp 5' insertion of unknown function. Similar inserts have also been detected in nearly full length *Dehalobacter*-related sequences including SJA-19 amplified from a trichlorobenzene-utilizing consortium(38), SHA-67 from a 1,2-dichloropropane-utilizing consortium(32), and d154 from trichloroethene-contaminated groundwater(22). The sequence of 1,2-DCB RFLP Type 2 insert most closely resembles that from d154 (96% identity over 115 bases), whereas the rest of the sequence resembles that of FTH2 (Figure 2.2). A qPCR study of a *Dehalobacter* sp. in 1,2-dichloroethane dehalogenating enrichment culture indicates that it harbors up to four 16S rRNA gene copies per genome(14), so it is plausible that a *Dehalobacter* sp. in the 1,2-DCB enrichment cultures also contains multiple 16S rRNA genes, one of which contains a 5'-insertion.

A 16S rRNA gene clone library from a purified 1,2-DCB dehalogenating culture had only two non-*Dehalobacter* sequences, one resembling *Desulfovibrio* spp., the other belonging to the genus *Sedimentibacter*. Sulfate reducers are common contaminants in anaerobic cultures, and *Sedimentibacter* spp. have commonly been found cultures involved in reductive dehalogenation(9, 36, 40, 41), thus, there may be specific interactions between *Sedimentibacter* spp. and reductive dehalogenators. We are presently attempting further purification of the 1,2-

DCB dehalogenating culture with the goal of obtaining an axenic 1,2-DCB dehalogenating *Dehalobacter* sp.

Despite our inability to culture MCB-dehalogenating organisms, the demonstration by qPCR of MCB-dependent growth of *Dehalobacter* spp. to a significant fraction of total bacteria is strong evidence that this genus plays a role in MCB dehalogenation in these microcosms, and apparently there can be at least two different phylotypes capable of this reaction which are distinct from those from DCB-utilizing cultures (Figure 2.2).

Dehalobacter restrictus PER-K23 was the first organism described to be able to dehalogenate tetrachloroethene to *cis*-dichloroethene(19), and was found to use only H_2 as the electron donor and chlorinated ethenes as electron acceptors. Since that time, formate has also been shown to serve as an electron donor for another *Dehalobacter* sp. isolate(35), and other chlorinated electron acceptors used by pure and mixed cultures of *Dehalobacter* spp. include 1,1,1-trichloroethane(35), 1,2-dichloroethane(14), 4,5,6,7-tetrachlorophthalide(40), β-hexachlorocyclohexane(36), and trichloromethane(13). Additionally, *Dehalobacter*-related sequence SJA-19 was found in a trichlorobenzene-dehalogenating consortium(38), and in light of the data presented here, it is possible *Dehalobacter* spp. played a role in the reductive dehalogenation of DCBs to MCB in that culture.

This publication demonstrates that *Dehalobacter* spp. are involved in the reductive dehalogenation of chlorobenzenes, and it is of interest that the two strains described in a culture dehalogenating tetrachlorophthalide, another aromatic compound, have 16S rRNA gene sequences most closely related to those from 1,2-DCB, 1,4-DCB, and MCB utilizing cultures described here. Thus, evidence is accruing that *Dehalobacter* spp., specialized dehalogenators

like *Dehalococcoides* spp., can play important roles in reductive dehalogenation at chloroaromatic-contaminated sites.

Acknowledgements

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CHAPTER THREE

CHARACTERIZATION OF THREE *DEHALOBACTER* SPP. CULTURES THAT REDUCTIVELY DEHALOGENATE DIFFERENT DICHLOROBENZENE ISOMERS

Abstract

Previously, we described three cultures highly enriched with *Dehalobacter* spp. that reductively dehalogenated each of the three dichlorobenzene (DCB) isomers to mainly monochlorobenzene (MCB). In this study, *Dehalobacter* spp. enriched on either 1,2-DCB or 1,3-DCB were isolated from enrichment cultures and denoted *Dehalobacter* sp. strain 12DCB1 and 13DCB1, respectively. Extracts from a *Sedimentibacter* sp. isolated from the 1,2-DCB enrichment allowed growth of *Dehalobacter* sp. strains 12DCB1 or 13DCB1 at 10^{-8} dilutions, but were not required for growth afterwards. A third *Dehalobacter* sp. from a 1,4-DCB dehalogenating enrichment culture was not isolated but was shown to be dominant in that culture and is denoted as strain 14DCB1. Strains 12DCB1 and 13DCB1 required acetate, vitamins cyanocobalamin and thiamine, and a chlorinated electron acceptor for growth and utilized H_2 and formate as electron donors. Besides the DCB isomers on which they were enriched, strain 13DCB1 could grow on 1,2-DCB and strain 14DCB1 could use 1,2-DCB slowly. Each of these cultures has distinct capabilities, and these results provide insight into the diversity of *Dehalobacter* spp. that dehalogenate chlorobenzenes.

Introduction

Dichlorobenzene isomers (DCBs) have long been used in the commercial production of dyes, pesticides, and as industrial solvents (6). While DCBs can be degraded aerobically (13), they readily form dense non-aqueous phase liquids and migrate to anaerobic zones. Costly treatment measures such as excavation or the pumping large of quantities of oxygen into anaerobic environments may not be feasible, thus taking advantage of anaerobic microorganisms that reductively dehalogenate DCBs and derive energy from this process is a potential bioremediation strategy. Moreover, understanding the fate of DCBs in anaerobic zones could help elucidate the most feasible treatment strategies for contaminated sites.

Only the *Dehalococcoides* spp. have been shown to dehalogenate chlorinated benzenes in pure culture. *Dehalococcoides* sp. CBDB1 dehalogenates chlorobenzenes with three or more chlorines to 1,3,5-trichlorobenzene (TCB) and DCBs (2), and *Dehalococcoides ethenogenes* strain 195 dehalogenates hexachlorobenzene (HCB) to 1,2,3,5-tetrachlorobenzene (TeCB) and 1,3,5-TCB (8). Another isolate and member of the *Chloroflexi*, bacterium DF-1 dehalogenates HCB to 1,3,5-TCB (26). In addition to members of the *Chloroflexi,* five 16S rRNA clones from a *Dehalococcoides*-containing mixed culture dehalogenating TCBs had 98.8-99.4% sequence identity to *Dehalobacter restrictus* 16S rRNA genes (25), but it was not determined whether organisms harboring these genes played a role in the reductive dehalogenation of TCBs.

Recently, our laboratory described three different *Dehalobacter*-containing enrichment cultures that dehalogenated individual DCB isomers (19), however, no pure cultures of DCB dehalogenating organisms have yet been described. An enrichment using butyrate as the electron donor and 1,2-DCB as the electron accepter was further purified through a process that included minimizing organic nutrients that support growth of contaminants, removal of methanogens

using the inhibitor bromoethanesulfonate (BES), and removal of hydrogenotrophic acetogens by continued culture using butyrate as the electron donor (which poises the H_2 concentrations below those used by acetogens (21)), followed by a 10^{-6} dilution into growth medium with H_2 as the electron donor. According to quantitative PCR studies, the dominant phylotype in the resulting H_2/1,2-DCB culture was *Dehalobacter* spp., and a 16S rRNA gene clone library was dominated by clones most closely related to *Dehalobacter* sp. FTH2 (27), though clones most closely related to *Desulfovibrio* spp. and *Sedimentibacter* spp. were also detected and are often associated with dehalogenating cultures (24, 28). Further dilution of this culture did not remove the contaminants. Similar purifications were done for enrichments using 1,3-DCB and 1,4-DCB, except that mevinolin, an inhibitor of archaeal lipid synthesis, was used to remove methanogens from the 1,3-DCB culture because BES was inhibitory to dehalogenation (19).

In this study, we describe the isolation of *Dehalobacter* spp. from cultures using 1,2-DCB and 1,3-DCB, and further characterize a *Dehalobacter* sp. that dominated 1,4-DCB dehalogenating enrichment cultures. The strains have been labeled *Dehalobacter* sp. strain 12DCB1, *Dehalobacter* sp. strain 13DCB1, and *Dehalobacter* sp. strain 14DCB1, respectively and each organism has a unique DCB dehalogenation spectrum. Strains 12DCB1 and 13DCB1 are the first examples of pure cultures capable of dehalogenating DCBs to MCB (and trace amounts of benzene), and the first *Dehalobacter* spp. shown to dehalogenate chlorobenzenes.

Materials and Methods

Chemicals

Dichlorobenzenes, monochlorobenzene, and benzene were purchased from Sigma Aldrich at the highest available purity (1,3-DCB: 98%, 1,2-DCB and 1,4-DCB: 99%, MCB:

99.9% HPLC grade). 1,4-DCB was dissolved in hexadecane to make a 4 M solution. All gases were purchased from Airgas East (Elmira, NY).

Cultivation of *Dehalobacter* spp. and *Sedimentibacter* sp.

Early enrichment procedures have been described previously (19), but briefly, 50 mL CBDB1 medium was added to 160 mL serum vials and amended with 12 mM $NaHCO_3$, 2 mM sodium acetate, 20 mg/L Casamino Acids, a vitamin solution containing 0.05 mg/L Vitamin B_{12} (17), and either 0.8 mM Na_2S or 1 mM amorphous FeS (5). 1,2-DCB and 1,3-DCB were added neat to a hexadecane layer (1 mL per 100 mL medium) and 1,4DCB was added as a 4 M solution in hexadecane, allowing large amounts to be added while keeping the aqueous concentration low (19). Cultures were incubated at 30 ˚C in the dark, shaking at 300 RPM in a New Brunswick rotary shaker.

Isolated colonies of *Sedimentibacter* sp. were obtained during attempts to isolate the 1,2-DCB dehalogenators using the roll tube technique (16). 7 mL anaerobic CBDB1 medium and 0.15 g agar were combined in 22 mL glass crimp-top tubes and autoclaved. The molten mixture was flushed with N_2-CO_2 (70%-30%) and amended with 12 mM $NaHCO_3$, 2 mM sodium acetate, 0.8 mM Na_2S, 60 mg/L Casamino Acids (CAAs), and a vitamin solution containing 0.05 mg/L Vitamin B_{12} (17). Tenfold serial dilutions were inoculated into the molten agar and immediately solidified by rolling the tube horizontally in an ice bath. 1 mM 1,2-DCB dissolved in hexadecane and 1 mL 80% H_2/20% CO_2 were added after the suspension had solidified and the tubes were upright. Agar roll tubes were incubated at 30 ºC, static, in the dark until the appearance of colonies.

Sedimentibacter sp., isolated from an agar roll tube containing a 10^{-5} dilution (see Results), was grown in 1 L CBDB1 medium amended as above except 500 mg/L CAAs was added approximately every 10 days for 30 days of incubation. *Sedimentibacter* sp. cell extract was obtained from this culture via centrifugation, resuspension of the pellet in 20 mL of distilled H_2O, passage 3x through a French pressure cell, and anaerobic filter sterilization. This extract was added (2% v/v) to 10^{-8} dilutions of 1,2-DCB and 1,3-DCB enrichment cultures.

Analytical procedures

DCBs, MCB, and benzene were measured using headspace analyses of 0.1 mL samples using flame-ionization detection gas chromatography (FID/GC) as described previously (9). Concentrations of chlorobenzenes are nominal rather than aqueous.

DNA extraction, PCR, quantitative PCR, and primers

Genomic DNA was extracted using the FastDNA Spin for Soil Kit (MP Biomedicals, LLC, Solon, OH) according to the manufacturer's protocol. DNA was extracted from 1.6 mL of culture and eluted in 55 µL kit-provided sterile, deionized water. PCR amplifications were performed using universal 16S rRNA gene primers 27f and 1492r or *Dehalobacter* specific 16S rRNA primers 110f and 1273r (19), and PCR products were sequenced at the Life Sciences Core Laboratory Center at Cornell University.

Quantitative PCR (qPCR) was performed as previously described (19) to estimate populations of *Dehalobacter* and total bacteria using *Dehalobacter*-specific primers Dhb 477f 5'-GATTGACGGTACCTAACGAGG-3' and Dhb 647r 5'-TACAGTTTCCAATGCTTTACGG-3' (11), and universal bacterial primers 331f 5'-

TCCTACGGGAGGCAGCAGT-3' and 797f 5'-GGACTACCAGGGTATCTAATCCTGTT-3' (18). The reaction mixture (25 µL total volume) contained 12.5 µL iQ SYBR Green Super Mix (BioRad, Hercules, CA) 200 nM of both forward and reverse primers, and 1 µL template DNA.

Sedimentibacter-specific 16S rRNA primers were designed and used to test purity of 1,2-DCB and 1,3-DCB dehalogenating enrichment cultures. Two forward primers and one reverse primer were developed: Sed74f 5'-TCGGATATGCTTAGTGGC-3', Sed224f 5'-TAGAGGTACAGGATGAGTTCG-3', and Sed1058r 5'-AGGAGAGGGCACATCTCTG-3'.

Electron Donor and Acceptor Experiments

Potential electron donors for the three cultures were examined by adding sterile anaerobic solutions to a concentration of 5 mM with the exception of ethanol and glucose (4 mM) with the appropriate DCB as electron acceptors. Potential electron acceptors other than haloorganics were added at a concentration of 2 mM along with H_2 as an electron donor. Oxygen as electron acceptor was tested by added 3 mL air to vial headspaces with H_2 as electron donor. Electron donors were considered positive if they allowed significant reductive dehalogenation of the DCB substrate. Growth with various electron acceptors was assayed by qPCR of the cultures using *Dehalobacter*-specific primers.

Doubling Time Estimation

Doubling times for *Dehalobacter* spp. strains were estimated by plotting the accumulation of MCB from dehalogenation of individual DCB isomers. A trend line was fitted through the period of exponential MCB production in triplicate cultures, akin to exponential bacterial growth, and used to estimate doubling times. To avoid biases associated with the

possibility dehalogenation could become uncoupled to cell growth, calculations were made at early time points during the incubation, before cultures had consumed 1 mmol/L DCB.

Nucleotide Sequence Accession Numbers

The nucleotide sequences discussed have been deposited into GenBank, accession numbers JQ918080-918083.

Results

Amorphous FeS increases 1,2-DCB dehalogenation in enrichment cultures

Dehalobacter-containing enrichment cultures that dehalogenated individual DCB isomers were described previously (19). 1,2-DCB dehalogenating enrichment cultures were first reduced with Na_2S, but growth was slow and unreliable. Since sulfide toxicity was a potential cause of poor growth, cultures were instead reduced with amorphous FeS (5) in which sulfide is sequestered as a black precipitate, which resulted in considerably faster dehalogenation of 1,2-DCB (Figure 3.1) and more reliable transfers of activity. 1,3-DCB and 1,4-DCB dehalogenating enrichment cultures also showed more rapid dehalogenation when amorphous FeS replaced Na_2S.

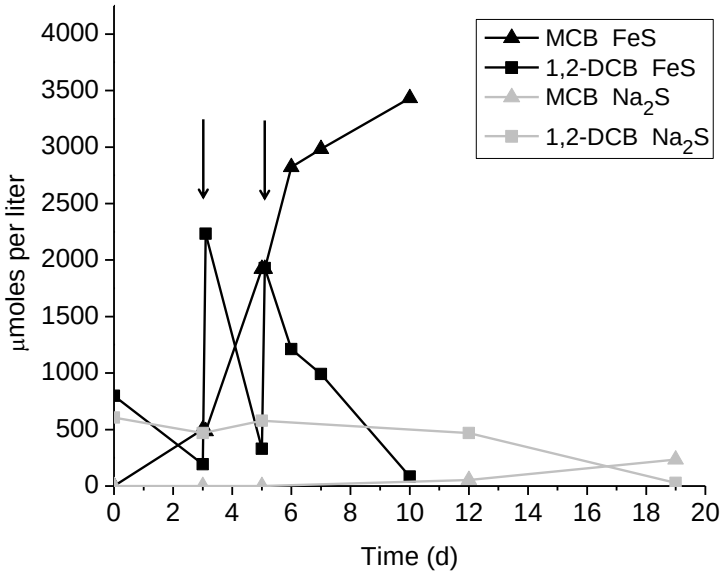

Figure 3.1. Reductive dehalogenation of 1,2-dichlorobenzene (squares) to monochlorobenzene (triangles) in cultures reduced with amorphous FeS precipitates (black) or Na_2S (gray). Results from single culture vials are presented, but duplicate vials produced similar results. Arrows indicate additional amendments of 1,2-DCB.

Isolation of 1,2-DCB and 1,3-DCB Dehalogenating *Dehalobacter* sp. Strains 12DCB1 and 13DCB1

As previously reported, *Dehalobacter* sp. 16S rRNA genes outnumbered those of contaminating organisms by approximately two orders of magnitude (10^8-10^9 vs. 10^6-10^7 16S rRNA genes/mL) in purified 1,2-DCB enrichment cultures, and dominated a 16S rRNA gene clone library (80 of 82 clones) that also contained one clone each of a *Sedimentibacter* sp. and a *Desulfovibrio* sp. (19). Tenfold serial dilutions into liquid medium were repeatedly performed on the purified 1,2-DCB culture. However, DCB dehalogenation was not detected in dilutions greater than 10^{-6}, despite the estimated presence of $\geq 10^8$ dehalogenators/mL in the original inoculum, and contaminating organisms were still present in the DCB-dehalogenating diluted

cultures. Higher dilutions (10^{-7}, 10^{-8}, and 10^{-9}) did not show DCB dehalogenation in numerous dilution attempts.

In another attempt to isolate of 1,2-DCB dehalogenators, 1,2-DCB-amended agar roll tubes were used to grow organisms from tenfold serial dilutions of the 1,2-DCB culture. After 25 days of incubation, tiny (~1 mm) colonies were visible in 10^{-5} dilution roll tubes. No MCB was detected in the tubes, indicating that DCB dehalogenation did not occur, and we presumed the colonies had grown with using the 60 mg/L CAAs in the medium. These colonies were inoculated into liquid CBDB1 medium amended with 200 mg/L CAAs, whereby a *Sedimentibacter* sp. (JQ918080) was isolated and identified by partial 16S rRNA gene sequence (>99% sequence identity to *Sedimentibacter* sp. C7 AY766466). No DCB dehalogenation was detected in any of the roll tubes incubated for longer periods. No *Dehalobacter* spp. were detected in the cultures using specific PCR primers (19), and the *Sedimentibacter* sp. cultures did not dehalogenate DCBs but did grow anaerobically on CAAs or pyruvate, similar to previously described strains (4) (data not presented). The cultures only contained rods longer and thicker than *Dehalobacter* spp. and we considered the culture axenic.

As *Sedimentibacter* spp. have been found in several different dehalogenating consortia (7, 24, 27, 28), we hypothesized this organism was providing one or more required nutrients to the *Dehalobacter* spp. in the culture, thereby explaining our inability to obtain growth in liquid dilutions past 10^{-6}. We amended 10^{-8} dilutions of the 1,2-DCB culture with either inocula of *Sedimentibacter* sp. cells or extracts of *Sedimentibacter* sp. from CAA-grown cells (Figure 3.2). 10^{-8} dilution cultures with no *Sedimentibacter* sp. addition did not dehalogenate 1,2-DCB after several weeks of incubation whereas cultures receiving the *Sedimentibacter* sp. inocula dehalogenated the entire DCB dose in less than seven days (Figure 3.2).

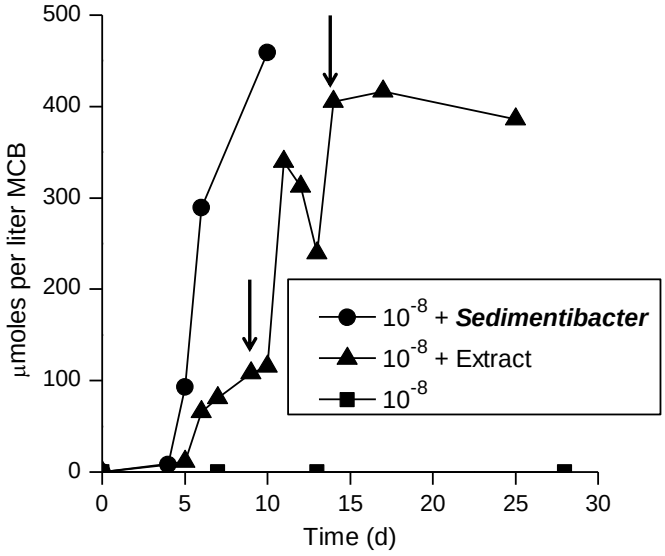

Figure 3.2. Monochlorobenzene (MCB) produced from 1,2-dichlorobenzene (DCB) dehalogenation in 10^{-8} dilution cultures amended with *Sedimentibacter* sp. co-inoculum (circles), *Sedimentibacter* sp. extract (triangles), or no additional amendment (squares). Arrows indicate additional amendments of *Sedimentibacter* sp. extract. Single representatives from "10^{-8} + *Sedimentibacter*" vials and "10^{-8}" vials are presented but were performed in triplicate with similar results. Quadruplicate "10^{-8} + Extract" vials were prepared though the culture presented was the only one to dehalogenate 1,2-DCB. DCB was amended at approximately 500 µmol/L and are not shown.

1,2-DCB dehalogenation occurred in only one of four replicate 10^{-8} dilutions receiving *Sedimentibacter* sp. extract and stalled after producing ca. 400 µmol/L MCB from 1,2-DCB dehalogenation (Figure 3.2). Additional amendments of *Sedimentibacter* sp. extract did not rescue the stalled culture. 1% transfers from the stalled culture into medium containing *Sedimentibacter* sp. extract behaved similarly with only one of four replicates dehalogenating 1,2-DCB. Subsequent transfers dehalogenated more reliably and, after three transfers, regained the ability to dehalogenate over 7 mmol/L 1,2-DCB to MCB and trace amounts of benzene (Figure 3.3A), and surprisingly, control cultures not receiving *Sedimentibacter* sp. extract also grew, although initially these cultures showed a lag in dehalogenation between 8-11 days

compared to cultures receiving extract (Figure 3.3). In subsequent transfers the lag was not observed.

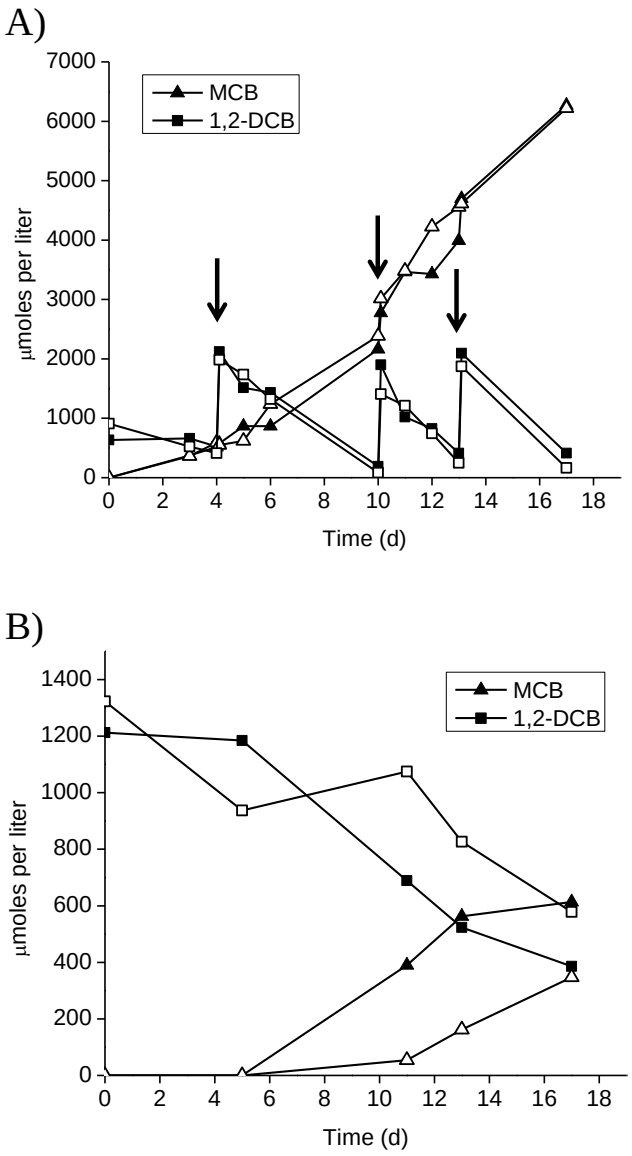

Figure 3.3. Dehalogenation of 1,2-dichlorobenzene (DCB) (squares) to monochlorobenzene (MCB) (triangles) by strain 12DCB1 in cultures amended with *Sedimentibacter* sp. extract (A) or without extract (B). Duplicate cultures are represented by open and closed symbols. Arrows indicate additional amendments of 1,2-DCB.

Highly enriched 1,3-DCB dehalogenating cultures underwent the same 10^{-8} dilution procedures as above, and dehalogenation was observed in four of five replicates amended with

Sedimentibacter sp. extract. Dehalogenation in these cultures lagged between 3-12 days compared to 10^{-8} dilutions receiving *Sedimentibacter* sp. inocula. Similar to the 1,2-DCB culture, after a lag, cultures of the 1,3-DCB dehalogenating organism also grew without *Sedimentibacter* sp. extract (data not presented).

We were concerned that the cultures derived from 10^{-8} dilutions with *Sedimentibacter* sp. extracts were not axenic, with particular concern that viable cells of *Sedimentibacter* sp. or another organism was present in a filter-sterilized extract preparation. To test this proposition, cultures derived from 10^{-8} dilution cultures containing *Sedimentibacter* sp. or *Sedimentibacter* sp. extract were amended with 500 mg/L CAAs and not given DCBs. Figure 3.4 shows total bacterial numbers increased over two orders of magnitude after 23 days in 1,2-DCB cultures amended with cells of *Sedimentibacter* sp., however, total bacterial 16S rRNA gene copies in cultures derived from extract-amended 10^{-8} dilution cultures did not increase and were approximately the same concentration as *Dehalobacter*-specific 16S rRNA gene copies, which did not increase because DCBs were omitted from the medium. 1,3-DCB cultures were analyzed with qPCR as above after 19 days with similar results (Figure 3.5).

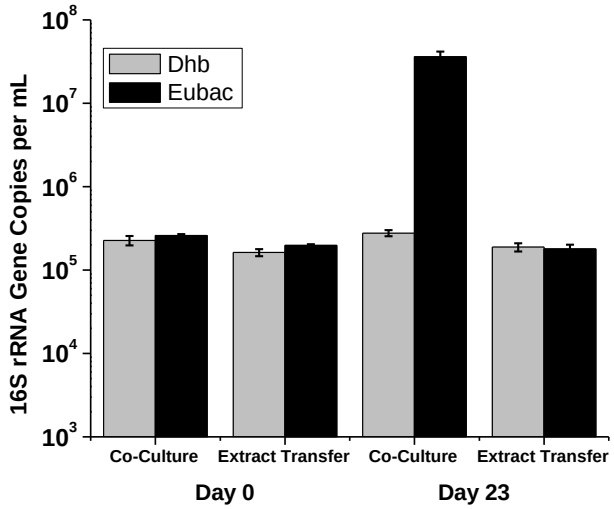

Figure 3.4. Quantitative PCR of *Dehalobacter*-specific and total bacterial 16S rRNA genes in cultures derived from a 1,2-dichlorobenzene (DCB) dehalogenating culture and amended with 500 mg/L Casamino Acids and *Sedimentibacter* sp. (Co-Culture) or *Sedimentibacter* sp. extract (Extract Transfer). DCBs were omitted from the culture medium. Error bars represent the standard deviation of the mean of duplicate DNA samples.

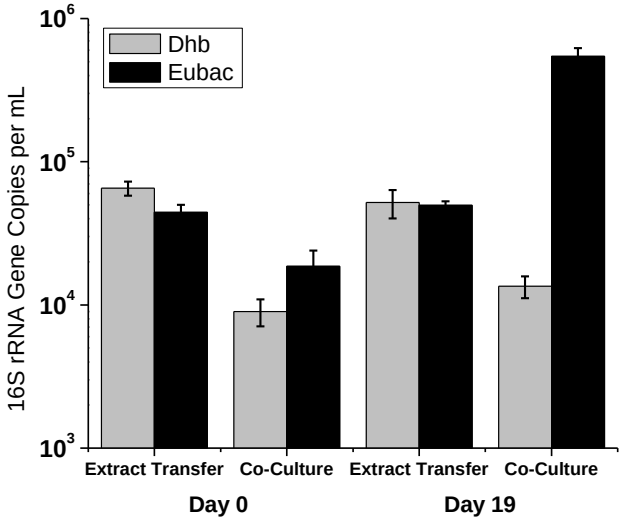

Figure 3.5. Quantitative PCR of *Dehalobacter*-specific and total bacterial 16S rRNA genes in cultures derived from a 1,3-dichlorobenzene (DCB) dehalogenating culture and amended with 500 mg/L Casamino Acids and *Sedimentibacter* sp. extract (Extract Transfer) or *Sedimentibacter* sp. (Co-Culture). DCBs were omitted from the culture medium. Error bars represent the standard deviation of duplicate DNA samples.

Microscopic analysis revealed only one cell morphology in either 1,2-DCB or 1,3-DCB dehalogenating cultures (Figure 3.6 A-D), and no PCR products were observed with *Sedimentibacter*-specific 16S rRNA gene primers in these cultures (data not presented). 1,2-DCB and 1,3-DCB dehalogenating *Dehalobacter* spp. were assumed to be pure cultures and are denoted *Dehalobacter* sp. strain 12DCB1 and *Dehalobacter* sp. strain 13DCB1, respectively. In our previous study (19), a 1,2-DCB enrichment culture 16S rRNA gene clone library detected two different *Dehalobacter* sp. 16S rRNA sequences that were identical except for an ca. 100 base pair 5'- insertion found in 41% of the *Dehalobacter* sp. clones. 16S rRNA gene sequences amplified from strain 12DCB1 using 27f and 1492r with and without the 5'- insertion (accession numbers JQ918082 and JQ918083) were identical with the partial sequences denoted 1,2-DCB RFLP Types 1 and 2 (JN051265 and JN051264) described in our previous study (19). The 16S rRNA sequence amplified from strain 13DCB1 (JQ918081) was identical with the partial sequence 1,3-DCB_Gen8 (JN051266) from our previous study. The 16S rRNA genes from strains 12DCB1 and 13DCB1 were 98% identical (1371/1400 nucleotide identity) to each other. Strain 12DCB1 was over 99% identical (1516/1525 nucleotide identity) to a 4,5,6,7-tetrachlorophthalide dehalogenating *Dehalobacter* sp. FTH2 AB294743 (27) while strain 13DCB1 was over 99% identical (1532/1547 nucleotide identity) to an uncultured *Dehalobacter* sp. clone SJA-19 AJ009454 detected in a trichlorobenzene dehalogenating consortium (25).

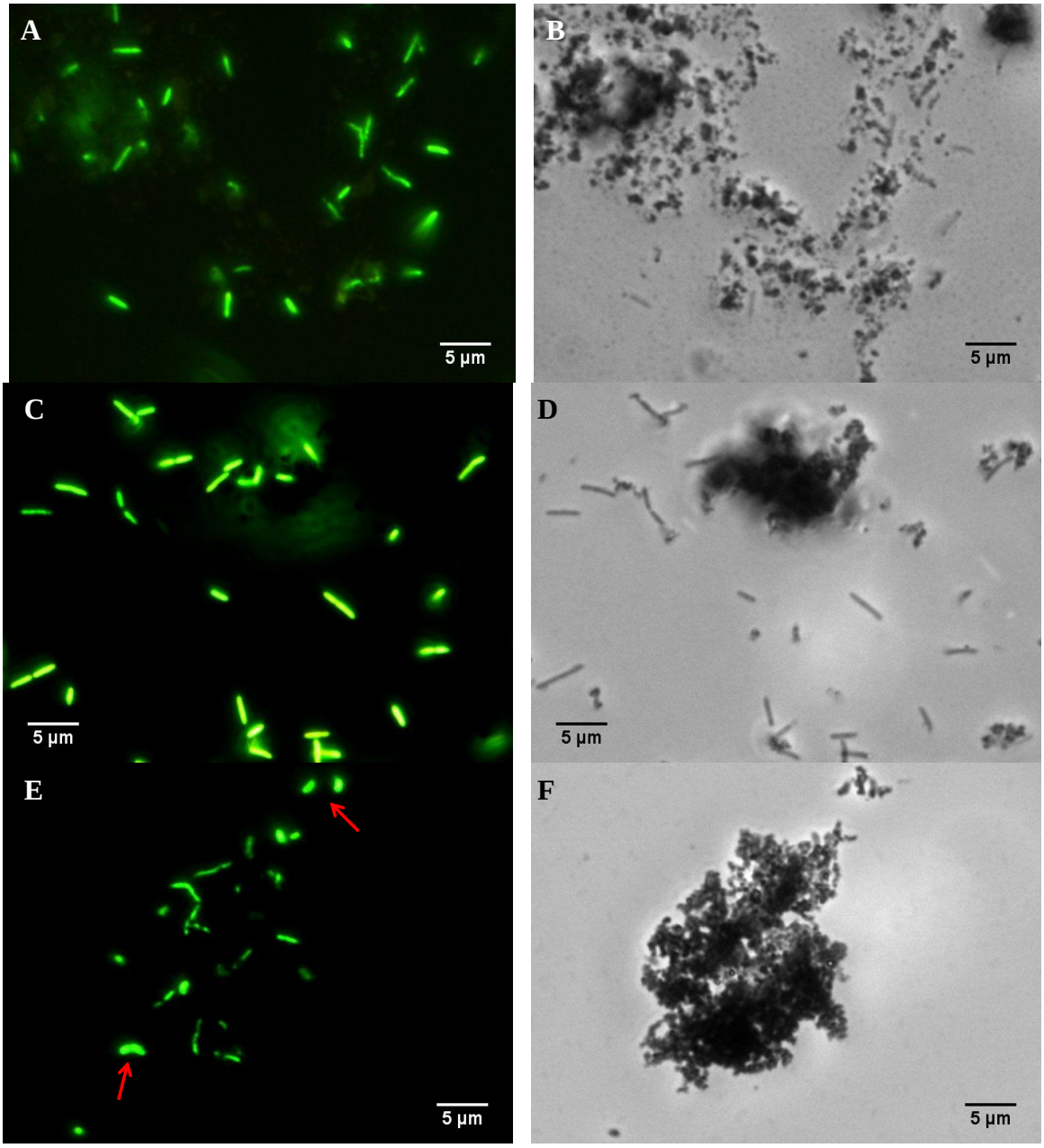

Figure 3.6. Acridine orange fluorescent staining (A, C, E) and phase contrast micrographs (B, D, F) of *Dehalobacter* sp. strains 12DCB1 (A & B), 13DCB1 (C & D), and 14DCB1 (E & F). All of the tested strains have been visualized in variable association with amorphous FeS precipitates added to cultures as a reducing agent. Red arrows in panel E point to probable culture contaminants, tentatively identified as *Desulfovibrio* sp.

1,4-DCB Dehalogenating *Dehalobacter* sp. Enrichment Culture

A highly enriched 1,4-DCB dehalogenating culture was described previously (19). Unlike 1,2-DCB and 1,3-DCB dehalogenating cultures, the slow-growing1,4-DCB cultures showed inconsistent growth at high dilutions and were not tested with *Sedimentibacter* sp. inocula or culture extracts. Instead, CAAs were omitted from the culture medium, which resulted in a ca. 4 day lag in dehalogenation, which was not found in subsequent transfers (data not presented). Tenfold serial dilutions dehalogenated out to 10^{-6} in the absence of CAAs. While initial results indicated the lack of growth of *Sedimentibacter* in cultures lacking 1,4-DCB and amended with CAAs, more recently we detected a motile curved rod in those cultures. DNA extraction from these cultures and PCR amplification with universal 16S rRNA primers followed by direct sequencing of PCR product tentatively identified a *Desulfovibrio* sp. (Figure 3.6 E), so the 14DCB1 culture is not axenic. The 16S rRNA gene sequence from the culture containing strain 14DCB1 (JN051267) was described previously (19) and differed by only two nucleotides (935/937) from the 16S rRNA genes of strain 12DCB1.

Strain Characteristics

Phase contrast microscopy and acridine orange fluorescent staining were used to visualize the three *Dehalobacter* sp. strains. Each culture was dominated by rod-shaped organisms presumed to be *Dehalobacter* spp. that measured 2.2 ± 0.3 by 0.51 ± 0.09 µM (n=10) (strain 12DCB1), 2.9 ± 1.0 by 0.68 ± 0.06 µM (n=10) (strain 13DCB1), and 2.0 ± 0.8 by 0.43 ± 0.05 µM (n=10) (strain 14DCB1) (Figure 3.6). In all cultures, cells were variably imaged in association with amorphous FeS precipitates added as reducing agent, though specific interactions between *Dehalobacter* spp. and FeS precipitates were not investigated.

All three cultures have adapted, after transient lag periods, to medium lacking CAAs, and have been routinely transferred. *Dehalobacter* sp. strains 12DCB1 and 13DCB1 required acetate, H_2, a chlorinated electron acceptor, and vitamins cyanocobalamin and thiamine for growth in CBDB1 medium (1). Strains 12DCB1 and 13DCB1 dehalogenated at temperatures between 15 ºC and 37 ºC, and strain 14DCB1 in enrichment culture dehalogenated at 22 ºC but was unable to dehalogenate at 15 ºC. Temperatures between 22 ºC and 15 ºC were not tested. No dehalogenation by any of the three strains occurred at 10 ºC (data not shown).

Utilization of DCBs as electron acceptors.

Of the DCB isomers, only 1,2-DCB was dehalogenated by strain 12DCB1, with an estimated doubling time from MCB production of 8.6 ± 0.2 h (n=3) at 30 ºC (Figure 3.7). Strain 13DCB1 could use either 1,2-DCB or 1,3-DCB with somewhat slower estimated doubling times, 13.5 ± 1.8 h (n=3) on 1,3-DCB at 30 ºC (Figure 3.8A) and 11.0 ± 1.9 h (n=3) on 1,2-DCB at 30 ºC (Figure 3.8B). To investigate whether 1,2-DCB utilization by the 1,3-DCB culture was carried out by a strain different from 13DCB1, such as 12DCB1, we examined the 16S rRNA gene sequences of cultures grown on 1,2-DCB using both universal bacterial and *Dehalobacter*-specific primers (see methods) and found they were identical with the 13DCB1 sequence.

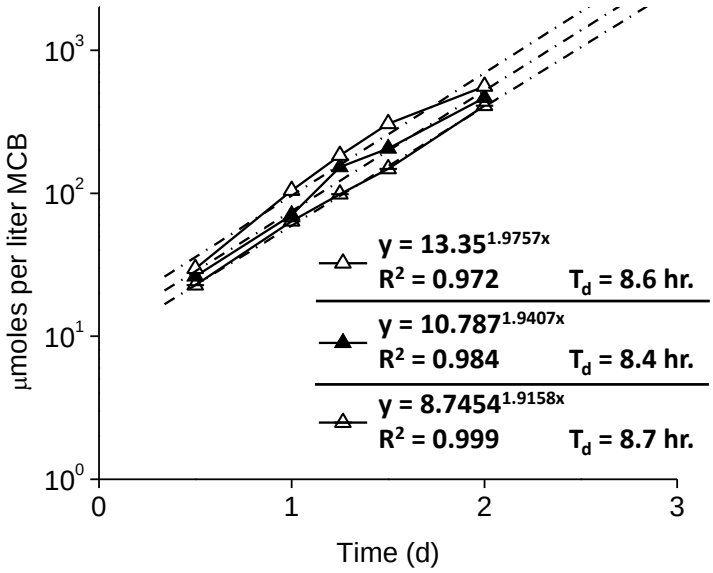

Figure 3.7. Doubling time estimation at 30 ºC of *Dehalobacter* sp. strain 12DCB1 calculated during exponential monochlorobenzene (MCB) production from 1,2-dichlorobenzene (DCB) dehalogenation in triplicate cultures. Trend lines are shown as dashed lines. 1,2-DCB is not shown.

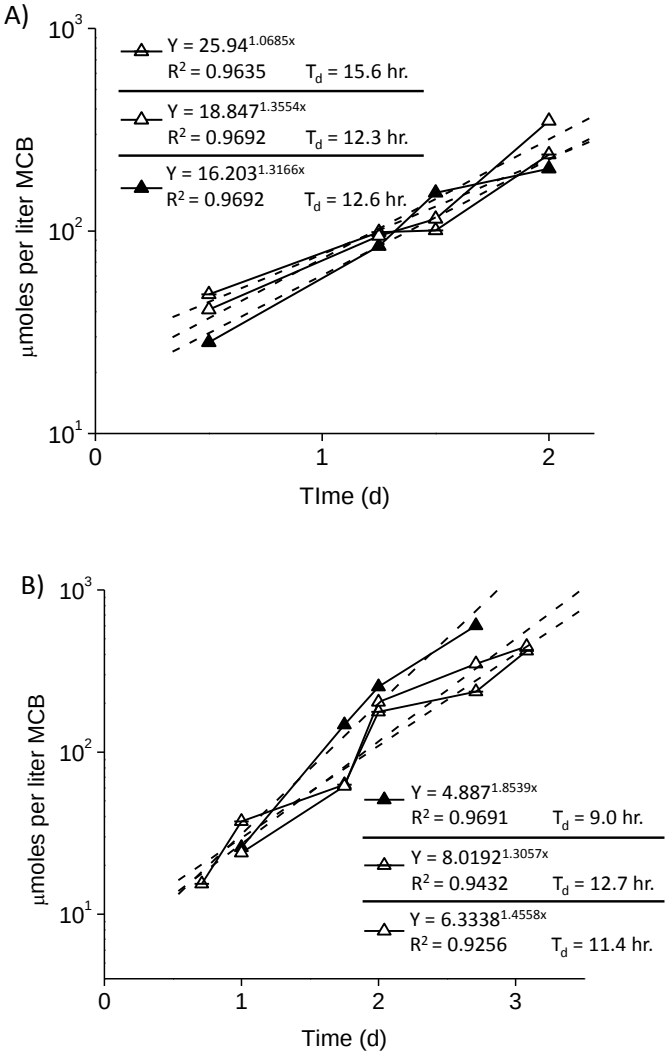

Figure 3.8. *Dehalobacter* sp. strain 13DCB1 doubling time estimation at 30 ºC calculated during exponential monochlorobenzene (MCB) production when dehalogenating 1,3-dichlorobenzene (DCB) (A) or 1,2-DCB (B) in triplicate cultures. Trend lines are shown as dashed lines. DCBs are not shown.

Besides dehalogenating1,4-DCB to MCB with an estimated doubling time of 17.5 ± 1.9 h (n=3) (Figure 3.9), strain 14DCB1 even more slowly dehalogenated 1,2-DCB to MCB (Figure 3.10). In duplicate cultures, 1,4-DCB (1 mmol/L total nominal starting concentration) was dehalogenated to 782 and 567 µmol/L MCB after 10 days, while cultures given 1,2-DCB (1 mmol/L) produced 34 and 13 µmol/L MCB after 10 days, and 152 and 160 µmol/L MCB after

48 days. Consistent with previous results, qPCR of DNA samples taken after 51 days showed 16S rRNA genes in 1,4-DCB-containing cultures increased by approximately two orders of magnitude (19) while those containing 1,2-DCB increased by less than one order of magnitude, suggesting minimal growth. No increase in *Dehalobacter* spp. 16S rRNA genes was observed in controls without DCBs (data not presented).

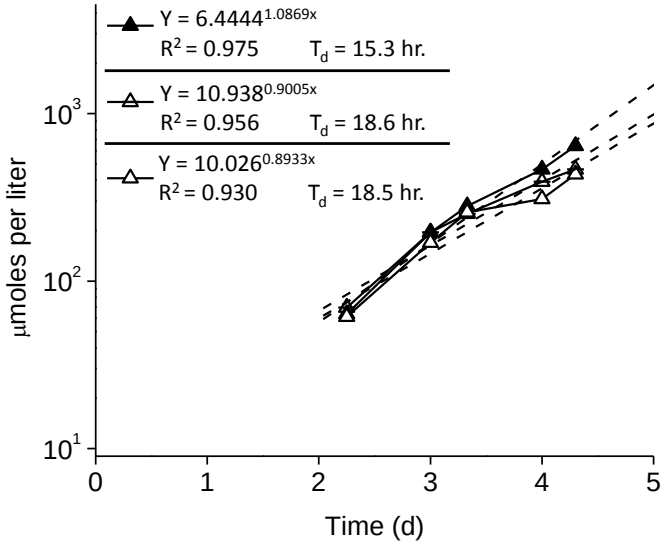

Figure 3.9. Doubling time estimation at 30 ºC of *Dehalobacter* sp. strain 14DCB1 calculated during exponential monochlorobenzene (MCB) production from 1,4-dichlorobenzene (DCB) dehalogenation in triplicate cultures. Trend lines are shown as dashed lines. 1,4-DCB is not shown.

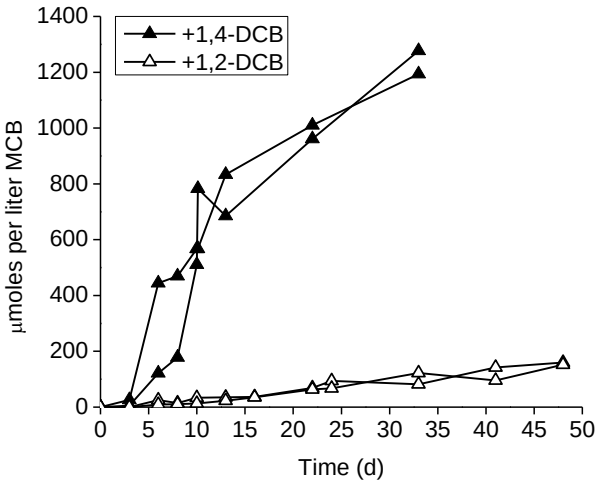

Figure 3.10. Monochlorobenzene (MCB) production from 1,4-dichlorobenzene (DCB) dehalogenation (closed triangles) or 1,2-DCB (open triangles) by *Dehalobacter* sp. strain 14DCB1 in duplicate cultures. DCBs are not shown.

Electron donor and non-halogenated electron acceptor utilization

Dehalobacter sp. strains 12DCB1 and 13DCB1 and the enrichment culture dominated by strain 14DCB1 were tested with several electron donors (H_2, formate, methanol, ethanol, glucose, succinate, glycerol, butyrate, acetate, pyruvate, and fumarate). All strains readily dehalogenated their respective DCB isomers when H_2 was provided as electron donor. When H_2 was substituted with formate, all three strains also dehalogenated their respective DCB isomer to MCB. Strains 13DCB1 and 14DCB1 continued to dehalogenate DCBs upon transfer with formate (data not presented) while strain 12DCB1 exhibited variable dehalogenation with formate, and activity could not be reliably transferred to fresh culture medium. Strain 14DCB1 also dehalogenated with ethanol as an electron donor, but a second morphotype was visible in that culture suggesting syntrophy. Thus neither formate nor ethanol can be claimed as an electron donor for strain 14DCB1 in the absence of an axenic culture. DCB dehalogenation was not observed with any of the other tested electron donors. qPCR with specific primers showed

no growth of any *Dehalobacter* sp. strain occurred when fumarate, nitrate, nitrite, sulfate, sulfite, glycine, or oxygen replaced DCBs as electron acceptor (data not presented). In strain 12DCB1 and 13DCB1 cultures, no bacterial growth was detected using qPCR with universal bacterial primers or via microscopic analysis with alternate electron donors or acceptors (data not presented). When sulfate replaced 1,4-DCB as electron acceptor in the mixed culture containing strain 14DCB1, microscopic analysis showed curved rods, likely *Desulfovibrio* sp., dominated cultures.

Discussion

In our previous study (19), we developed three separate enrichment cultures on each of the DCB isomers. Partial 16S rRNA gene sequences amplified using *Dehalobacter* sp. specific primers indicated that strain 13DCB1 had a 16S rRNA gene sequence distinct from those in strains 12DCB1 and 14DCB1, which were nearly identical.

Each of the *Dehalobacter* spp. presented here had a distinct DCB dehalogenation spectrum. Despite having nearly identical 16S rRNA gene sequences, strain 12DCB1 could not use 1,4-DCB, and 14DCB1 only showed low activity on 1,2-DCB, though neither strain 12DCB1 or 14DCB1 could dehalogenate 1,3-DCB. Strain 13DCB1 rapidly using either 1,2-DCB or 1,3-DCB, but not 1,4-DCB. Notably, mixed cultures in Nowak et al. (20) dehalogenated chlorobenzene isomers to predominantly MCB, and when these mixed cultures were adapted to either 1,2-DCB, 1,3-DCB, or 1,4-DCB, the adapted mixed cultures exhibited the same dehalogenation spectrum of DCBs as the strains presented here (e.g. the mixed culture adapted to 1,4-DCB dehalogenated 1,4-DCB rapidly and 1,2-DCB slowly and the 1,3-DCB-adapted culture could use 1,2-DCB or 1,3-DCB). That study did not identify microorganisms responsible for

chlorobenzene dehalogenation but *Dehalobacter* spp. 16S rRNA gene sequences were present in a version of that culture adapted to trichlorobenzenes (25). Moreover, other organisms, including *Dehalococcoides* spp., have not been shown to dehalogenate DCBs and the dehalogenation spectra of DCB-adapted cultures matched the spectra presented for strains 12DCB1, 13DCB1, and 14DCB1, We are presently examining the abilities of the three DCB strains to reductively dehalogenate other chloroorganics.

Our previous results (19) showed that the enriched 12DCB1 culture was numerically dominated by *Dehalobacter* spp. with over 10^8 cells per mL, assuming three copies of 16S rRNA genes per genome (10). However, we were unable to obtain growth in liquid dilutions beyond 10^{-6}, and no growth at all in agar roll tube dilutions. In the latter, we fortuitously obtained colonies of a *Sedimentibacter* sp. in roll tube dilutions of the 12DCB1 culture into medium containing CAAs as a nutrient. *Sedimentibacter* spp. have been found in numerous dehalogenating consortia including those dehalogenating chlorophenols (3, 28), chloroethenes (12), and 4,5,6,7-tetrachlorophthalide (27). In two separate cases, a *Dehalobacter* sp. dehalogenating β–hexachlorocyclohexane (24) and a *Dehalococcoides* sp. dehalogenating tetrachloroethene (PCE) to *trans*-dichloroethene (7), the dehalogenator was described as requiring the presence of a *Sedimentibacter* sp., for growth.

Because of these precedents (7, 24), we examined whether co-inoculation with the isolated *Sedimentibacter* sp., or addition of *Sedimentibacter* sp. extract, would allow growth of 10^{-8} dilutions of cultures containing strains 12DCB1 or 13DCB1. Both treatments allowed growth and we concentrated on the extract-containing cultures since the co-inoculated cultures would not be axenic. Surprisingly, extract-containing cultures grew better over time and eventually did not require addition of *Sedimentibacter* sp. extract at all. While we have no

confirmed explanation for this phenomenon, we speculate that *Sedimentibacter* sp., growing on low levels of CAAs in the cultures from which dilutions were made, produced one or more nutrients used by *Dehalobacter* sp. in the culture. In 10^{-7} dilutions or higher, *Sedimentibacter* sp. was no longer present, potentially depriving the small number of remaining *Dehalobacter* sp. cells of those nutrients and the *Dehalobacter* spp. were unable to make the transition to producing the nutrients themselves. Upon growth and transfer into medium with extract and limiting amounts of the hypothesized nutrients (growth was initially poor), *Dehalobacter* sp. began synthesizing the nutrients themselves and transfers into medium dehalogenated at high rates in extract-free medium. Clearly, there are alternative scenarios that may explain this phenomenon. Since both strains 12DCB1 and 13DCB1 exhibited the same phenomenon of initially requiring *Sedimentibacter* extracts after high dilutions, it appears that this was not an isolated event. Moreover, both cultures subsequently adapted to growth without CAAs. The slow adaptation of *Dehalococcoides ethenogenes* strain 195, which initially required extracts of mixed cultures (17), to grow in defined medium (14) may be a similar phenomenon.

Whatever the explanation for this phenomenon, we believe that the 1,2-DCB and 1,3-DCB cultures are axenic, which is supported by microscopic examination and the inability to detect growth by qPCR in cultures lacking DCBs (Figures 3.4 & 3.5) in contrast to the enriched cultures presented previously (19), which showed more than an order of magnitude increase in total bacteria when lacking DCBs, presumably due to growth of fermentative heterotrophs. That no bacterial growth was obtained with a variety of electron donors and acceptors indicates the lack of common contaminants like *Desulfovibrio* carrying out anaerobic respiration in 12DCB1 and 13DCB1 cultures. Strain 14DCB1 does contain *Desulfovibrio* as a contaminant, presumably growing on H_2 and traces of oxidized sulfur compounds.

It is more difficult to say that the cultures are single clones of *Dehalobacter* spp. The cultures went through several transfers and 10^{-6} dilutions on a single substrate followed by a 10^{-8} dilution, which is expected to be to extinction, supported by the lack of growth of some in the vials at this dilution. After a similar dilution scheme, the genome sequence of *Dehalococcoides ethenogenes* strain 195 showed no evidence of polymorphisms expected from multiple clones (22). One particular possibility, that the growth of strain 13DCB1 on 1,2-DCB was due to the presence of the growth of small initial numbers of strain 12DCB1, was eliminated by the finding that the 16S rRNA gene sequence of that culture was the same whether grown on 1,2-DCB or 1,3-DCB.

An alternative purification strategy was applied to the 1,4-DCB enrichment culture in that CAAs (the primary source of fermentable organic nutrients in these cultures, likely supporting a basal level of contaminants) were removed from the medium followed by a 10^{-6} dilution. Because of the presence of at least one other organism, we cannot conclude that formate and ethanol, both of which supported dehalogenation and growth, were used by strain 14DCB1. Moreover, when sulfate replaced 1,4-DCB as electron acceptor, the culture was dominated by motile curved rods, likely *Desulfovibrio* sp. as this organism was detected in 1,4-DCB cultures using and is known for reducing sulfate.

Dehalobacter spp. are known to grow on a limited range of substrates, using only halogenated organic compounds as electron acceptors and H_2 and occasionally formate as electron donors (15, 23), and the cultures presented here fit that pattern. *Dehalobacter restrictus* PER-K23 required acetate as a carbon source, the amino acids arginine, histidine, and threonine, and the vitamins thiamine and vitamin B_{12} (15). The three strains described here can grow in the absence of amino acids but did require thiamine and vitamin B_{12}. Strain PER-K23 grew on PCE

with a doubling time near 19 h, as estimated by product formation (15), and the doubling times of these cultures on DCBs, 8.6-17.5 h, are similar, as are the growth temperatures. The inability of these cultures to grow below 15^{o} C may limit their usefulness for groundwater bioremediation in northern latitudes unless lower temperature variants are found.

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CHAPTER FOUR

DEHALOGENATION OF CHLOROBENZENES, CHLORETHENES, AND CHLOROTOLUENES BY THREE *DEHALOBACTER* SPP.

Abstract

Three enrichment cultures containing *Dehalobacter* spp. were developed that dehalogenate each of the dichlorobenzene (DCB) isomers to monochlorobenzene (MCB), and the strains using 1,2-DCB (12DCB1) or 1,3-DCB (13DCB1) are now considered isolated. In this study, we examined the dehalogenation capability of each strain to use chlorobenzenes with three or more chlorines, tetrachloroethene (PCE), or dichlorotoluene (DCT) isomers. Strain 12DCB1 preferentially dehalogenated singly flanked chlorines, and did not dehalogenate doubly flanked or unflanked chlorines. It dehalogenated pentachlorobenzene to MCB, with little buildup of intermediates, and benzene, apparently produced via a cometabolic reaction, accounted for approximately half of measurable end-products. Strain 13DCB1, which could use either 1,3-DCB or 1,2-DCB, demonstrated the widest dehalogenation spectrum of electron acceptors tested, and dehalogenated every chlorobenzene isomer except 1,2,3,5-TeCB and 1,4-DCB. Notably, strain 13DCB1 dehalogenated the recalcitrant 1,3,5-trichlorobenzene isomer to MCB, and qPCR of 16S rRNA genes indicated growth of strain 13DCB1 was supported by this dehalogenation. Strain 14DCB1 exhibited the narrowest range of substrate utilization, but was the only strain to dehalogenate *para* substituted chlorines. Strains 12DCB1 and 13DCB1 dehalogenated PCE to *cis*-dichloroethene, and all strains dehalogenated 3,4-DCT to monochlorotoluene. These findings show that *Dehalobacter* spp., like *Dehalococcoides* spp., are versatile dehalogenators and should be considered when determining the fate chlorinated organics at contaminated sites.

Introduction

Chlorobenzenes and chloroethenes are common environmental pollutants that have been extensively released into the environment due to their use in the production of pesticides, dyes, industrial solvents, and as intermediates in the manufacturing of organic chemicals (3). While many chlorinated organic compounds can be aerobically biodegraded to CO_2, chlorinated organic compounds readily form dense non-aqueous phase liquids (DNAPLs) and migrate to anaerobic zones in sediment and groundwater. Understanding the role of microorganisms in these environments and their potential to break down chlorinated compounds is important for determining the environmental fate of these compounds and for bioremediation strategies at contaminated sites. As such, anaerobic microbial degradation of chlorinated compounds has been studied extensively, and chlorobenzenes and chloroethenes have been shown to be completely reductively dehalogenated to benzene and ethene, respectively, by different dehalogenating microorganisms (4, 12, 21, 24).

Certain *Dehalococcoides* spp. have been shown to dehalogenate chlorobenzenes with three or more chlorines (1, 7, 33) and usually remove doubly flanked chlorines if they are available. *Dehalococcoides ethenogenes* strain 195 dehalogenated hexachlorobenzene (HCB) to 1,2,3,5-tetrachlorobenzene (TeCB) and 1,3,5-trichlorobenzene (TCB) as end products (7), and strain DF-1, a genus closely related to *Dehalococcoides*, dehalogenated HCB to 1,3,5-TCB via pentachlorobenzene (QCB) and 1,2,3,5-TeCB intermediates (33). Given higher chlorinated benzenes, *Dehalococcoides* sp. strain CBDB1 produced a mixture of 1,3,5-TCB, 1,3-dichlorobenzene (DCB), and 1,4-DCB (1). Additionally, many *Dehalococcoides* spp. strains have been shown to reductively dehalogenate tetrachloroethene (PCE) or trichloroethene (TCE) to vinyl chloride (VC) and ethene (4, 5, 21).

Dehalobacter spp. have also been shown to reductively dehalogenate a wide variety of chlorinated compounds including PCE and TCE which are dehalogenated to *cis*-1,2-dichloroethene (*cis*-DCE) (14, 32). 16S rRNA gene sequences with 98.8-99.4% sequence identity to *D. restrictus* were found in *Dehalococcoides*-containing mixed cultures dehalogenating TCBs to MCB (31), though no further studies investigated the role of *Dehalobacter* in this process. More recently, enrichment cultures containing *Dehalobacter* spp. were shown to reductively dehalogenate all DCB isomers to MCB, and *Dehalobacter* spp. in sediment microcosms dehalogenated MCB to benzene (9, 24). In these studies, quantitative PCR showed *Dehalobacter* spp. 16S rRNA gene copies/mL increased in enrichment cultures and microcosms only when chlorobenzenes were included in the medium, establishing a role for *Dehalobacter* in the dehalogenation of DCBs (in enrichment cultures) and MCB (in microcosms) (24). *Dehalococcoides* 16S rRNA gene sequences were not detected in any of the 16S rRNA gene clone libraries in these studies.

In this study, we tested dehalogenation of all chlorobenzene congeners with three or more chlorines, as well as various dichlorotoluenes and PCE, with three different *Dehalobacter* spp. *Dehalobacter* sp. strain 12DCB1 was isolated from a 1,2-DCB dehalogenating enrichment culture, and dehalogenated 1,2-DCB to MCB but not the other two DCB isomers (Chapter 3). *Dehalobacter* sp. strain 13DCB1 was isolated from a 1,3-DCB dehalogenating enrichment culture and could also use 1,2-DCB. *Dehalobacter* sp. strain 14DCB1, characterized in a highly enriched mixed culture, was the only strain that dehalogenated 1,4-DCB and also utilized 1,2-DCB, though much more slowly. These results demonstrate diverse dehalogenation capabilities of three *Dehalobacter* spp. strains, and in this study we demonstrate that these differences can be extended to their utilization of other chlorinated organic compounds.

Materials and Methods

Chemicals.

All chlorinated benzenes except for 1,2,3,4-tetrachlorobenzene (TeCB), all chlorinated ethenes, 2,5-dichlorotoluene (DCT), 2,6-DCT, 3,4-DCT, and 3-monochlorotoluene (MCT) were purchased from Sigma Aldrich at the highest available purity (hexachlorobenzene (HCB): 97%, pentachlorobenzene (QCB): 98%, 1,2,4,5-TeCB: 98%, 1,2,3,5-TeCB: 97.5%, 1,2,3-trichlorobenzene (TCB): 99%, 1,2,4-TCB: 99%, 1,3,5-TCB: 99%, 1,3-dichlorobenzene (DCB): 98%, 1,2-DCB: 99%, 1,4-DCB: 99%, monochlorobenzene: HPLC Grade 99.9%, benzene: 99%, tetrachloroethene: 99.9% HPLC grade, trichloroethene: 99.5% spectrophotometric grade, 1,2-*cis*-dichloroethene: 97%, 2,5-DCT: 98%, 2,6-DCT: 99%, and 3,4-DCT: 97%). 1,2,3,4-TeCB, 2,3-DCT, and 2,4-DCT were purchased from Acros Organics at the highest available purity (1,2,3,4-TeCB: 98%, 2,3-DCT: 98%, 2,4-DCT: 99%). 2- and 4-MCT were purchased from Fluka Analytical at the highest available purity (99% and 99.9% respectively). Gases were purchased through Airgas East (Elmira, NY).

Culturing Conditions.

Pure cultures of *Dehalobacter* sp. strains 12DCB1 and 13DCB1 as well as the highly enriched mixed culture containing *Dehalobacter* sp. strain 14DCB1 were cultured in a mineral salts medium designed for *Dehalococcoides* sp. CBDB1 (1) as previously described (24). 50 mL medium was added to 160 mL serum vials and medium headspaces were flushed with N_2-CO_2 (70%-30%) and amended with 12 mM $NaHCO_3$ as a pH buffer, 2 mM sodium acetate as a probable carbon source, 1 mM amorphous iron sulfide precipitates as the reductant, and a vitamin solution (21). 5 psi H_2-CO_2 (80%-20%) overpressure (ca. 15 mmol/L H_2) was provided

as electron donor. 20 mg/L Casamino Acids was provided to cultures in some of the presented experiments, though all cultures could grow in the absence of Casamino Acids with minimal increases in lag times. DCB isomers were dissolved in 0.5 mL sterile hexadecane and added to cultures at a total nominal concentration of 1 mmol/L and aqueous concentrations of DCBs, MCB, and benzene were estimated as previously described (24). 1,4-DCB was first dissolved in acetone to make a 4 M solution and then added to cultures containing hexadecane. Cultures were incubated at 30 ˚C in the dark, shaking at 300 RPM in a New Brunswick rotary shaker.

Dehalogenation tests.

Dehalobacter sp. strains 12DCB1 and 13DCB1 and the enrichment culture containing *Dehalobacter* sp. strain 14DCB1 were typically amended with individual chlorinated compounds. Dichlorotoluene isomers (2,3-DCT, 2,4-DCT, 2,5-DCT, 2,6-DCT, and 3,4-DCT) and PCE were dissolved in sterile hexadecane at a total nominal aqueous concentrations of 1 mmol/L.

Chlorobenzenes with three or more chlorines were added to cultures in the absence of hexadecane to better allow detection of intermediate products. Cultures received total nominal concentrations of 150 µmol/L 1,2,4-TCB, 180 µmol/L 1,2,3-TCB (3 M solution in acetone), 300 µmol/L 1,3,5-TCB (1M solution in acetone), and 90 µmol/L 1,2,3,4-TeCB (1M solution in acetone) or added as a solid. Due to their low solubility in acetone or hexadecane, 1,2,3,5-TeCB, 1,2,4,5-TeCB, QCB, and HCB were added to sterile medium as crystals (20-30 mg per vial).

Analytical Procedures.

TCBs, DCBs, MCB, DCTs, MCT, benzene, toluene, and methane were detected using headspace analysis by injecting 0.1 mL culture headspace samples into a Perkin-Elmer 8500 gas chromatograph with a Restek Rtx-35 0.5 mm OD x 60 m macrocapillary column and flame ionization detector as previously described (9). TCBs could be detected in headspace analyses, but because of their low vapor pressures which were sensitive to small temperature changes, they could not be accurately quantified. Chloroethenes were detected by injecting 0.1 mL culture headspace samples into a Perkin-Elmer 8500 gas chromatograph outfitted with a stainless steel column (3.2 mm by 2.44 m) packed with 1% SP-1000 on 60/80 Carbopack-B (Supelco, Inc.) as previously described (8).

DNA Extraction and Quantitative PCR.

The FastDNA SPIN Kit for Soil (MP Biomedicals, LLC, Solon, OH) was used to extract genomic DNA from pure cultures and enrichment cultures per the manufacturer's protocol. DNA extracted from 1.6 mL culture was eluted into 55 µL sterile deionized water and used for downstream applications.

Quantitative PCR (qPCR) was used to estimate *Dehalobacter* spp. and total bacterial 16S rRNA gene copies per mL of cultures amended with a variety of individual electron acceptors using *Dehalobacter* specific primers from Grostern et al. (11), Dhb 477f 5'-GATTGACGGTACCTAACGAGG-3' and Dhb 647r 5'-TACAGTTTCCAATGCTTTACGG-3', or universal bacterial primers from Nadkarni et al. (23), Eubac 331f 5'-TCCTACGGGAGGCAGCAGT-3' and Eubac 797r 5'-GGACTACCAGGGTATCTAATCCTGTT-3'). Reaction mixtures (25 µL total volume) contained 12.5 µL iQ SYBR Green Super Mix (BioRad, Hercules, CA), 1 µL template DNA,

and 200 nM each of forward and reverse primers. Cycling conditions using a MyiQ Single Color Real Time PCR Detection System (BioRad) were as follows: 10 min. at 95 °C, 35 cycles of 15 s at 95 °C and 1 min at 62°C, followed by melting curve analysis from 60 to 95 °C. Quantification of *Dehalobacter* and total bacterial 16S rRNA genes was achieved by analyzing serial dilutions of known quantities of plasmids containing partial *Dehalobacter* sp. or *Bacillus subtilis* 16S rRNA genes as previously described (24).

Results

Dehalogenation capabilities of strain 12DCB1.

Dehalobacter sp. strain 12DCB1 dehalogenated predominantly singly flanked *ortho* chlorines on chlorobenzenes and chlorotoluenes. Strain 12DCB1 dehalogenated 1,2,3-TCB and 1,2,3,4-TeCB to MCB and trace amounts of benzene (Table 4.1) with no DCBs or TCBs detected as intermediate products. Pentachlorobenzene (QCB) was initially dehalogenated to MCB and low concentrations of 1,3-DCB, followed by slow accumulation of benzene at rates that did not increase over time (Figure 4.1). Crystals of QCB were still visible at the end of the incubation. 1,2,4-TCB, 1,2,3,5-TeCB, and 1,2,4,5-TeCB were dehalogenated to a mixture of 1,3-DCB, 1,4-DCB and MCB (Table 4.1). Strain 12DCB1 did not dehalogenate 1,3-DCB, 1,4-DCB, 1,3,5-TCB, or HCB.

Of the dichlorotoluene (DCT) isomers tested with strain 12DCB1, only 3,4-DCT was dehalogenated to an unknown monochlorotoluene (MCT) isomer. Specific MCT isomers, which have nearly identical boiling points, could not be resolved by the GC column we used. MCT was not detected when strain 12DCB1 was given 2,3-DCT, 2,4-DCT, 2,5-DCT, or 2,6-DCT, and

DCTs were still detected in culture vial headspaces after incubations of at least 30 days. PCE was dehalogenated to TCE and *cis*-DCE (Table 4.1).

Table 4.1. Utilization of chlorinated benzenes, toluenes, and ethenes by *Dehalobacter* sp. strain 12DCB1.

Dehalobacter sp. strain 12DCB1

Substrate	**Starting amount in culture**	**Endproducts after 30 ± 3 days incubation (µmol/L)**	
1,2-DCB	1 mmol/L in hexadecane	MCB (985 ± 72) Benzene (4 ± 1)	n=3
1,2,3-TCB	180 µmol/L in acetone	MCB (168 ± 8)	n=2
1,2,4-TCB	300 µmol/L neat	MCB (145 ± 24) 1,3-DCB (207 ± 97) 1,4-DCB (31 ± 11)	n=2
1,2,3,4-TeCB	90 µmol/L in acetone	MCB (52 ± 8)	n=2
1,2,3,5-TeCB	~25 mg solid crystals	MCB (100 ± 70) 1,3-DCB (93 ± 7) 1,4-DCB (1297 ± 125)	n=3
1,2,4,5-TeCB	~25 mg solid crystals	MCB (245 ± 71) 1,4-DCB (195 ± 16)	n=2
QCB	~25 mg solid crystals	Benzene (112 ± 7) MCB (661 ± 41) 1,3-DCB (92 ± 7)	n=2
PCE	1 mmol/L in hexadecane	*cis*-1,2-DCE (19 ± 12) TCE(225 ± 71)	n=2
3,4-DCT	1 mmol/L in hexadecane	MCT	n=3

HCB; 1,3,5-TCB; 1,3-DCB; 1,4-DCB; 2,3-DCT; 2,4-DCT; 2,5-DCT; and 2,6-DCT were not dehalogenated by strain 12DCB1

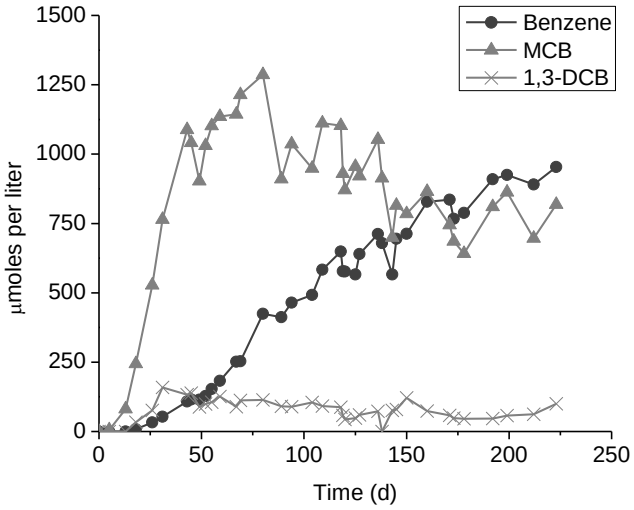

Figure 4.1. Dehalogenation of pentachlorobenzene (QCB) to 1,3-dichlorobenzene (DCB), monochlorobenzene (MCB) and benzene by *Dehalobacter* sp. strain 12DCB1. An individual experiment is presented, but is representative of triplicate vials.

Dehalogenation capabilities of *Dehalobacter* sp. strain 13DCB1.

Dehalobacter sp. strain 13DCB1, which was capable of using both 1,3-DCB and 1,2-DCB, dehalogenated singly flanked, doubly flanked, and unflanked *meta*-substituted chlorines on chlorobenzenes and chlorotoluenes. 1,2,3-TCB and 1,3,5-TCB were both dehalogenated to MCB. Trace amounts of benzene were occasionally produced, though only during dehalogenation of the DCBs or TCBs (data not presented). HCB, QCB, 1,2,3,4-TeCB, 1,2,3,5-TeCB, 1,2,4,5-TeCB, and 1,2,4-TCB were dehalogenated to mixtures of 1,4-DCB and MCB with higher amounts of 1,4-DCB in all cases (Table 4.2). Strain 13DCB1 dehalogenated HCB slowly, and HCB crystals were still visible in the culture medium after 80 days of incubation. Detectable end products from HCB dehalogenation accounted for only ca. 10% of added HCB. Intermediates including TCBs or 1,2-DCB were not detected during dehalogenation of any chlorobenzene isomer. Strain 13DCB1 did not dehalogenate 1,4-DCB.

We examined in greater detail the utilization of 1,3,5-TCB, an endproduct of HCB dehalogenation by *Dehalococcoides* spp.(7, 33). Low concentrations of 1,3-DCB (<15 µmol/L)

were variably detected at early time points of cultures amended with 1,3,5-TCB, but were not detected at later time points (data not presented). Cultures of strain 13DCB1 amended with three doses of ~300 µmol/L 1,3,5-TCB produced 942 ± 14 µmol/L MCB after 11 days incubation (Figure 4.2A). DNA samples from these cultures were subsequently analyzed using qPCR whereupon *Dehalobacter* sp. strain 13DCB1 16S rRNA gene copy number increased over two orders of magnitude (from $3.73 \times 10^4 \pm 9.66 \times 10^3$ to $1.24 \times 10^6 \pm 1.58 \times 10^5$ 16S rRNA gene copies/mL). Parallel cultures that had dehalogenated ~2000 µmol/L 1,3-DCB to MCB (comparable to the concentration of chlorines removed when ~900 µmol/L 1,3,5-TCB is dehalogenated to MCB) showed a similar increase in 16 S rRNA gene copies (from $1.69 \times 10^4 \pm 2.03 \times 10^3$ to $4.70 \times 10^6 \pm 3.58 \times 10^5$ gene copies/mL) (Figure 4.2). When 1,3,5-TCB or 1,3-DCB was not included in the medium, 16S rRNA gene numbers did not increase (data not presented).

Strain 13DCB1 dehalogenated 2,3-DCT, 3,4-DCT, and 2,4-DCT to undetermined MCT isomers, and trace amounts of toluene were detected in cultures dehalogenating 2,3-DCT. As with strain 12DCB1, strain 13DCB1 dehalogenated PCE to a mixture of TCE and *cis*-DCE (Table 4.2).

Table 4.2. Utilization of chlorinated benzenes, toluenes, ethenes by *Dehalobacter* sp. strain 13DCB1.

Dehalobacter sp. strain 13DCB1

Substrate	**Starting amount in culture**	**Endproducts after 30 ± 3 days incubation (µmol/L)**	
1,2-DCB	1 mmol/L in hexadecane	MCB (923 ± 186)	n=3
1,3-DCB	1 mmol/L in hexadecane	MCB (962 ± 101)	n=3
1,2,3-TCB	180 µmol/L in acetone	MCB (49 ± 5)	n=3
1,2,4-TCB	150 µmol/L neat	MCB (16 ± 2) 1,4-DCB (136 ± 21)	n=2
1,3,5-TCB	300 µmol/L in acetone	MCB (287 ± 64)	n=3
1,2,3,4-TeCB	~25 mg solid crystals	MCB (60 ± 47) 1,4-DCB (377 ± 244)	n=2
1,2,3,5-TeCB	~25 mg solid crystals	MCB (26 ± 7) 1,4-DCB (1269 ± 305)	n=3
1,2,4,5-TeCB	~25 mg solid crystals	MCB (57 ± 15) 1,4-DCB (253 ± 55)	n=2
QCB	~25 mg solid crystals	MCB (61 ± 13) 1,4-DCB (140 ± 14)	n=2
HCB	~25 mg solid crystals	MCB (46 ± 3) 1,4-DCB (116 ± 13)	n=2
PCE	1 mmol/L in hexadecane	*cis*-1,2-DCE (504 ± 179) TCE (137 ± 17)	n=2
2,3-DCT	1 mmol/L in hexadecane	MCT, toluene	n=3
2,4-DCT	1 mmol/L in hexadecane	MCT	n=3
3,4-DCT	1 mmol/L in hexadecane	MCT	n=3
1,4-DCB, 2,5-DCT, and 2,6-DCT were not dehalogenated by strain 13DCB1			

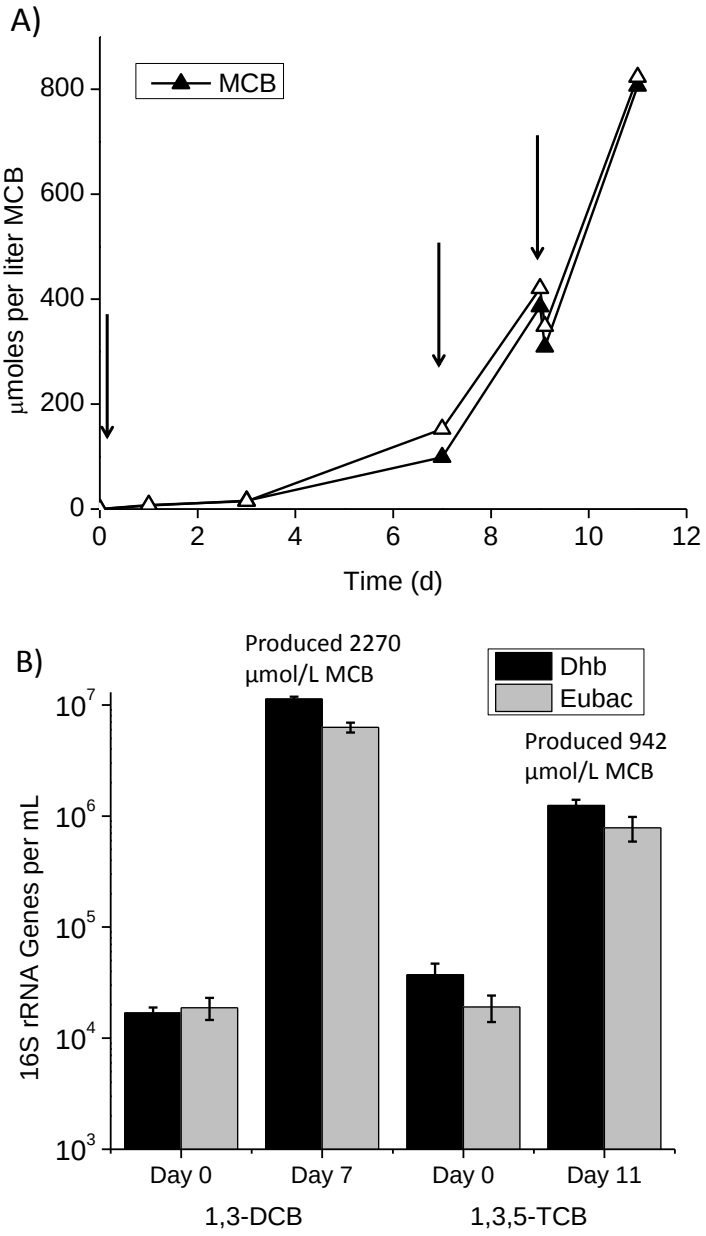

Figure 4.2. 1,3,5-trichlorobenzne (TCB) dehalogenation to MCB by strain 13DCB1 in duplicate cultures (A). TCB was not quantifiable and no DCBs were detected. Open and closed symbols represent duplicate cultures. qPCR of *Dehalobacter* spp. 16S rRNA genes and total bacterial 16S rRNA genes in cultures amended with 1,3-DCB or 1,3,5-TCB (B). Arrows in (A) indicate 300 µmol/L amendments of 1,3,5-TCB. Error bars represent the standard deviation of the mean in triplicate DNA samples.

Dehalogenation capabilities of *Dehalobacter* sp. strain 14DCB1.

Strain 14DCB1 was the only strain tested that dehalogenated *para*-substituted chlorines on chlorobenzenes. Singly flanked chlorines were dehalogenated slowly by strain 14DCB1 and it is unclear whether this dehalogenation supported growth (Chapter 3). 1,2,3-TCB and 1,2,3,4-TeCB were partially dehalogenated after approximately 30 days with 1,2-DCB and MCB observed as dehalogenation products. 1,2,4-TCB and 1,2,4,5-TeCB were dehalogenated to 1,3-DCB and MCB (Table 4.3). Strain 14DCB1 did not dehalogenate 1,3-DCB, 1,3,5-TCB, 1,2,3,5-TeCB, QCB, or HCB.

Table 4.3. Utilization of chlorinated benzenes and toluenes by *Dehalobacter* sp. strain 14DCB1.

Dehalobacter sp. strain 14DCB1

Electron Acceptor	**Starting amount in culture**	**Endproducts after 30 ± 3 days incubation (µmol/L)**	
1,2-DCB	1 mmol/L in hexadecane	MCB (166 ± 4)	n=2
1,4-DCB	1 mmol/L (1,4-DCB dissolved in acetone) in hexadecane	MCB (746 ± 111)	n=3
1,2,3-TCB	180 µmol/L in acetone	MCB (33 ± 14) 1,2-DCB (6 ± 3)	n=3
1,2,4-TCB	150 µmol/L neat	MCB (28 ± 21) 1,3-DCB (104 ± 14)	n=3
1,2,3,4-TeCB	~25 mg solid crystals	MCB (70 ± 8) 1,2-DCB (19 ± 1)	n=2
1,2,4,5-TeCB	~25 mg solid crystals	MCB (89 ± 34) 1,3-DCB (30 ± 1)	n=3
3,4-DCT	1 mmol/L in hexadecane	MCT	n=3
HCB; QCB; 1,2,3,5-TeCB; 1,3,5-TCB; 1,3-DCB; 2,3-DCT; 2,4-DCT; 2,5-DCT; 2,6-DCT; and PCE were not dehalogenated by strain 14DCB1			

Chlorotoluenes were supplied as electron acceptors to strain 14DCB1 as above, and only 3,4-DCT was dehalogenated to MCT. Notably, the *para*-substituted 2,5-DCT isomer was not dehalogenated in culture incubations exceeding 30 days. Unlike strains 12DCB1 and 13DCB1, strain 14DCB1 did not dehalogenate PCE.

Discussion

Dehalobacter sp. strain 12DCB1 dehalogenated QCB, 1,2,3,4-TeCB, 1,2,3-TCB, and 1,2-DCB to MCB and varying amounts of benzene (likely produced via cometabolism) as end-products, indicating preferential dehalogenation of singly flanked *ortho* chlorines on chlorobenzenes. Intermediates in the dehalogenation of the more highly chlorinated benzenes were not detected indicating efficient utilization of those intermediates. In the case of QCB dehalogenation, benzene accumulated slowly and eventually surpassed the levels of MCB in these cultures (Figure 4.1), a pattern not seen when strain 12DCB1 was provided with other chlorobenzenes. Moreover, the production of low concentrations of 1,3-DCB from QCB dehalogenation, which must have resulted from dehalogenation of a doubly flanked chlorine, is difficult to explain but suggests that dehalogenation was not 100% specific to singly flanked chlorines. Strain 12DCB1 did not dehalogenate HCB, which had only doubly flanked chlorines, or 1,3,5-TCB, 1,3-DCB, or 1,4-DCB in which none of the chlorines were flanked.

Strain 12DCB1 produced more 1,3-DCB than 1,4-DCB from 1,2,4-TCB indicating a preference for carbon 1, but also produced significant amounts of MCB, which cannot be produced if only singly flanked chlorines were utilized. Moreover, the only products detected from 1,2,4,5-TeCB were 1,4-DCB and MCB, with MCB in higher amounts. We have no explanation for this phenomenon, but, similar to benzene production from MCB when growing on 1,2-DCB (24), MCB may be produced cometabolically in this case.

Strain 12DCB1 could remove a chlorine from 3,4-DCT, in which both chlorines are singly flanked, but could not dehalogenate 2,3-DCT despite the fact that chlorine 3 is singly flanked, indicating interference by the methyl group. It could not use any of the MCT isomers, or 2,4-DCT, which lacks singly flanked chlorines. PCE was dehalogenated to *cis*-DCE by strain

12DCB1, but this process occurred slowly and accumulated TCE, in contrast with *D. restrictus* strain PER-K23, which accumulates only small amounts of TCE when using PCE (15).

Dehalobacter sp. strain 13DCB1, which could use either 1,2-DCB or 1,3-DCB, was the most versatile of the three cultures at using chlorobenzenes, dehalogenating singly flanked chlorines as well as doubly flanked and unflanked *meta*-substituted chlorines on chlorobenzenes. Strain 13DCB1 was the only strain that could use HCB, albeit slowly. When strain 13DCB1 dehalogenated HCB, QCB, and 1,2,3,4-TeCB, 1,4-DCB accumulated as the main end product over MCB suggesting that it preferentially dehalogenated one doubly flanked chlorine (on QCB and 1,2,3,4-TeCB) or two doubly flanked chlorines (on HCB) over singly flanked chlorines. Thus the ability to use both singly flanked and *meta*-substituted chlorines led to less complete dehalogenation of these chlorobenzenes than the ability to use only singly-flanked chlorines, as found in strain 12DCB1.

1,3,5-TCB is the product of dehalogenation of HCB or QCB by organisms with a preference for doubly flanked chlorines, and has been detected as an endproduct in several mixed cultures (6, 13, 22) and pure cultures containing *Dehalococcoides* spp. and relatives (7, 17, 33). Dehalogenation of 1,3,5-TCB to 1,3-DCB and MCB has been observed before in mixed communities (2, 16, 20, 25), but a causative organism has not been identified. *Dehalobacter* sp. strain 13DCB1 dehalogenated 1,3,5-TCB to MCB with little detectable buildup of 1,3-DCB, consistent with its ability to use *meta*-substituted chlorines, and qPCR studies indicated this dehalogenation supported growth of strain 13DCB1 (Figure 4.2B). This pattern for 1,3,5-DCB utilization is different from that recently described for an enrichment culture (16) in which there was significant buildup of 1,3-DCB as an intermediate.

Strain 13DCB1 could dehalogenate 3,4-DCT to MCT, similar to strain 12DCB1. Utilization of 2,4-DCT by strain 13DCB1 could be attributed to its ability to use *meta*-substituted chlorines, though dehalogenation of 2,3-DCT to MCT and trace amounts of toluene is difficult to explain, although the *meta* position of the 3 chlorine versus the methyl group may play a role. Strain 13DCB1 utilized PCE, producing mostly *cis*-DCE and low concentrations of TCE, unlike strain 12DCB1 and similar to strain PER-K23(14).

Strain 14DCB1 had the narrowest spectrum of chlorobenzene utilization and did not use PCE. Its activities against chlorobenzenes appeared to be a combination of its ability to use *para*-substituted chlorines coupled with slow use of *ortho* chlorines. Thus it converted 1,2,4-TCB mainly to 1,3-DCB, the product of *para* dechlorination at chlorine 1, a singly flanked chlorine, but also produced some MCB, which could be the product of *para* utilization of carbon 4 followed by *ortho* utilization of the resulting 1,2-DCB. That strain 14DCB1 could not use QCB or HCB indicates that it cannot *para* dechlorinate doubly flanked chlorines or the singly flanked chlorines in QCB. Similar to strain 12DCB1, 3,4-DCT was the only DCT isomer used by strain 14DCB1 which may be due to its slow utilization of singly flanked chlorines or perhaps, assuming chlorine 4 was utilized, its *para* position relative to the methyl group. 2,5-DCT was not utilized suggesting interference by the methyl group.

Studies of anaerobic dehalogenation of highly chlorinated benzenes in microcosms and mixed cultures have shown different patterns, some showing a preference for doubly flanked chlorines (6, 22), others for singly flanked chlorines (26), and others with mixed pathways (25). Of interest is the study of Ramanand et al. (26) in which QCB was converted nearly stoichiometrically to MCB, similar to strain 12DCB1. Intermediates expected from dehalogenation of doubly flanked chlorines such as 1,3,5-TCB were not detected, and the

intermediates that were detected accumulated to low levels and included 1,2,3,4-TeCB, 1,2-DCB, and 1,4-DCB. These results indicate the presence of *ortho*, *meta*, and *para* dehalogenation patterns similar to those found in the three *Dehalobacter* spp. strains presented. Dehalogenation of MCB to benzene, as we detected in microcosm studies (9, 24) was not detected. Ramanand et al. (26) also demonstrated dehalogenation of a mixture of 3,4 DCT, 2,5-DCT and 2,3,6-trichlorotoluene. All three chlorotoluenes were degraded with buildup of MCTs and eventual conversion to toluene, indicating the presence of organisms capable of using MCTs, an activity not found in the cultures studied here.

These studies have demonstrated the ability of *Dehalobacter* spp. to utilize more highly chlorinated benzenes, and between the three cultures, all chlorobenzene congeners with two or more chlorines were utilized (Figure 4.3), as well as some dichlorotoluenes. These substrates are added to those already known for *Dehalobacter* spp. including chloroethenes (14), chloroethanes (10, 28), chloromethanes (18, 19), chloropropanes (27), hexachlorocyclohexane (29), 4,5,6,7-tetrachlorophthalide (34), PCBs and dioxins (35), and DCBs and MCB (24). Thus *Dehalobacter* spp. show a dehalogenation versatility comparable to *Dehalococcoides* spp., the other genus known to be specialized for reductive dehalogenation.

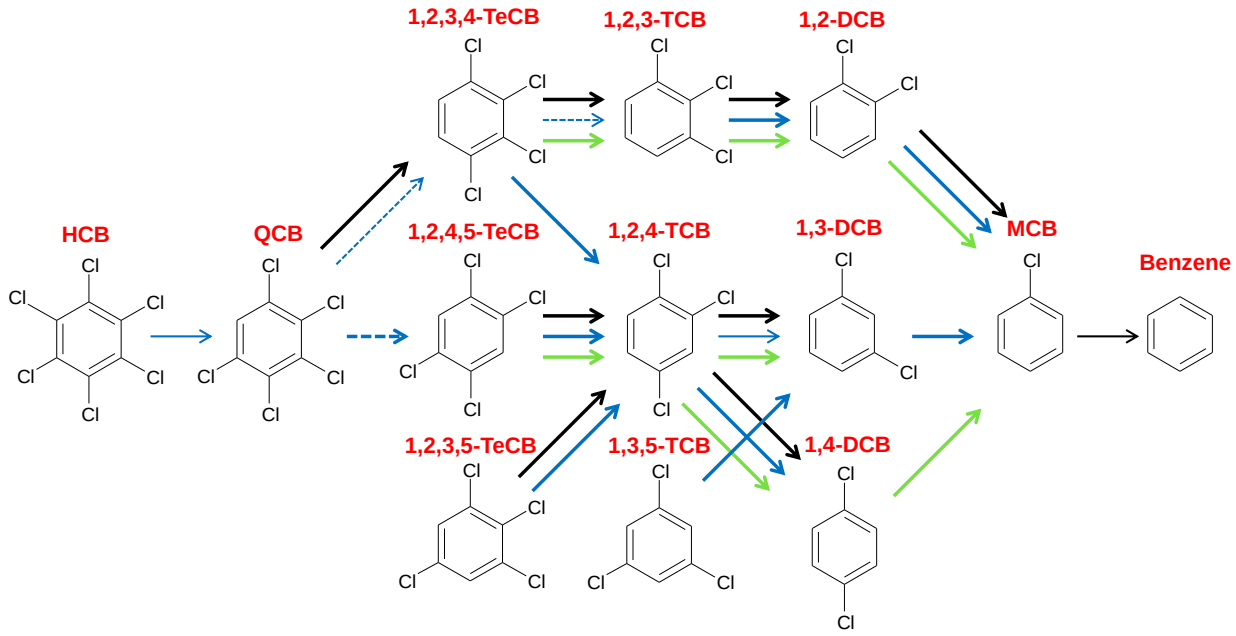

Figure 4.3. Model of chlorobenzene dehalogenation by *Dehalobacter* sp. strains 12DCB1 (black arrows), 13DCB1 (blue arrows), and 14DCB1 (green arrows). Dashed lines indicate unknown pathways. Thin arrows indicate dehalogenation pathways that occurred at low rates.

At this point, the role of *Dehalobacter* spp. in chlorobenzene dehalogenation at contaminated sites is not known, but this genus should be considered when studying these sites. It should be pointed out that 16S rRNA gene PCR primers originally based on *Dehalobacter restrictus* strain PER-K23 (30), the only strain available at the time, will not capture the phylotype that includes *Dehalobacter* spp. strains 12DCB1, 14DCB1, and FTH2 (34), or the phylotype that contains FTH2. More recently developed 16S rRNA gene primers (11, 24) do capture these phylotypes and should be used.

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CHAPTER FIVE

DIVERSE *DEHALOBACTER* SPP. ARE INVOLVED IN REDUCTIVE DEHALOGENATION OF MONOCHLOROBENZENE TO BENZENE IN MICROCOSMS

Abstract

Reductive dehalogenation of monochlorobenzene (MCB) to benzene has been reported only rarely. Here we present results from microcosms constructed from two different sediment samples (CWD and SC) that dehalogenated MCB to benzene and were taken within 1 km of each other at a site where MCB was used as a chemical feedstock. Dilution of enriched SC material to 1% vol/vol in FeS-reduced CBDB1 growth medium initially showed MCB dehalogenating activity, but this activity ceased before the first MCB dose was consumed. This activity was not restored by adding complex nutrients, vitamins, or co-inoculation with *Sedimentibacter* sp. to the culture medium. Dehalogenation of MCB occurred when SC microcosm slurries were inoculated into medium containing commercial potting mix, which suggested a surface was beneficial for MCB dehalogenation and greatly reduced reliance upon contaminated sediment to perpetuate MCB dehalogenation activity. We investigated the presence of a threshold at low MCB concentrations in both microcosm types and observed that MCB was rapidly dehalogenated when concentrations were greater than 100 µmol/L, but dehalogenation stalled when MCB levels approached 20-40 µmol/L despite the thermodynamic favorability of the reaction. 16S rRNA gene analysis revealed that distinct *Dehalobacter* spp. phylotypes populated the two sediment samples when enriched using MCB as an electron acceptor, the main phylotypes from CWD falling in the FTH1 clade as previously reported, whereas the phylotypes from SC microcosms belonged to the FTH2 clade. These studies are an important first step in

establishing MCB-benzene dehalogenation activity in enrichment culture and further indicate 16S rRNA gene sequence is not a good predictor of *Dehalobacter* spp. dehalogenation spectra.

Introduction

Monochlorobenzene (MCB) is a potential human carcinogen and has been used in industrial manufacturing processes for decades. Specifically, MCB has been used in the production of phenol, as a solvent for the production of paint, degreasers, and adhesives, and was one of the main precursors of the pervasive pesticide DDT (8). Like many chemicals used for industrial applications, MCB has been released into the environment and poses a threat to humans and wildlife living near contaminated sites. MCB is readily degradable under aerobic conditions (17); however, due to its high density and hydrophobicity, MCB tends to migrate downward and is commonly found in anaerobic zones including sediments and deep in groundwater. Dehalogenation of chlorinated benzenes with two or more chlorines in both pure and mixed cultures has been shown (2, 10, 15, 18, 26), however most of these examples fall short of complete chlorobenzene dehalogenation to benzene.

In mixed culture studies by Nowak et al. (24), cultures provided with several different chlorobenzene isomers produced MCB and trace amounts of benzene as end products. In this study, benzene was likely produced via a cometabolic reaction, as it accounted for less than 1% of end products and accumulated only when cultures were dehalogenating higher chlorinated benzenes. A similar phenomenon of benzene production in trace amounts was observed during studies of DCB dehalogenation in microcosms constructed with material from Robins Air Force Base, GA (25). Nijenhuis et al. (23) used ^{13}C-labeled MCB to investigate degradation

capabilities in a contaminated aquifer and detected trace amounts of ^{13}C –labeled benzene after incubations of more than 100 days.

More recently, Fung et al. (12) constructed microcosms using sediment from a site historically contaminated with chlorobenzenes and demonstrated MCB dehalogenation to benzene at unprecedented levels. In these studies, microcosms were initially given DCB isomers, which were dehalogenated first to MCB followed by MCB dehalogenation to benzene. Subsequent feedings of MCB were consumed more rapidly, indicative of a growth-promoting biological process. In later experiments, naïve sediment microcosms amended with MCB were inoculated with actively dehalogenating microcosm slurry and dehalogenated MCB to benzene with a reduced lag phase (12).

A role for *Dehalobacter* spp. in MCB-benzene dehalogenation was presented in Chapter 2 of this work. In these studies, several unique *Dehalobacter* spp. 16S rRNA sequences were detected in clone libraries of MCB-dehalogenating microcosms suggesting a diverse population of *Dehalobacter* spp. resided in contaminated sediment samples. 16S rRNA gene clone libraries did not contain *Dehalobacter* spp. sequences when derived from microcosms or enrichment cultures not amended with chlorobenzenes, and *Dehalococcoides* spp. were not detected in any of the clone libraries performed in these studies. Moreover, qPCR studies with *Dehalobacter* spp. specific and universal bacterial 16S rRNA gene primers showed MCB-dehalogenating microcosms contained over three orders of magnitude higher numbers of *Dehalobacter* spp. 16S rRNA gene copies than microcosms not amended with MCB, and those numbers in amended sediments were a significant percentage of total bacterial 16S rRNA gene copies. While enrichment cultures dehalogenating each of the three DCB isomers to MCB were readily established (22), we were unable to transfer MCB activity to growth medium.

Here we present results from MCB-dehalogenating microcosms established from two different sediment samples from the Chambers Works site. In both samples, minimum thresholds for MCB dehalogenation were found. While establishing MCB-benzene dehalogenation activity in sediment free enrichment cultures was not successful, activity transfers into medium containing commercial potting mix were achieved which greatly reduced reliance upon sediments to perpetuate MCB-benzene dehalogenation activity. *Dehalobacter* spp. were detected in microcosms constructed from both sediment samples using 16S rRNA gene clone libraries and quantitative PCR, although they represented distinctly different phylotypes.

Material and Methods

Chemicals.

All chemicals were purchased from Sigma Aldrich at the highest available purity (monochlorobenzene: HPLC Grade 99.9%, benzene: 99.8%). Gases were purchased from Airgas East (Elmira, NY).

Sediment samples and microcosms.

Sediment was obtained from two different chlorobenzene-contaminated sites located within 1 km of each other in the DuPont Chambers Works site, Salem County, NJ, and denoted Chambers Works ditch (CWD) sediment and Salem Canal (SC) sediment. Both samples were water saturated in close proximity to the Delaware River. CWD sediment, which was used in our previous studies (11, 12, 22), did not contain detectable amounts of chlorobenzenes or other organic compounds other than methane and was collected at various times as needed by scooping sediment from the ditch into glass containers. CWD sediment used for these experiments was

collected in December 2008 and March 2010. Salem Canal is contiguous with the Delaware River, and SC sediment was harvested in March 2009 as three 4" diameter cores into the sediment surface down to a depth of between 18-21" (total volume of each core was approximately 3 L). SC sediment cores were divided into three sections corresponding to depth (surface-6", 6"-12", and 12"-18"); each section was approximately 1 L in volume. 40-70 µmol/L MCB was detected in each SC core section and the 6"-12" core section was used for the experiments presented here. CWD sediment appeared sandy, contained small pebbles and seashells, and was medium-dark brown in color. SC sediment was black in color and became darker with depth, more fine-grained and homogeneous, and did not contain many pebbles or shells. CWD and SC sediments were stored in the dark at 4 °C in 1 L glass bottles with no protection from oxygen, though sediment used for experiments was taken from beneath the surface and away from the sides of the container and was dark in color.

Microcosms were constructed inside an anaerobic glove box with 20 g (wet wt.) sediment combined with 50 mL anaerobic deionized water in 160 mL serum vials sealed with Teflon-coated butyl rubber serum stoppers. Before any additions, microcosm headspaces were flushed with N_2-CO_2 (70%-30%) on the benchtop to remove residual H_2 present in the glove box atmosphere. Microcosms were amended with 12 mM $NaHCO_3$ (pH buffer), 200 mg/L yeast extract (nutrient supplement and electron donor), and 0.5-1.0 mmol/L MCB and incubated at 30 °C in the dark either static or at 300 RPM in a New Brunswick rotary shaker. As previously described (22), all MCB and benzene concentrations presented are nominal (i.e. ignoring partitioning to headspace) to facilitate mass balance and volumetric productivity calculations.

Transfer of MCB-dehalogenating activity to growth medium.

Several different strategies to transfer dehalogenation activity to CBDB1 mineral basal medium (1) were attempted. Medium additions included 12 mM $NaHCO_3$, 1 mM amorphous FeS precipitate as reducing agent, 2 mM sodium butyrate and/or 200 mg/L yeast extract were added as electron donor, a vitamin solution (19), and 0.5-1.0 mmol/L MCB. Cultures were incubated static at 30 ˚C in the dark. In some experiments, 7 g commercial potting mix or 1.0 g perlite (both purchased from a local Lowe's Home Improvement store (Mooresville, NC)) was added to CBDB1 medium with the above additions. Potting mix components vary by region, but the locally purchased mix likely contained reed-sedge peat, composted forest components, perlite, ammonium, nitrate, phosphate salts as fertilizers, a wetting agent, and lime.

MCB threshold experiments.

Actively dehalogenating microcosm headspaces were flushed with N_2-CO_2 (70%-30%) for approximately five minutes to remove a considerable fraction of MCB, benzene, and methane from them, shaken vigorously by hand, and headspaces were flushed again for approximately five more minutes. 12 mM $NaHCO_3$, 200 mg/L yeast extract, and 500 µmol/L MCB were added to microcosms immediately following headspace flushing. Electron donor was kept in excess in the form of repeated amendments of yeast extract, and microcosms were incubated shaking at 30 ˚C in the dark.

Thermodynamics calculations.

The Gibbs free energy value for chlorobenzene dehalogenation (represented by the following equation: MCB + H_2 → Benzene + H^+ + Cl^-) under standard biological conditions (25

˚C, 1 atm H_2, pH = 7, 1 M solutes) was taken from Dolfing and Harrison (6), and used in calculations to determine thermodynamic favorability of MCB dehalogenation in microcosms using the following equation (28):

$$\Delta G' = \Delta G^{\circ\prime} + 5.7 \log \frac{[\text{Benzene}][Cl^-]}{[\text{MCB}][H_2]}$$

Where ΔG˚' = -139.6 kJ/reaction, "5.7 log" is the transformation of "RTln" for calculation in kJ, and [X] denotes the concentration or partial pressure of a reactant or product X.

We did not directly measure H_2 or Cl^- in microcosms, but since we wanted to determine whether MCB dehalogenation to benzene was favorable at low MCB concentrations, we assumed values of H_2 and Cl^- that, if inaccurate, would skew the calculation towards less thermodynamically favorable. Since microcosms were routinely amended with yeast extract as electron donor (to provide H_2 in the form of a fermentation product), we chose 10^{-5} atm H_2 to use in our calculations as some fatty acids produced during yeast extract fermentation can poise H_2 concentrations as low as 10^{-5} atm (9). SC sediment contained 20-40 mM endogenous Cl^- (DuPont Corporation, personal communication), and microcosms in the presented experiments had dehalogenated at least 2 mmol/L MCB to benzene (releasing at least 2 mmol/L Cl^- into the microcosm) before experiments began, so we chose 50 mM Cl^- to use in our calculations. MCB-benzene dehalogenation in microcosms stalled when aqueous MCB concentrations approached 15-22 µM (see Results) so we chose a lower limit of 10 µM, and concentrations of benzene in these microcosms were variable and did not affect dehalogenation at the levels measured in the microcosm headspaces, thus 1 mM benzene was chosen for calculations. Microcosms were buffered with $NaHCO_3$ and CO_2 which poised pH near 7.

DNA extraction, primers, and clone library preparation.

DNA was extracted from SC microcosms using the FastDNA Spin for Soil Kit (MP Biomedicals, LLC, Solon, OH) per the manufacturer's instructions, eluted into 55 μL deionized water, and used for downstream applications.

A clone library of an SC microcosm was prepared using DNA extracted from the microcosm followed by PCR amplification of *Dehalobacter* spp. 16S rRNA genes using *Dehalobacter* spp.-specific primers Dhb 110f and 1273r (Table 2.3). Cloning, restriction digests, and sequencing methods have been previously described (22).

Quantitative PCR.

Quantitative PCR (qPCR) was used to estimate total bacteria and *Dehalobacter* spp. in microcosms using primers 331f and 797r (total bacteria) (21) and 477f and 647r (Dhb) (13) as described previously (22) (Table 2.3). Quantification of 16S rRNA was achieved by analyzing dilution series of known quantities of plasmids containing either *Dehalobacter* sp. or *Bacillus subtilis* 16S rRNA gene fragments. DNA concentrations were estimated using the ND-1000 spectrophotometer (Nanodrop, Wilmington, DE).

Analytical procedures.

Methane, MCB, and benzene were measured by injecting 0.1 mL headspace samples into a Perkin Elmer 8500 gas chromatograph equipped with a macrocapillary column and a flame ionization detector as described previously (12).

Results

MCB dehalogenation to benzene in CWD and SC microcosms.

Substantial MCB dehalogenation to benzene occurred in both CWD and SC microcosms (Figures 5.1 & 5.2) though the lag before dehalogenation was longer in SC microcosms. The SC sediments contained 2-3 mM sulfate (DuPont Corporation, personal communication) which may have contributed to longer lags in methanogenesis and MCB dehalogenation to benzene. Consistent with previous studies (12), the lag phase in these microcosms could be shortened if an actively dehalogenating microcosm was used to "inoculate" naïve microcosms (Figures 5.1 & 5.2). In the case of the SC microcosms, a fresh dose of yeast extract as electron donor at Day 38 caused a nearly immediate increase in methanogenesis in both microcosm types, but there was still a 30 day additional lag in benzene formation in the naïve microcosms, whereas the inoculated microcosms produced benzene without additional lag.

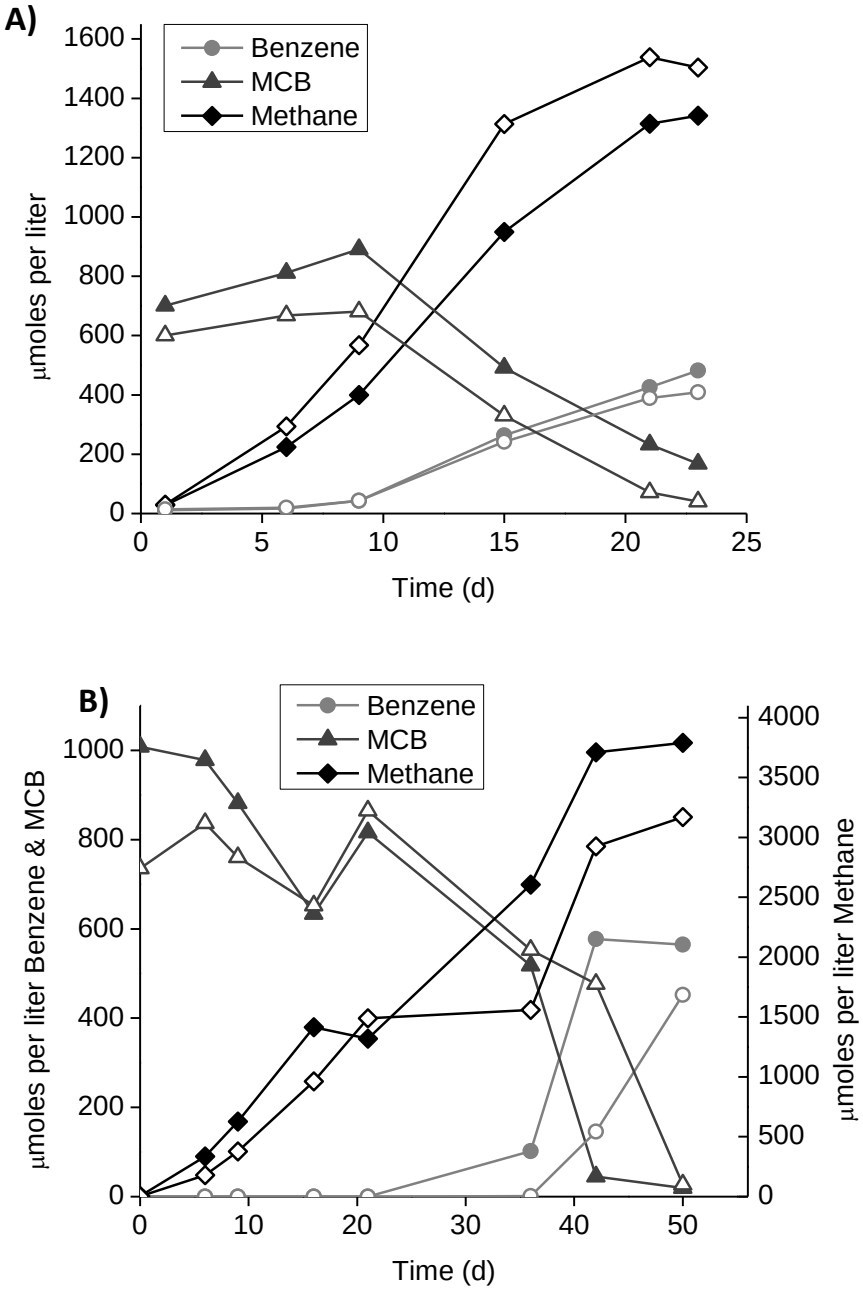

Figure 5.1. MCB dehalogenation to benzene in CWD sediment microcosms inoculated with actively dehalogenating sediment slurry (A) or uninoculated (B). Duplicate microcosms are represented by open and closed symbols.

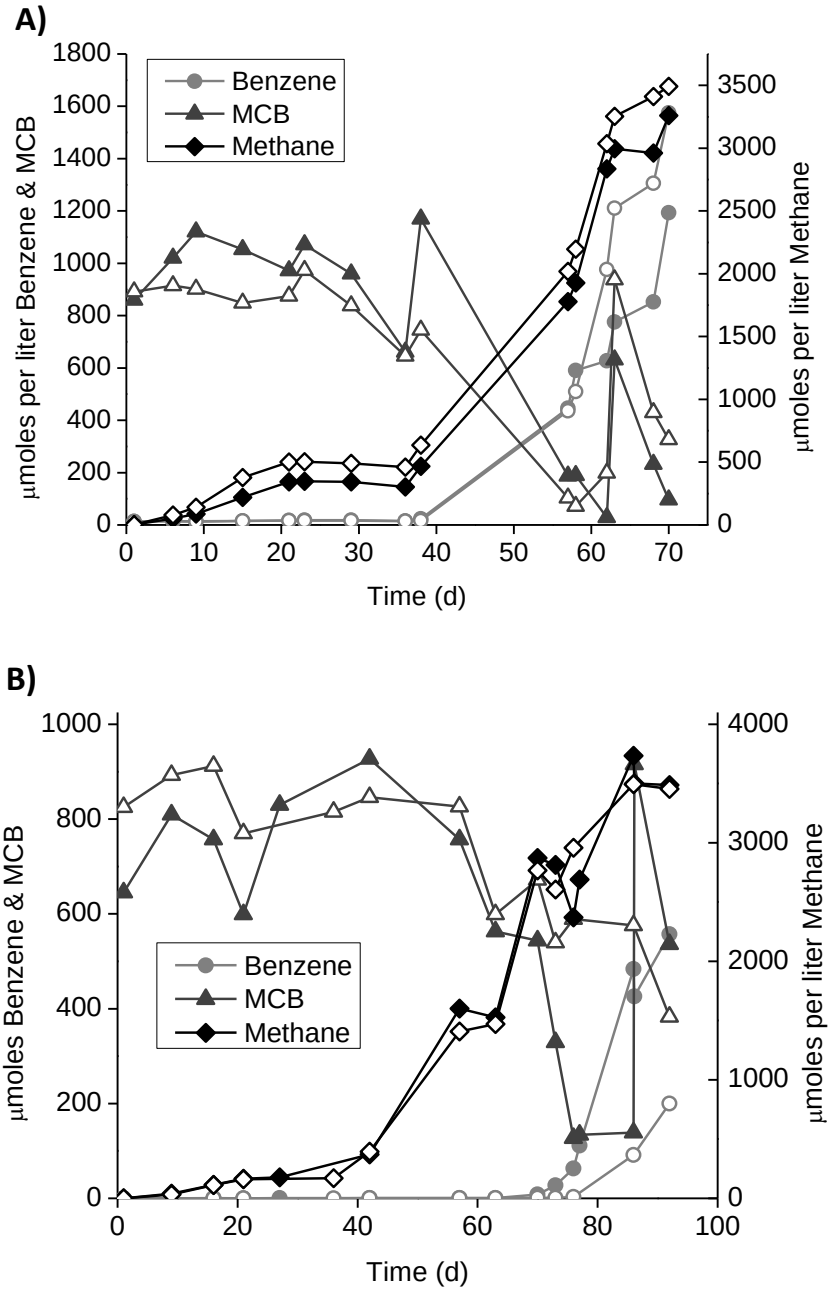

Figure 5.2. MCB dehalogenation to benzene in SC sediment microcosms inoculated with actively dehalogenating sediment slurry (A) or uninoculated (B). Duplicate microcosms are represented by open and closed symbols.

Strategies for enrichment of MCB dehalogenating *Dehalobacter* spp.

We attempted to establish MCB-dehalogenating enrichment cultures from both CWD and SC microcosms. In preliminary experiments, different reducing agents (Na_2S, dithiothreitol (DTT), Ti(III) NTA, amorphous FeS precipitate, sodium 2-mercaptoethanesulfonate (coenzyme M), or L-cysteine) were added to CWD sediment microcosms to investigate whether dehalogenation activity would be affected. Na_2S slowed dehalogenation in microcosms compared to control microcosms without any exogenous reducing agents, consistent with previous results with DCB-dehalogenating enrichment cultures (22). Microcosms amended with all of the other tested reducing agents performed similarly to control microcosms (data not presented). FeS precipitate as reducing agent was chosen for use in MCB-benzene dehalogenating enrichment cultures as it allowed for the fastest dehalogenation in our previous studies on DCB dehalogenators (22) (Chapters 3).

Typical enrichment cultures were inoculated with 1% (vol/vol) microcosm slurry to culture medium. CWD microcosms did not dehalogenate MCB in medium with these standard additions, so amendment of digestor sludge supernatant (used to cultivate *Dehalococcoides ethenogenes* strain 195 (19)), *Sedimentibacter* sp. inocula, or *Sedimentibacter* sp. culture extract (Chapter 3) were tested, however MCB dehalogenation to benzene did not occur in all cases (data not presented).

Since MCB dehalogenation did not occur when 1% slurry inocula from CWD microcosms were transferred to culture medium, we investigated the extent to which microcosms could be diluted before losing activity. One MCB-dehalogenating CWD microcosm was sacrificed to make two 40% dilution vials (20 mL microcosm slurry combined with 30 mL culture medium), and two 10% dilution vials (5 mL microcosm slurry combined with 45 mL

culture medium). MCB was dehalogenated to benzene in "40% dilution" CWD cultures, though no benzene formation was detected in the "10% dilution" CWD cultures (Figure 5.3 A&B).

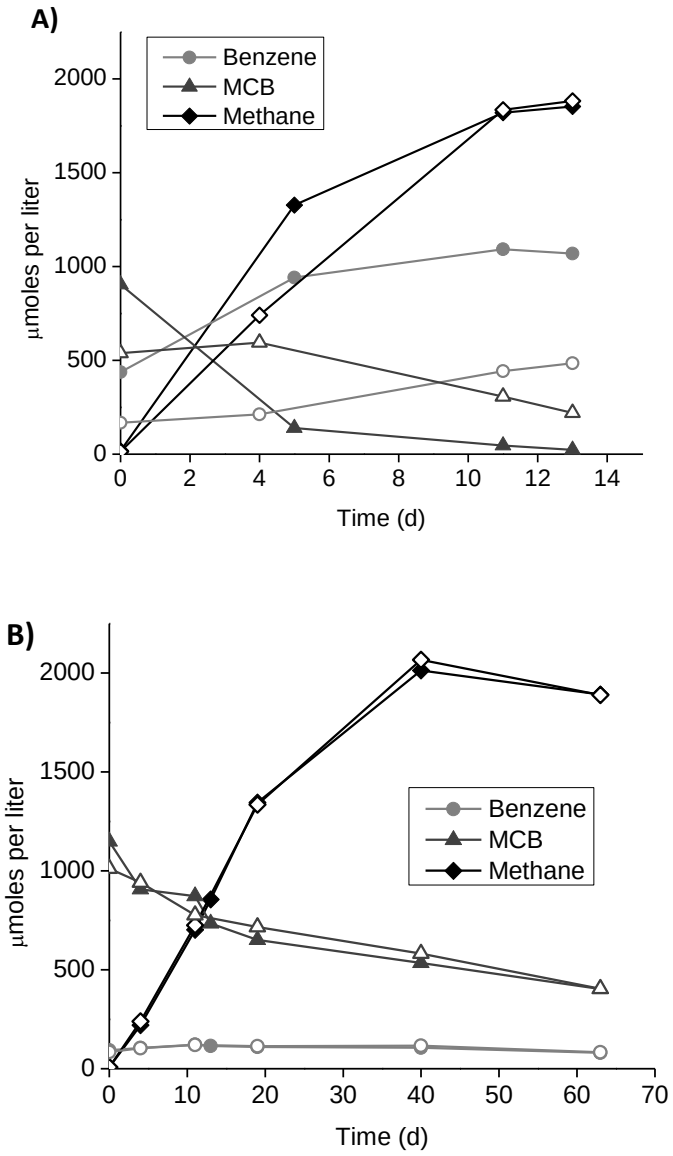

Figure 5.3. Cultures containing 40% CWD microcosm material (A) dehalogenated MCB to benzene while those with 10% CWD microcosm material (B) did not dehalogenate MCB. Open and closed symbols represent duplicate experiments.

In contrast to CWD transfer cultures, 10% transfers from active SC microcosms into medium provided with the standard amendments listed above could dehalogenate MCB to benzene (data not presented). Moreover, 1% SC microcosm slurry transfers into medium also showed MCB-benzene dehalogenation activity (Figure 5.4), but these cultures stalled before consuming the initial MCB dose, a pattern found in numerous transfers from active SC sediments into medium, with ca. 300 µmol/L MCB remaining.

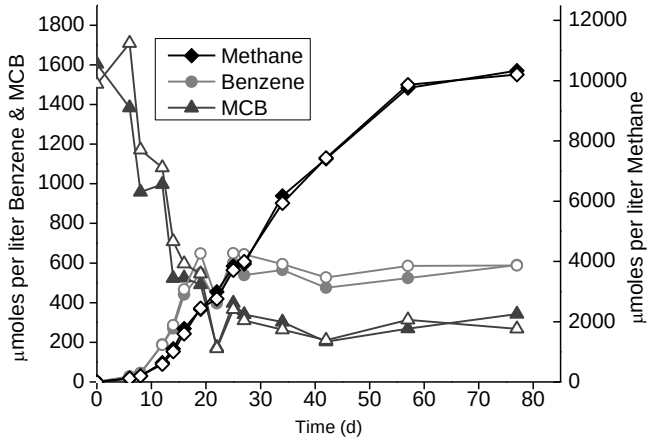

Figure 5.4. 1% (vol/vol) SC microcosm slurry transfers into growth medium dehalogenated MCB to benzene before stalling. Open and closed symbols represent duplicate cultures.

Cessation of growth or activity in culture medium can be caused by nutrient depletion, buildup of a toxic product, or from having reached a thermodynamic limit. As such, attempts to rescue stalled cultures included additional amendments of yeast extract, B vitamins, *Sedimentibacter* sp. inoculation (Chapter 3), purging headspaces to reduce benzene concentration, adding ascorbic acid or humic acids as potential radical scavengers, adding more MCB, and adding DCBs. None of these attempts to rescue the MCB dehalogenation stall were successful. However, when DCBs were added to cultures, they were consumed to below our

detection limit of ca. 4 µmol/L and MCB concentrations increased in these vials, but there was no further increase of benzene (data not presented), in contrast with results in microcosms.

Another potential explanation for our inability to obtain sediment-free cultures was that the MCB dehalogenators required a surface to grow on provided by the sediments. Since SC sediments were obtainable in limited amounts and contained MCB and possibly other toxic chemicals, we sought a better defined addition obtainable in large or unlimited amounts. Attempts to use 1 mm glass beads or playground sand to sustain 1% transfers were unsuccessful, whereas commercial potting mix added at 7 g per 50 mL culture medium allowed for consumption of MCB with concomitant benzene production after a ca. 20 day lag, and more rapid consumption of a second MCB dose (Figure 5.5). Moreover, we have now transferred this culture five times into fresh medium amended with potting mix. Preliminary tests using perlite, an inorganic component of the commercial potting mix, and supernatant from a water extraction of potting mix provided in amounts approximately equivalent to their amounts in potting mix, did not support MCB dehalogenation.

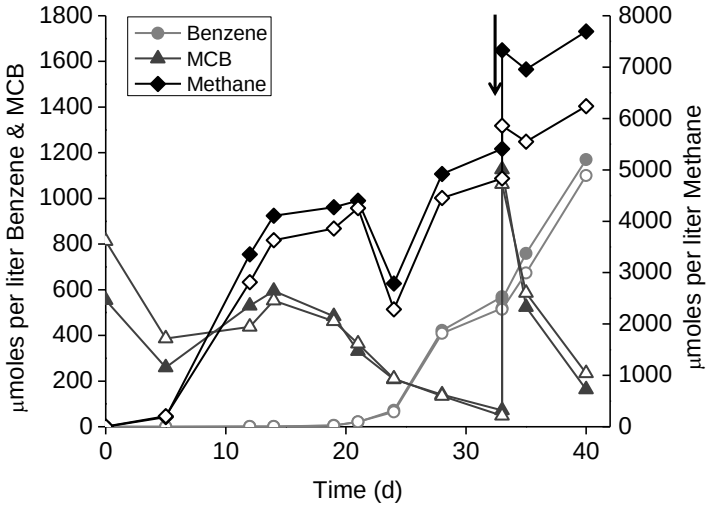

Figure 5.5. MCB dehalogenation to benzene in growth medium amended with potting mix and inoculated with 1% (vol/vol) SC microcosm slurry. Duplicate microcosms are presented and represented as open and closed symbols. The arrow represents amendment of MCB.

Efforts to limit methanogenesis in potting mix microcosms with methanogen inhibitor bromoethanesulfonate (BES) have not been effective, since BES inhibited methanogenesis and MCB dehalogenation to similar extents, resembling our previous results with 1,3-DCB-dehalogenating enrichment cultures (22) (data not presented).

MCB threshold in CWD and SC microcosms.

During DCB and MCB dehalogenation in microcosm studies, we observed that MCB never seemed to be completely consumed in later stages of a consumption curve, whereas DCBs were routinely consumed below our limit of detection in microcosms and cultures. To further investigate this phenomenon, headspaces of CWD and SC microcosms that had dehalogenated >2 mmol/L MCB were flushed to remove most of the benzene and methane that had accumulated followed by amendment with MCB. Multiple amendments of yeast extract were supplied to ensure electron donor was not limiting, and microcosms were monitored for more than four weeks. In both CWD and SC microcosms, MCB was rapidly dehalogenated to benzene until less than ca. 100 µmol/L MCB remained, and stalled completely when MCB nominal concentrations reached 20 and 23 µmol/L (15 and 17 µM aqueous concentration) in CWD microcosms and 35 and 38 µmol/L (26 and 28 µM aqueous) in SC microcosms. Another dose of MCB was given to the stalled microcosms with similar results, and dehalogenation again stalled when 34 and 36 µmol/L MCB remained in CWD microcosms and 42 and 50 µmol/L MCB remained in SC microcosms (Figure 5.6).

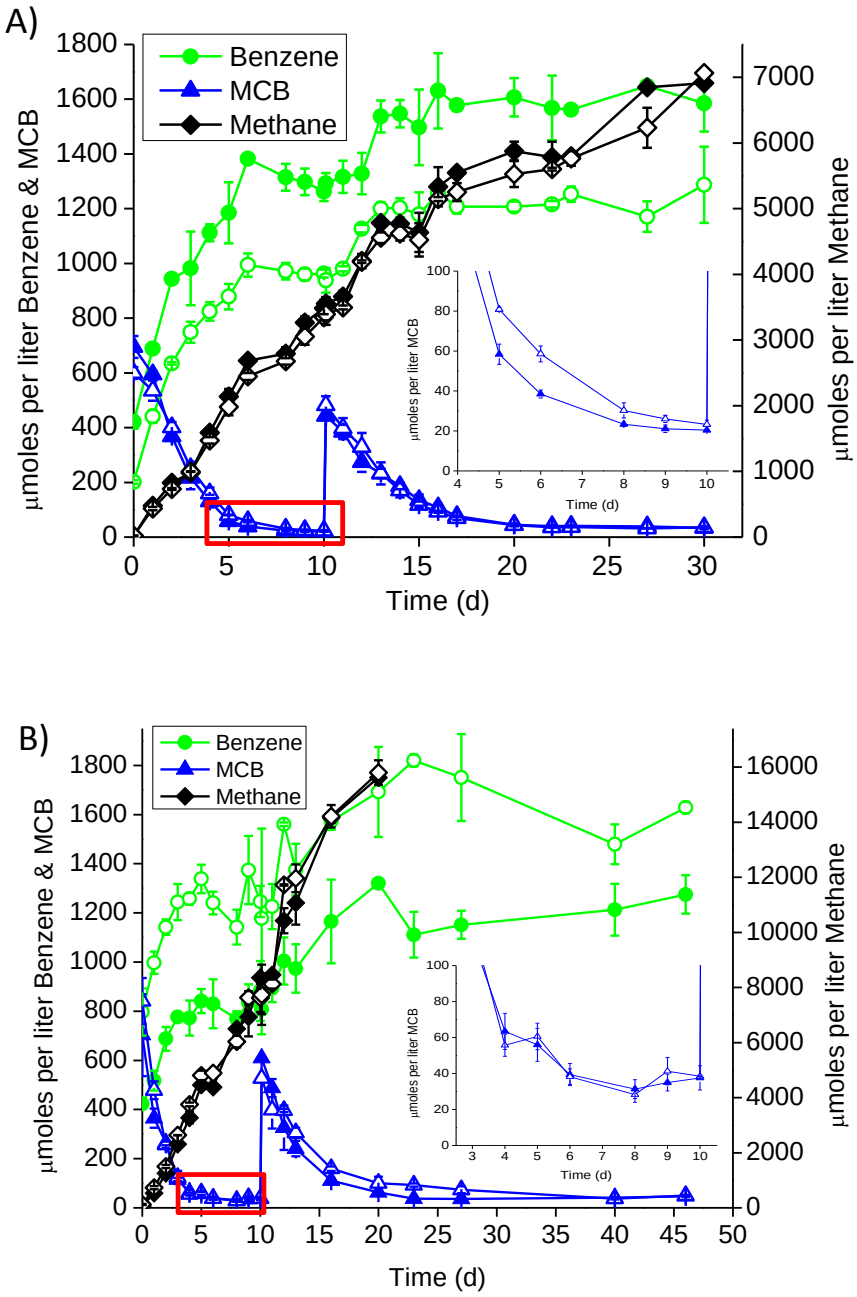

Figure 5.6. MCB dehalogenation to benzene in CWD (A) and SC (B) microcosms. MCB is consumed rapidly until concentrations fall below ca. 100 µmol/L. The boxed portions of MCB curves are shown as insets. MCB nominal concentrations remained stable at approximately 35 µmol/L in CWD microcosms and 45 µmol/L in SC microcosms at the end of incubation. Duplicate microcosms are presented for each experiment and are represented as open and closed symbols. Error bars represent the standard deviation of the mean of three headspace samples. Methane concentration in (A) exceeded the upper limits of our detection after 20 days.

As mentioned in Materials and Methods, SC sediments contained a low endogenous concentration of MCB, and control SC microcosms amended with yeast extract accumulated large amounts of methane but did not consume endogenous MCB even when "inoculated" with a slurry from an actively dehalogenating SC microcosm. A similar test was performed using CWD sediment, in which microcosms were amended with 50 µmol/L MCB and yeast extract, and no dehalogenation was detected in incubations of more than 70 days (data not presented). As another example, we present an experiment on benzene degradation in SC sediments. In these studies, multiple doses of benzene were consumed by the sediments. Methane was not detected for the first 50 days during benzene consumption, presumably because sulfate and possibly other electron acceptors in the sediments were being used. After this time, benzene consumption slowed down and methane began to accumulate. No MCB dehalogenation was observed for incubations surpassing 500 days (Figure 5.7). These studies were only performed with SC microcosms as CWD microcosms did not degrade benzene when incubated for more than 100 days and did not contain endogenous MCB.

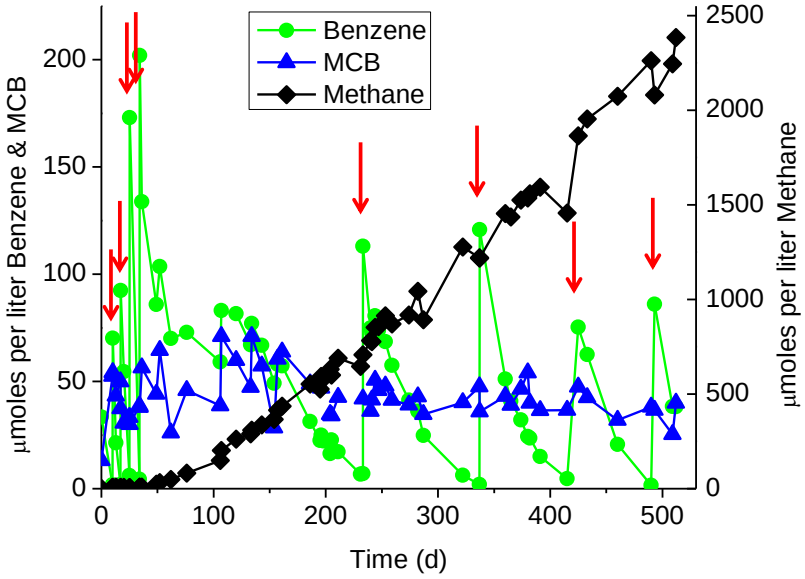

Figure 5.7. Benzene degradation in SC sediment occurred rapidly until sulfate was consumed, then slowed when microcosms became methanogenic. SC microcosms contained between 40-70 µmol/L endogenous MCB. Arrows indicate additional amendments of benzene. One experimental vial is presented and is representative of duplicate microcosms.

MCB dehalogenating SC microcosms contain *Dehalobacter* spp.

Previous work supported a role for *Dehalobacter* spp. in CWD microcosms dehalogenating MCB to benzene using qPCR and clone library analyses (22). Here too, qPCR using *Dehalobacter* specific 16S rRNA gene primers showed *Dehalobacter* spp. 16S rRNA gene copy number per mL increased in SC microcosms (n=2) from $6.4 \times 10^4 \pm 4.6 \times 10^4$ to $5.3 \times 10^8 \pm 5.6\ 10^7$ after dehalogenating more than 4 mmol/L MCB to benzene. *Dehalobacter* spp. 16S rRNA gene copies per mL remained at $4.5 \times 10^4 \pm 2.7 \times 10^4$ when MCB was not present in SC microcosms (data not presented).

16S rRNA gene clone library analysis of *Dehalobacter* spp. sequences from two CWD microcosms fell into two main groups in our previous studies (22). One group shared >99% nucleotide identity with *Dehalobacter restrictus* and the other group shared >99% sequence

identity with tetrachlorophthalide-dehalogenating strain FTH1 (31). In contrast, *Dehalobacter* spp. 16S rRNA genes from a 22 member clone library of an MCB-dehalogenating SC microcosm all shared >99% sequence identity with *Dehalobacter* sp. strains 12DCB1 and 14DCB1 (22)(Chapters 3 and 4) and tetrachlorophthalide-dehalogenating strain FTH2 (31) (Figure 5.8).

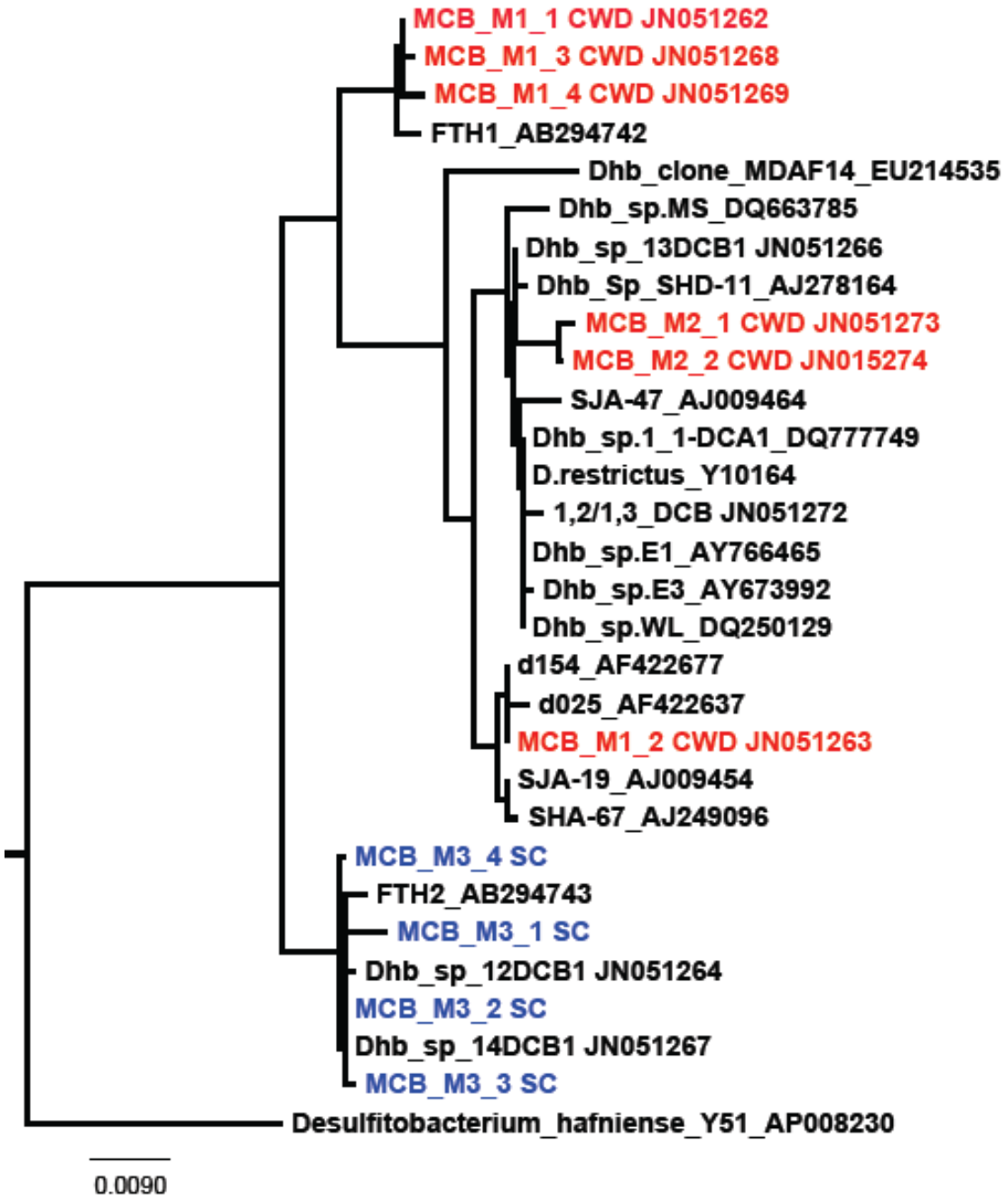

Figure 5.8. Phylogenetic tree for *Dehalobacter* spp. 16S rRNA genes from an SC sediment microcosm (shown in blue) that reductively dehalogenated monochlorobenzene (MCB) to benzene. Unique microcosms used to generate clone libraries are denoted as M1, M2, and M3. Microcosms M1 and M2 were both constructed with CWD sediments (shown in red), though sediment was collected at different times (M1 in June 2007, M2 in December 2008), and these sequences were determined previously (22). Microcosm M3 was constructed with SC sediment collected in May 2009. The 16S rRNA gene from *Desulfitobacterium hafniense* Y51 was used as an outgroup.

Discussion

Here we have demonstrated MCB dehalogenation to benzene in two sediment samples from the same historically contaminated site. CWD and SC sediments were dissimilar in certain aspects, specifically that SC sediments contained a considerable amount of endogenous sulfate and detectable MCB while CWD sediments did not contain detectable MCB and produced methane immediately upon amendment of electron donor.

Multiple attempts to rescue stalled 1% SC microcosm transfers into growth medium failed including amendment of *Sedimentibacter* sp. extracts as members of this genus have been found in association with dehalogenating communities on several occasions (3, 4, 22, 30-32). Concentrations as low as 200 µM benzene have been shown to increase lag periods in some benzene degrading systems (7, 29) so headspaces of stalled cultures were flushed to remove benzene that accumulated during dehalogenation. This, too, did not rescue stalled cultures and it is unlikely benzene toxicity affects these dehalogenators as microcosms with more than 5 mmol/L benzene continued to dehalogenate MCB at high rates in previous microcosm studies (11, 12).

DCBs were completely dehalogenated when provided to stalled cultures and equivalent concentrations of MCB accumulated, yet further dehalogenation of MCB to benzene did not occur after the initial MCB-benzene dehalogenation stall (data not presented), indicating that this phenomenon is distinct from the threshold phenomenon found in microcosms. These findings indicate that organisms responsible for DCB dehalogenation were either not limited for a nutrient or inhibited at the same time that MCB dehalogenators were unable to metabolize MCB, suggesting fundamental differences between these processes, despite evidence that they are

carried out by members of the same genus, *Dehalobacter*. Further investigations should address whether the organisms dehalogenating DCBs in stalled cultures were distinct from the *Dehalobacter* spp. dehalogenating MCB.

We found that MCB dehalogenation activity could be reliably transferred from SC microcosms into culture medium containing commercial potting mix. Potting mix typically contains composted plant materials, inorganic macronutrients, and perlite, and we have begun to investigate potential stimulating components of potting mix on MCB potting mix transfer cultures. Preliminary experiments testing the addition of water-soluble potting mix components or providing the porous high surface area constituent perlite to transfer cultures did not demonstrate stimulation of dehalogenation activity.

An interesting finding was that the rate of MCB dehalogenation was concentration dependent below 100-200 µM aqueous MCB, slowing and eventually reaching thresholds at 15-40 µM. One potential explanation for minimum thresholds for anaerobic processes is that they are approaching a thermodynamic limit that no longer allows energy conservation (27). Thermodynamic calculations (see Materials and Methods) estimated the free energy of MCB to benzene dehalogenation in either sediment microcosm type was approximately -121.7 kJ/reaction at the beginning of the experiments (MCB = 500 µM, benzene = 134 µM) and approximately -107.1 kJ/reaction after the dehalogenation stalled (MCB = 10 µM as a lower limit, benzene = 1000 µM). These free energy values suggest MCB dehalogenation is quite favorable at threshold MCB concentrations. The values for DCB dehalogenation to MCB would be 8-14 kJ/reaction less negative and DCBs are dehalogenated below our detection limit of ca. 4 µmol/L (12, 22).

That CWD or SC microcosms did not dehalogenate low initial doses of MCB further suggests poor utilization by *Dehalobacter* spp. at these low levels. The endogenous concentration of MCB in the SC sediments did not significantly decrease despite numerous additions of benzene, a potential electron donor that we have shown in other studies (Liang et al., unpublished results) can support dehalogenation of high MCB concentrations. Moreover, poor utilization of low concentrations of MCB as a substrate may have played a role in the slow start of MCB dehalogenation in some of our previous studies (12) and perhaps not allowed establishment of MCB dehalogenators in the studies of others, since significant MCB dehalogenation to benzene had not been detected previously (23, 24).

A similar threshold phenomenon at contaminated sites may be a partial explanation for the persistence of MCB in anaerobic contaminated sites like the Bitterfeld site in Germany (5) and the SC site examined in this work, though MCB concentrations at the Bitterfeld site were as high as 270 µM compared to approximately 40 µM at the SC site. The microcosms presented here dehalogenated MCB to benzene at high rates until concentrations fell below 100 µM, however other factors at the Bitterfeld site may have contributed to higher endogenous MCB concentrations than those measured from SC sediments.

16S rRNA clone library analyses of two different MCB-dehalogenating CWD microcosms (constructed with sediment collected at different times) detected two distinct *Dehalobacter* phylotypes, one that grouped with *Dehalobacter restrictus* strain PER-K23 (14) and the other with a tetrachlorophthalide dehalogenating *Dehalobacter* strain FTH1 (31). In contrast, all of the *Dehalobacter* 16S rRNA clones from an MCB-dehalogenating SC microcosm were of a single phylotype most closely related to our previously described cultures using 1,2-DCB and 1,4-DCB (22) as well as *Dehalobacter* strain FTH2 (31). That CWD and SC

sediments harbored at least three different *Dehalobacter* sp. phylotypes capable of MCB-benzene dehalogenation, together with our previous studies of 16S rRNA genes in DCB dehalogenators (22), further implies 16S rRNA sequence is not a good predictor of dehalogenation phenotype, similar to the *Dehalococcoides* spp. (2, 20).

These findings have significance for devising anaerobic bioremediation strategies for MCB. Anaerobic MCB oxidation has not been observed, thus reductive dehalogenation of MCB to benzene is the critical step required for remediation of chlorinated benzenes in anaerobic zones. Though benzene is a known carcinogen, many studies have investigated anaerobic benzene degraders and benzene has been shown to be degraded under methanogenic conditions as well as nitrate, sulfate, or Fe(III) reducing conditions (16, 29). Obtaining viable MCB dehalogenating transfers in medium containing commercial potting mix is an important first step in establishing transferrable MCB-dehalogenating cultures, obviating the need for the limited amounts of sediment to perpetuate the cultures and allowing significant scale up. Evidence suggesting that MCB dehalogenation stalls after reaching a threshold at low concentrations may impact the treatment of contaminated sites and alternative means of completely remediating MCB, perhaps with a final aerobic step, may better serve some sites if further investigations reinforce these findings.

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CHAPTER SIX

REDUCTIVE DEHALOGENATION OF CHLOROBENZENES: CONCLUSIONS AND FUTURE DIRECTIONS

This work has established a role for *Dehalobacter* spp. in the dehalogenation of every chlorinated benzene isomer and expanded the list of known electron acceptors for the *Dehalobacter* spp. *Dehalobacter* sp. strains 12DCB1 and 13DCB1 were isolated from contaminated Chambers Works ditch (CWD) sediments within the Chambers Works site in New Jersey after enrichment on 1,2-DCB and 1,3-DCB, respectively. *Dehalobacter* sp. strain 14DCB1 was also derived from CWD sediment and studied in a highly enriched mixed culture amended with 1,4-DCB (Chapters 2 & 3). Strains 12DCB1, 13DCB1, and 14DCB1 could dehalogenate when formate replaced H_2 as electron donor (Chapter 3), a finding that has been reported with one other *Dehalobacter* spp. isolate (7). All strains required vitamins B_{12} and thiamine, acetate, and a chlorinated compound for growth (Chapter 3) consistent with previous *Dehalobacter* studies (2, 6), and each strain had unique dehalogenation spectra with chlorobenzenes, chlorotoluenes, and tetrachloroethene (Chapter 4).

Monochlorobenzene (MCB) dehalogenation to benzene was studied primarily in sediment microcosms (Chapter 5) as attempts to transfer this activity to sediment-free culture medium were not successful. Two different sediment types from the Chambers Works site, CWD and Salem Canal (SC) sediments, were used to construct microcosms and both showed MCB-benzene dehalogenation activity. A threshold below which MCB was not dehalogenated was examined and found to be at similar levels in both microcosms types despite the thermodynamic favorability of this reaction. Quantitative PCR and 16S rRNA gene clone library

analysis showed *Dehalobacter* spp. were main players in MCB dehalogenation, and the different sediments harbored unique *Dehalobacter* spp.

One of the major impediments of *Dehalobacter* spp. research has been the lack of a publically available sequenced genome. We have generated draft genomes of *Dehalobacter* sp. strains 12DCB1, 13DCB1, and 14DCB1 and putatively annotated at least 43, 32, and 46 reductive dehalogenase (RDase) genes, respectively, in each strain (Heather Fullerton, unpublished results). Interestingly, strain 14DCB1 harbored the most RDases despite our finding that this strain dehalogenated the fewest tested chlorinated electron acceptors (Chapter 4). The abundance of RDases in these *Dehalobacter* sp. genomes suggests testing additional chlorinated electron acceptors including chlorinated phenols, ethanes, methanes, propanes, biphenyls, and dibenzo-dioxins, among others, may further expand dehalogenation spectra for these organisms.

Protein studies have investigated and identified RDases using different methods (1, 3-5), and these strategies could be applied to the *Dehalobacter* strains described here to identify specific RDases responsible for dehalogenation of chlorobenzenes. Proteomic analyses could be used to identify RDases and other important and highly expressed proteins in these *Dehalobacter* strains, as well as allow for comparisons either between strains or within a strain grown on different substrates.

MCB dehalogenating *Dehalobacter* spp. from CWD and SC sediment microcosms behaved differently and were phylogenetically distinct (Chapter 5), thus interesting comparisons may be found with DCB dehalogenators from the different sediment types as well. Enrichment cultures derived from SC microcosms have been transferred at least four times with individual DCB isomers and dehalogenate either 1,2-DCB, 1,3-DCB, or 1,4-DCB to MCB and trace amounts of benzene. Preliminary results indicated each of these enrichment cultures contained

unique *Dehalobacter* spp., and these sequences did not group with the DCB-dehalogenating *Dehalobacter* spp. enriched and isolated from CWD sediments. Characterization of DCB-dehalogenating *Dehalobacter* spp. from SC sediment could add yet another layer to the diversity of chlorobenzene dehalogenating *Dehalobacter* spp., and comparative studies of CWD and SC sediment isolates could provide interesting insights into the different reductive dehalogenases in these organisms.

This work has shown *Dehalobacter* spp. are present in chlorobenzene-contaminated sediments, and the unique 16S rRNA sequences detected in enrichment cultures and microcosms suggest 16S rRNA sequence is not a good predictor of dehalogenation spectra. This work has established a role for *Dehalobacter* spp. in the dehalogenation of chlorobenzenes, and our data support that *Dehalobacter* spp. should be considered when developing bioremediation strategies and determining the fates of chlorobenzene-contaminated sites.

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