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QUANTITATIVE ENVIRONMENTAL MONITORING OF PCE DECHLORINATORS IN A CONTAMINATED AQUIFER AND PCE-FED REACTOR

presented by

Michael Robert Aiello

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of the requirements for the

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Major Professor's Signature

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Date

QUANTITATIVE ENVIRONMENTAL MONITORING OF PCE DECHLORINATORS IN A CONTAMINATED AQUIFER AND PCE-FED REACTOR

By

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ABSTRACT

QUANTITATIVE ENVIRONMENTAL MONITORING OF PCE DECHLORINATORS IN A CONTAMINATED AQUIFER AND PCE-FED REACTOR

By

Michael Robert Aiello

Understanding the mechanisms by which natural attenuation of halogenated compounds occurs is important to the fields of bioremediation and environmental toxicology. Of the halogenated organic compounds in the environment, the chlorinated ethenes, PCE, TCE, -DCE, VC are of importance. *Dehalococcoides ethenogenes* and *Dehalococcoides* spp. strain FL2 are the only isolates capable of dechlorinating PCE to ethene. The objective of this research was to utilize Real Time PCR (RTm PCR) to detect and quantify specific chlorinated ethene degraders in environmental systems. RTm PCR was capable of detecting and quantifying the *Dehalococcoides* and *Desulfuromonas* groups of PCE dechlorinators. 16S and *tceA* gene primers and probes specific to these groups of organisms were designed and tested to assure specificity for the organisms of interest. PCE contaminated aquifer core samples were used to test the ability of RTm PCR to detect the key dechlorinators in an environmental sample. The population of *Dehalococcoides* was quantified in a large-scale reactor along with the mRNA of its dehalogenase through several feeding cycles. RTm PCR was shown to be an accurate and efficient quantitative tool in the detection of dechlorinating microorganisms.

DEDICATION

To everyone who actually cared, you know who you are and I know who you are. Thank you for sticking it out with me. You are truly the only people that matter.

ACKNOWLEDGMENTS

Special thanks goes out to my mentor Dr. Tiedje who has put a great deal of time and energy into making my work successful. In addition, thanks goes out to Dr. Löffler who has put in a lot of time reading my work and providing useful insight into my projects.

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KEY TO SYMBOLS AND ABBREVIATIONS

PCE; Tetrachloroethene

TCE; Trichloroethene

cis-DCE and *trans*-DCE; *cis*-1,2-dichloroethene and *trans*-1,2-dichloroethene

VC; Vinyl chloride

RDases; Reductive dehalogenases

RTm PCR; Real Time Polymerase Chain Reaction

CFUs; Colony forming units

RDP; Ribosomal Database Project

Dsf.; *Desulfuromonas*

Dhc.; *Dehalococcoides*

CHAPTER 1

Background

Halogenated organic compounds are an important class of environmental pollutants partly because they are produced industrially in large quantities and released into the environment (8). Moreover, many of these compounds are produced through abiotic and biotic processes (9). These compounds, whether man-made or naturally produced, pose a risk to both humans and the environment, due to the their toxicity and worldwide distribution in nearly every habitat (9). Understanding the mechanisms by which natural attenuation of halogenated compounds occurs is important to the fields of bioremediation and environmental toxicology.

Recently, the U.S. Environmental Protection Agency (US EPA) recognized, through a 1999 directive, monitored natural attenuation as an appropriate remediation option for sites contaminated with halogenated compounds (40). This directive details the many approaches to natural attenuation carried out by remediation approaches; the EPA in evaluating sites for natural attenuation require evidence that the biodegradative process is destroying the contaminant (40). Natural attenuation, a remediation approach acting without human intervention, occurs via several mechanisms in the environment (40, 45). A major process of natural attenuation is biological degradation; however, processes such as dispersion, dilution, volatilization, etc. can also contribute to a decrease in the chemical concentration. Bioremediation is a promising and cost-effective technology to clean up contaminated sites, and

hence is a major focus of current research efforts. The biological mechanisms that can lead to the degradation of halogenated aliphatic and aromatic compounds are: oxidative dehalogenation, hydrolytic dehalogenation, dehydrohalogenation, and reductive dehalogenation (8, 45). The focus of this research is on chloroethenes, a group of compounds that is most effectively degraded by the process of reductive dehalogenation.

Anaerobic reductive dehalogenation is unique because it occurs in the absence of oxygen. It should be noted, however, that reductive dehalogenation is capable of occurring in aerobic environments as well (8, 16). Chlorinated compounds are highly oxidized and contain electron deficient carbon atoms, thus electrophilic attack by oxygenases is not favorable. With oxygen being a high-energy terminal electron acceptor and a co-substrate in oxygenase reactions, its absence poses a challenge to anaerobic microbial degradation.

Reductive dehalogenators have overcome this barrier. They are capable of receiving their energy from the anaerobic dehalogenation process by utilizing chlorinated compounds as terminal electron acceptors (2, 16). This growth-linked process, known as chloridogenesis, is characterized by high dechlorination rates compared to anaerobic cometabolic reduction (24). Thus, the stimulation of populations that utilize chlorinated compounds as a respiratory process is a very promising bioremediation strategy for compounds in anoxic subsurface environments.

Today a reliable method is needed to specifically measure dechlorinating activity via the quantification and monitoring of biodegrading microorganisms in

contaminated soil. While fluorescent *in situ* hybridization (FISH) is one method by which to quantify microorganisms in environment, this method is labor intensive and complex (4, 37). 16S rDNA based PCR is sufficient for detection and monitoring the presence of particular populations but it lacks quantitative information (24). Dilution plate counting, while an option for some organisms, is generally not applicable for most of the dechlorinators, because they are difficult to grow in pure culture on solid surfaces. The process of estimating dechlorinating activity in the environment by chemical assays such as gas chromatography (GC) surveys is even more complex, often taking several years to get a reliable figure (11).

Chlorinated ethenes

Of the halogenated organic compounds in the environment, the chlorinated ethenes are of significant importance. Tetrachloroethene (PCE), trichloroethene (TCE), *cis*–1,2- and *trans*-1,2-dichoroethene (*cis*-DCE, *trans*-DCE), and vinyl chloride (VC) are compounds that pose a risk to public health due to their toxicity. TCE is a suspected carcinogen and VC is a human carcinogen (5, 27, 40, 43). The wide use of PCE and TCE in dry cleaning and in the degreasing of machinery and electronic components, has led to their environmental release and caused PCE and TCE to become the most commonly found groundwater contaminants (8, 32, 40, 43, 45).

A major problem with remediating PCE- and TCE-contaminated aquifers is the dense non-aqueous phase liquids (DNAPLs) associated with their presence;

DNAPLs occur when chemical concentrations exceed water solubility. Pump and treat strategies, traditionally used, are long term and high cost method of removal (27, 40). The oxidized nature of PCE and TCE makes degradation through strictly oxidative processes difficult (27). (Co)-oxidation, however, has been shown to be effective for degradation of TCE, DCE and VC (20, 43). The availability of adequate oxygen for (co)-oxidation is often a major limiting factor to using this approach at many contaminated sites (20). Therefore, stimulation of in-situ anaerobic reductive dehalogenation is of great interest (40).

Reductive dechlorination of PCE and TCE occurs via a hydrogenolysis process, and involves the substitution of chlorine with a hydrogen atom (32, 45). Dechlorination occurs in a stepwise fashion replacing a single chlorine atom with a hydrogen atom (Figure 1) (45). This process can proceed to the product ethene, yet complete dechlorination is only known to occur with two species of *Dehalococcoides (Dhc.)*; *Dhc. ethenogenes* and strain FL2 (26, 24).

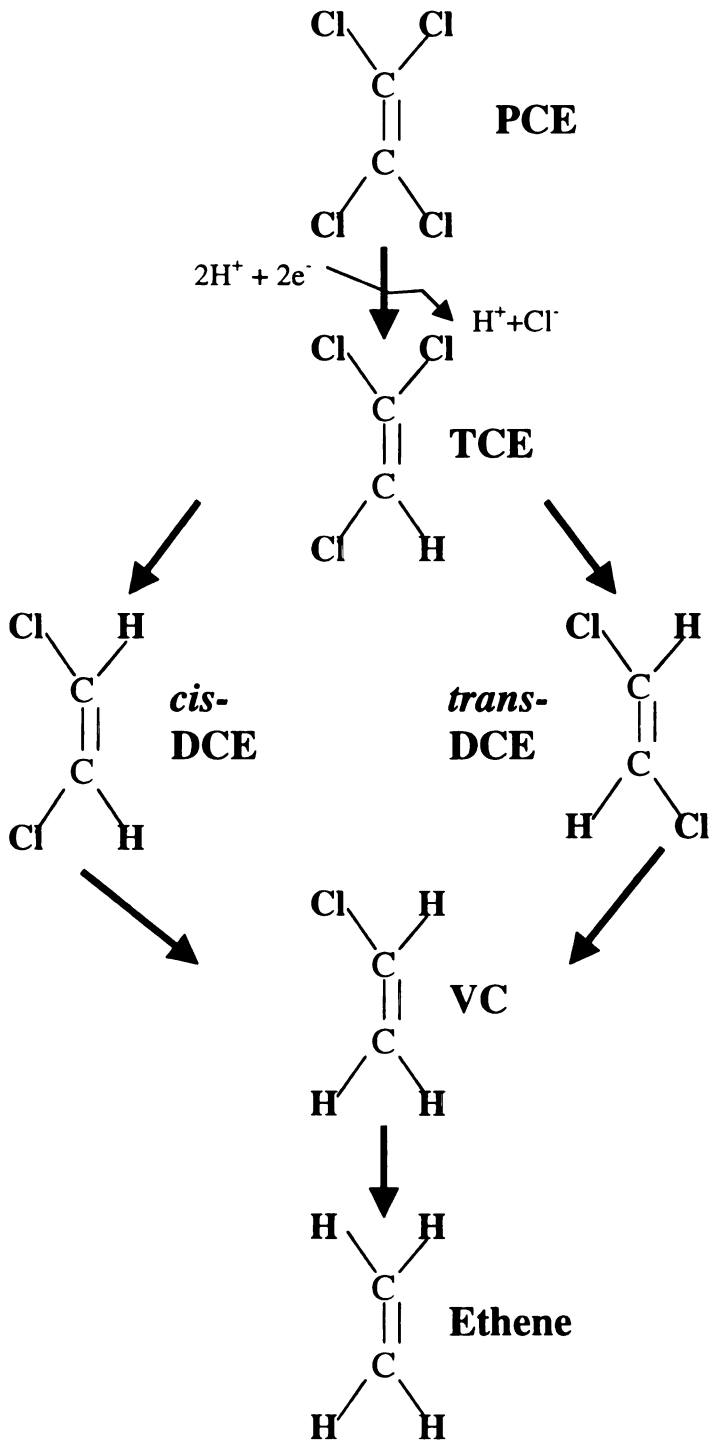

Figure 1. Stepwise dechlorination pathways of PCE to ethene, all intermediates are labeled. Each arrow (⟶) represents the following: $2H^+ + 2e^-$ are consumed and $H^+ + Cl^-$ released.

PCE dechlorinating groups

Several bacterial isolates are capable of reductive dechlorination of PCE to various endpoints while conserving energy from the process. These organisms are diverse, including low G+C gram positives, delta (δ), epsilon (ε) and gamma (γ) sub-groups of the Proteobacteria (Figure 2) (16, 38). All, except *Enterobacter* sp. strain MS-1, are strict anaerobes (16). Of the other Proteobacteria, *Dehalospirillum multivorans* (ε sub-group), *Desulfuromonas (Dsf.) chloroethenica*, *Dsf. michiganensis* strains BRS1 and BB1 (δ sub-group), and *Dehalobacter restrictus* (low G+C) transform PCE/TCE to *cis*-DCE as well (16, 24)]. Among the other PCE dechlorinators, the *Desulfitobacterium* spp. (low G+C) varies in their final products of PCE dechlorination (16). These can involve a single chlorine removal, PCE to TCE, for *Desulfitobacterium dehalogenans*, or dual chlorine removal, PCE to *cis*-DCE, for *Desulfitobacterium* spp. strains PCE1 and PCE-S (16). One group not found amongst the low G+C and Proteobacteria is *Dhc. ethenogenes* (16). *Dhc. ethenogenes* and *Dhc.* spp. strain FL2 are the only isolates thus far capable of dechlorinating PCE all the way to ethene, however, the last step, VC to ethene, is co-metabolic (16, 27). Of interest to this research were the *Dhc.* and *Dsf.* groups of PCE dechlorinators. This interest is due to the ability of *Dhc.* spp. to transform PCE to ethene, and *Dsf.* spp. for its ease of growth and rapid conversion of PCE to *cis*-DCE.

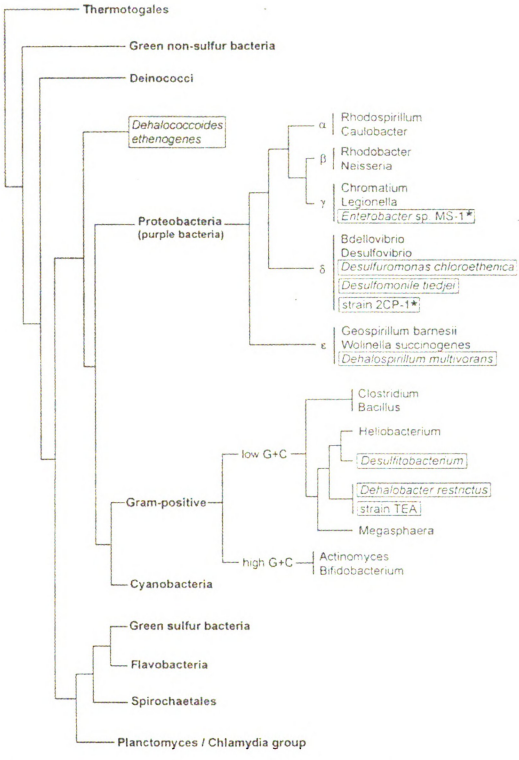

Figure 2. Phylogenetic affiliations, of the bacteria capable of reductive dechlorination (framed), as determined by 16S rDNA gene sequence analysis. Asterisks indicate the facultative anaerobes. Holliger et al. (1999)

Reductive dehalogenases (RDases) are the key enzyme systems involved in the dehalogenation process of the chlorinated ethenes (16). A few RDases have been purified and characterized that are involved in the dechlorination of various compounds (16, 31, 34). Those that are known to dechlorinate PCE, were isolated from *Dehalospirillum multivorans, Dehalococcoides ethenogenes, Desulfitobacterium* strain PCE-S, and *Dehalobacter restrictus* (16, 31).

The PCE-RDase from *Dehalospirillum multivorans* is cytoplasmically located and has an apparent molecular mass of 58 kDa (16, 34). Unlike *D. multivorans,* the other PCE-RDases isolated are membrane bound (16, 27, 31, 34, 39, 42). The proteins range in size from 51-65 kDa: these include the PCE-RDase from *D. ethenogenes, Dehalobacter restrictus*, and *Desulfitobacterium* spp. (16, 27, 39). Of particular interest to this work was the PCE and TCE-RDase from *Dhc. ethenogenes.* These enzymes are capable of dechlorinating PCE to TCE and TCE, DCEs' and VC to ethene, respectively (27). However, unlike other PCE-RDases, PCE is the only substrate for the PCE-RDase of *Dhc. ethenogenes* (26). The ability of this organism to dechlorinate PCE/TCE down to ethene is of particular importance to the bioremediation field. The gene encoding the TCE-RDase (*tceA*) of this bacterium has been cloned and sequenced (26). The gene was found to be unique, having only a limited similarity to PCE-RDase (*pceA*) of *D. multivorans* and no similarity to dehalogenase sequences from *Desulfitobacterium* spp. (26).

Dsf. spp. have different electron donor requirements than all chloroethene dechlorinators studied; *Dsf.* spp. utilize acetate but not H_2 as an electron donor

for the reductive dechlorination of PCE and TCE to *cis*-DCE (16, 19, 24). *Dsf.* spp. also shows very high rates of dechlorination, compared to other PCE dechlorinators (22). The PCE-RDase from *Dsf. michiganensis* strain BB1 is induced in the presence of PCE and has yet to be isolated and its gene identified (22).

Real Time Quantitative PCR

Real time PCR (RTm PCR) will be used as a quantification tool in this study. RTm PCR takes advantage of the 5'-3'nuclease activity of *Taq* DNA polymerase to digest a fluorescently labeled probe. This probe allows for the quantification of the initial nucleotide template (1, 3, 10, 35). The probe is specifically designed and carries a 5' reporter dye, such as 6-carboxyfluorescein (6-FAM), and a 3' quencher molecule, 6-carboxytetramethylrhodamine (TAMRA) (1). The RTm PCR reaction is similar to regular PCR in that a forward and a reverse primer, and *Taq* polymerase are used to initiate and complete the reaction. However, the addition of a labeled internal probe allows for the fluorescent detection of a completed polymerase reaction (Figure 3).

The presence of the TAMRA molecule on the 3' end of the probe quenches the fluorescent signal of the 6-FAM. Detectable fluorescence is produced when the polymerase cleaves the reporter dye from the 5' position on the probe (Figure 3) (35). This occurs only when the probe is hybridized to the target and the polymerase is moving in the 5'-3' direction from the forward primer. The probe is also designed to prevent its own elongation by the addition

of a phosphorylated 3' end (1). Since the process of extension only produces a fluorescent signal, repeated cycles of PCR will result in an exponential amplification of the product and the corresponding increase in fluorescent intensity (35). This exponential increase in intensity is then reported on an exponential plot showing the fluorescent intensity vs. cycle number (Figure 4). Fluorescence increases in earlier cycles with larger template concentrations (Figure 4). A standard curve was obtained by plotting cycle threshold (Ct), a chosen value above background fluorescence, versus template concentration (Figure 5).

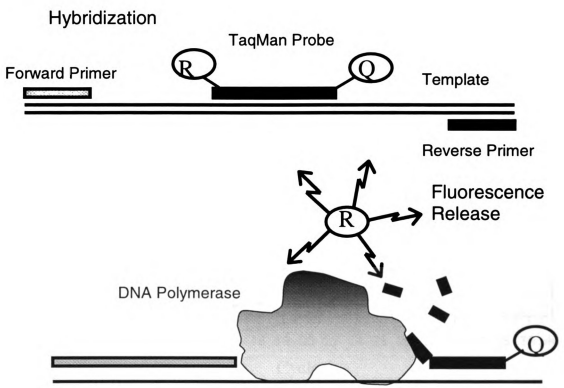

Figure 3. A schematic diagram of the RTm PCR reaction, which shows probe hybridization and eventual cleavage of the reporter dye from the probe by *taq*-polymerase. Release of the fluorescent dye causes a measurable increase in fluorescence.

The cycle threshold value (Ct) increases as template concentration (ngs of template, CFUs, gene copy number) decreases (Figure 5). RTm PCR is not only used to quantify DNA templates (genomic, plasmid, etc.), RTm PCR can also used to quantify mRNA by the addition of a reverse transcriptase reaction prior to RTm PCR (36). With high reproducibility and minimal standard deviations between replicates, RTm PCR is a accurate method for the quantification of organisms in pure and mixed cultures and in environmental samples (3, 10, 35, 37). RTm PCR is a powerful quantitative tool; its use can help advance the current knowledge of microbial populations in contaminated areas (7).

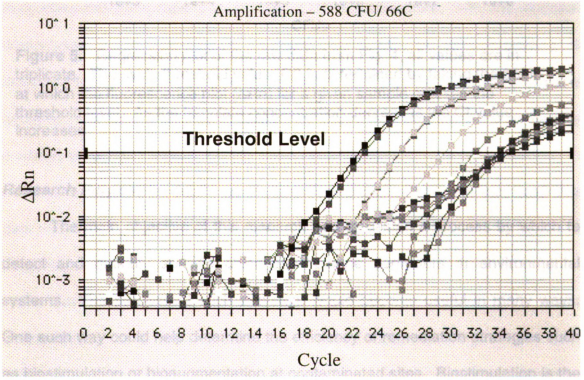

Figure 4. Raw data of an RTm PCR reaction, showing the increase in fluorescence over cycle number. The increase in fluorescence is recorded as a change in intensity (ΔRn). The threshold level is indicated. The different curves are fluorescence of different concentrations of template DNA.

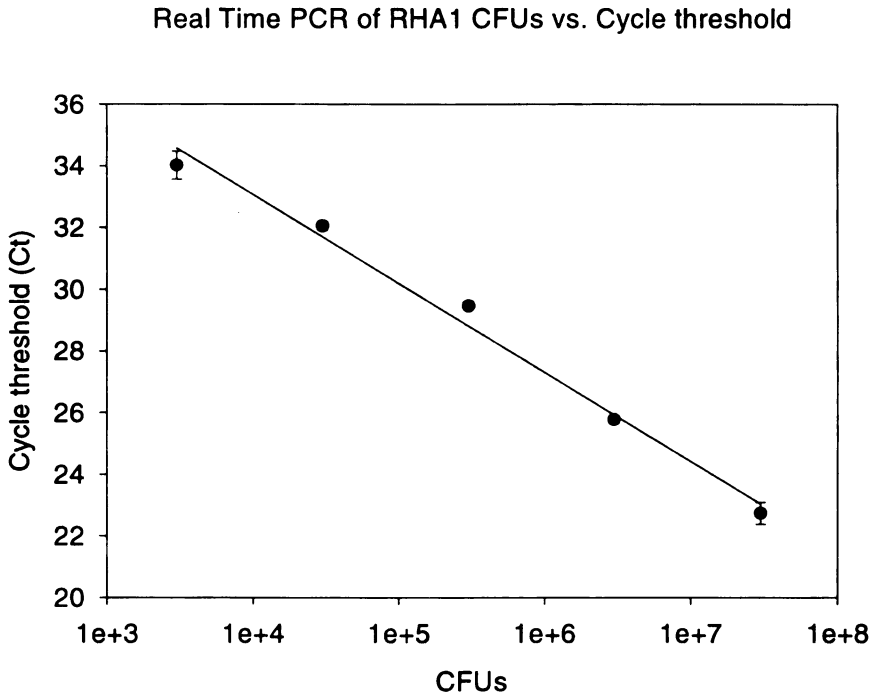

Figure 5. Regression plot of the raw data from Fig. 4, all values are in triplicate. The cycle threshold value (Ct) is determined by taking the point at which the fluorescence plot (ΔRn) for a given sample crosses the threshold level. As the template concentration decreases the Ct value increases.

Research Purpose

The main objective of this research was to design a process by which to detect and quantify specific chlorinated ethene degraders in environmental systems. The purpose of such a system would become useful in many ways. One such way could help determine the efficiency of remediation strategies such as biostimulation or bioaugmentation at contaminated sites. Biostimulation is the process of promoting the degradative capabilities of indigenous microorganisms through the addition of electron donors and other nutrients (21). Bioaugmentation, on the other hand, is the process of adding an extraneous

source of microorganisms and associated nutrients to aid the degradation of a specific environmental contaminant (21). The quantitative monitoring of specific dechlorinators in situ would prove useful as well. Such a tool would be helpful in assessing background populations in the environment prior to introduction of inocula. Being able to track and monitor organisms of interest in the environment is important to establishing cause and effect relationships; quantification would be a major improvement into this process and it can confirm growth and aid in quantitative modeling of the process in situ.

A system that looks at the microbiology of contaminated sites, not just their chemical composition is in need (11). Furthermore, with the concern surrounding the introduction of microorganisms into the environment, it is important that the fate of inocula is assessed (44). This assessment is not only necessary to determine their effectiveness at site remediation but potential for environmental harm as well.

The use of RTm PCR, with its quantitative capabilities, can help to fill this gap. RTm PCR poses a unique challenge. While used in the medical field, it is beginning to see application in environmental microbiology (1, 12, 17, 37).

Having a broader more complete understanding of the major dechlorinators at a contaminated site is useful when deciding on a proper remediation plan. Being able to determine if inocula of introduced organisms have grown and maintained a stable presence after introduction into a contaminated plot can aid in the assessment of remediation strategies in use. RTm PCR can provide this type of assessment.

CHAPTER 2

Development of Real Time PCR (RTm PCR) for the Detection and Quantification of the PCE/TCE Dechlorinating *Dehalococcoides* and *Desulfuromonas* Populations

Introduction

Tetrachloroethene (PCE) and trichloroethene (TCE) are hazardous compounds widely used as organic solvents and degreasers. Due to poor handling; i.e. leakage and inadequate disposal practices, these compounds have become some of the most common ground water contaminants (13, 26, 27). Their presence in underground sediments, soils, and aquifers has posed a unique challenge to their remediation. These compounds pose a unique risk to the surface waters surrounding the State of Michigan (21). This is in part due to the groundwater flow towards the surface waters (21). It has also been realized that natural degradation fluxes are likely insufficient to mitigate the impact of surface contaminant discharge (21). Thus, the "implementation of engineered strategies aimed at source and plume control will be required" (21). Which strategy should be used? It has long been realized that the pump and treat approaches are ineffective and costly when dealing with chlorinated solvents (13). With the process of reductive dechlorination being considerably faster than anaerobic cometabolic reduction and the possibility of complete detoxification, the stimulation of organisms to carry out this process for site remediation is

promising (24). The use of microorganisms to carry out the remediation process has been of considerable focus in the last several years (13, 21, 24, 26, 27).

Several bacterial enrichments have been found capable of reducing PCE to ethene (6, 13, 24, 27). However, only a few pure culture isolates capable of carrying out this process are known: *Dehalococcoides ethenogenes* strain 195, and strain FL2 (13, 24, 28). Another *Dhc.,* strain DCEH2, is known to carry out the same dechlorination process. It is currently maintained in highly enriched cultures but has yet to be isolated in pure culture (13, 24). The *Dehalococcoides* strains utilize hydrogen as their ultimate electron donor in the dechlorination process. In addition, other bacterial strains have been isolated that are capable of reducing PCE down to other end products; such as TCE and DCE's (6, 15, 18, 24, 28-30, 33). Of these organisms, the dechlorinating *Desulfuromonas* strains, which are capable of reducing PCE to *cis*-DCE, are also of interest due to their unique substrate requirements, the utilization of acetate as opposed to hydrogen as the electron donor (18, 24). They are the only PCE to *cis*-DCE dechlorinators detected at some sites (e.g. Bachman Road Site). Both groups of organisms appear promising for use in field bioremediation (13, 24)].

A recent pilot-scale project at the Bachman Road Site in Oscoda, MI was carried out. A major goal of the project was to evaluate the differences between biostimulation and bioaugmentation of a chlorinated ethene contaminated aquifer (21). This study was also being carried out to explore the effectiveness of a microbial inoculum being used for bioaugmentation. The Bachman Road Site had previously been evaluated for the presence of dechlorinating

microorganisms. From that work, a PCE to *cis*-DCE dechlorinating isolate, *Dsf. michiganensis* strain BRS-1, along with an enrichment culture containing a *Dhc.* species capable of dechlorinating *cis*-DCE to ethene were obtained (21, 24). To prepare the inoculum for the field study the two cultures were used to inoculate a fluidized bed reactor, fed with lactate and PCE. After inoculation and a short lag period, reductive dechlorination of PCE to ethene at an efficient rate was observed in the reactor. Periodic additions of PCE and lactate were made over a 12-month period, in order to obtain a sufficient amount of biomass. Amendments were made 8-10 times over the 12-month period.

The Bachman Road Site project was designed with a control plot and test plot, which are hydraulically separated from each other (Figure 6) (21). The control plot was left untouched for twenty weeks, and compared to the bioaugmentation plot (test plot) for chloroethene removal. Biostimulation was later initiated at the control plot by the continuous injection of lactate and nutrients (21). Directly monitoring the chlorinated ethenes, along with tRFLP analysis, provided insight into the dechlorination activities at the different plots (21). The data also provided insight into the relative presence and distribution of specific organisms (21). However, a quantitative measurement of the introduced dechlorinating organisms, as well as those organisms stimulated in the plots was lacking. A quantitative measurement would provide insight into the efficacy of the reactor inoculum as well as provide a numeric comparison of population density between plots (37). By gathering quantitative data, it is possible to gain an understanding of the environmental fate of the inoculum organisms (44).

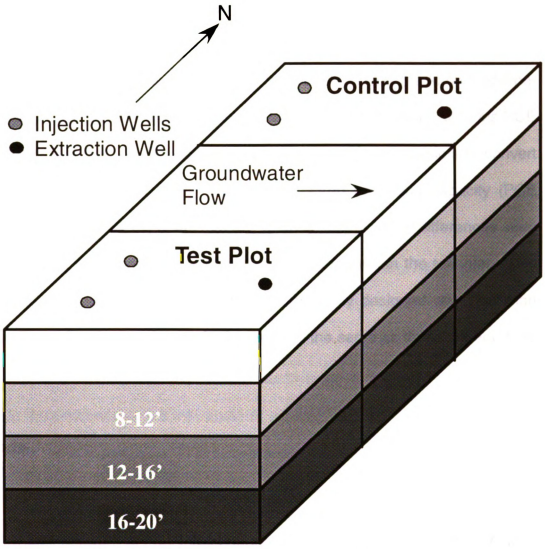

Figure 6. Schematic diagram of the Bachman Road Site; details include the relative location of the groundwater injection and extraction wells, in relation to the ground water flow. In addition the depth ranges (in feet), tested by RTm PCR are indicated.

Knowing two of the main dechlorinating groups present in the reactor inoculum, *Dhc.* sp. and *Dsf. michiganensis* strain BRS1; it was hypothesized that the bioaugmented plot would have a larger number of *Dhc.* sp., and *Dsf.* sp. than would the biostimulated plot. It was also hypothesized that the known ethene-producing populations, *Dhc.* will be in larger quantities than the *cis*-DCE producing population, i.e. *Dsf.*, due to the observed ability of the plots to convert PCE to ethene at a high rate. With more electron accepting capacity (PCE through VC) it would be expected that any quantitative microbial differences seen in the inoculum producing reactor should also be seen within the test plot. This difference is expected since the reactor was originally designed with Bachman aquifer material and amendments in the field are the same as those provided the reactor.

Methods

Chemicals

Chemicals were of the highest purity available and purchased from Aldrich (Milwaukee, WI) and Sigma Chemical Co. (St. Louis, MO). Forward and reverse primers were obtained form the Macromolecular Structure, Sequencing and Synthesis Facility (Michigan State University, East Lansing, MI). Fluorescent-labeled probes were obtained form PE Biosystems (Perkin Elmer Applied Biosystems, Foster City, CA).

Cultures

A culture of *Dhc.* spp. strain FL2 culture and extracted DNA along with *Dsf. michiganensis* strain BRS1, *Dsf. acetoxidans, Dsf. succinoxidans, Dsf. acetexigens, Dsf. thiophila* were provided by F. Löffler, Georgia Institute of Technology, Atlanta, GA. *Dehalospirillum multivorans* was provided by John Davis, Center for Microbial Ecology. *Dehalobacter restrictus* (DSMZ 9455) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; http://www.dsmz.de) and grown as recommended by the DSMZ. *Dsf. michiganensis* strain BRS1, *Dhc.* spp strain FL2, and *Dehalospirillum multivorans* were grown in a basal salts medium as outlined in Appendix 1 (23, 25) *Dsf. michiganensis* strain BRS1 was grown on 2.5 mM acetate and PCE (0.1 mM, in 5 ml hexadecane) with a N_2 /CO_2 (80/20) headspace. *Dhc.* spp. strain FL2 was grown in the presence of 0.5 mM acetate, 0.2 mM TCE and hydrogen (10 kPa). All cultures were grown at 25 °C in strictly anaerobic 160-ml serum bottles with a total volume of 100 ml. L-cystiene, 0.21 mM, and Na_2S were used as reductants.

Reactor and Bachman Road Site Sampling

EFX Systems, Lansing, MI, maintained the 75 L liquid glass column reactor in which the contents were slowly and continuously circulated. Dechlorination in the reactor was monitored via gas chromatography. Additional amendments of PCE and lactate were added as needed to maintain a steady state within the reactor. Nitrogen purged serum bottles, 160 ml, were used to collect 100 ml of reactor effluent. Microbial biomass was harvested by centrifugation.

The test plot used for the bioaugmentation experiment and an un-inoculated control plot that was amended later for the biostimulation experiment is shown in Figure 6. Aquifer cores were taken from four locations within the biostimulated (control) and bioaugmented (test) plots at the Bachman Site on May 24th and 25th, 2001 (21). One set of cores were downstream from the injection well and the second downstream from the extraction wells (21). Cores were divided into three depth ranges: 8-12', 12-16', and 16-20'; and were stored at 4 °C until analyzed.

DNA extraction and quantitation

Genomic DNA was extracted from reactor effluent and from pure cultures after harvest by centrifugation. DNA was isolated with the Qiagen Blood and Cell Kit (Qiagen Inc., Valencia, CA). The concentration of DNA was determined by absorbance at 260 nm. *Dhc.* spp. 16S and *tceA* gene copy numbers were estimated based on the genome size of 1.5 Mbp (41) and on the assumption that there is only 1 copy of the *tceA* and 16S rDNA gene per genome. For *Dsf. michiganensis* strain BRS1, a genome size of 2.5 Mbp and 1 copy of the 16S rDNA gene per genome was assumed based on the average genome size of similar organisms.

Aquifer core DNA was extracted using the Ultra Clean Soil DNA Kit (MO BIO, Solana Beach, CA.) following manufacturers instructions. 0.25 g of aquifer material was removed in triplicate from each core. DNA was extracted and later combined from a given core, so that the final mass of sediment sampled for each

core was 0.75 g. DNA concentration was determined at a wavelength of 260 nm. All samples were stored at –20 °C until use.

Primer Design and RTm PCR

16S rDNA gene sequences for *Dhc.* spp. strain FL2 (GenBank accession no. AF357918), *Dsf. michiganensis* strain BRS1 (GenBank accession no. AF357914), and the *tceA* reductive dehalogenase gene (GenBank accession no. AF228507) were utilized for primer/probe design. Primer/probe sequences for the above sequences were designed using Primer Express Software (Perkin Elmer Applied Biosystems, Foster City, CA). Potential sequences were subsequently submitted to BLAST and the PROBE-MATCH program of the Ribosomal Database Project II (RDP) to insure specificity within the groups of interest. When the entire amplicon matched only the dechlorinating strains of interest, the primer/probes were deemed specific.

The probe for the all primer/probe sets contained a FAM reporter dye and TAMRA quencher dye. A series of calibration reactions were performed, to determine ideal concentrations of the forward and reverse primer, and probe. All reactions utilized pure culture DNA for the particular strain of interest. Each 30 µl reaction contained the given concentration of primers and probe in addition to 1 X TaqMan buffer, 150 pmol of each dNTP, 1.5 units of AmpliTaq Gold DNA polymerase (Perkin Elmer Applied Biosystems, Foster City, CA). DNA concentrations per reaction varied and depended on the experiment being performed. All experiments were performed in triplicate with appropriate no-template control reactions. The reaction cycles were as followed: 2 min at 50 °C

for optimal AmpErase uracil-N-glycosylase enzyme activity, then denaturation at 95 °C for 10 min and 40 cycles of amplification of 15 s at 95 °C and 1 min at 60 °C of annealing and extension. AmpErase uracil-N-glycosylase degrades residual carry over RNA products that may be amplified during the PCR amplification phase. The PCR was performed in a spectrofluorimetric thermal cycler, ABI Prism 7700 Sequence Detection System (Perkin Elmer Applied Biosystems, Foster City, CA). Fluorescent data was gathered and analyzed by Sequence Detector Software v. 1.6 and 1.7 (Perkin Elmer Applied Biosystems, Foster City, CA). Plots were conducted and statistics were performed using SigmaPlot 2001.

Primer/probe specificity

To determine the specificity of the designed primer/probe sets for each dechlorinating group RTm PCR reactions were performed using pure cultures or cloned 16S rDNA and/or *tceA* DNA. To determine specificity for the PCE-dechlorinating *Dsf.* group, pure culture DNA was obtained and tested from *Dsf. michiganensis* strain BB1, *Dsf. acetoxidans, Dsf. succinoxidans, Dsf. acetexigens, Dsf. thiophila, Dehalobacter restrictus, Dhc.* sp. strain *FL2, Dehalospirillum multivorans,* and *Escherichia coli.* The dechlorinating *Dhc.* group 16S rDNA and *tceA* gene probes were tested against *Dsf. michiganensis* strain BRS1, *Dhc. sp.* strain FL2, *Dehalobacter restrictus*, *E. coli*, and *Dhc. ethenogenes, Dhc.* sp. strain CBDB1, and *Dhc. sp.* strain FL2 16S rDNA clones. Furthermore, a *tceA* reductive dehalogenase clone was also used for specificity testing of the *tceA* gene. All reactions were 30 µl in volume and contained the

same components as described above. DNA concentrations were constant at 30 ng per reaction.

Sensitivity of RTm PCR and standard curve development

To determine the sensitivity of the 16S rDNA and functional gene primer and probe sets, a 1:10 serial dilution (from 10^1 to 10^7 copies) of the 16S rDNA and functional genes were prepared in herring sperm DNA (1 $\mu g/ml^{-1}$ in water; Boehringer Mannheim, Indianapolis, Ind.) as a carrier. All reactions were performed in triplicate and the 95% confidence intervals were determined.

RTm PCR of core samples

RTm PCR was performed on DNA extracted from core samples. Between 100-130 ng of DNA was added to each reaction tube, and triplicate RTm PCR reactions were performed as described above. Primer/probe sets for the 16S rDNA genes of *Dehalococcoides* spp. and *Desulfuromonas* spp. were used. Results were converted to gene copy number per gram of sediment.

Results

Primer/probe design

Primer/probe sets for the 16S rDNA gene of *Dsf.* spp. and *Dhc.* spp., and *tceA* functional dehalogenase gene of *Dhc. ethenogenes* were developed (Table 1). After evaluation of different primer/probe sets, the best combinations were compared to phylogenetically related organisms to ensure specificity. *Dsf.* spp. probe/primer sequences were found to be specific for the dechlorinating *Dsf.*

strains of interest (Table 2). One unknown base within *Dsf. chloroethenica* probe sequence was resolved by replacing the base pair with the universal base inosine. Two to four mismatches were found within the probe sequence amongst the non-dechlorinating *Dsf.* strains. The forward primers contained two mismatches with the exception of *Dsf. thiophila*, which had three. The primer/probe sequences also showed little similarity to the other known PCE/TCE dechlorinators: *Dehalobacter* sp., *Dhc.* spp., and *Dehalospirillum* spp.

Dhc. spp. probe sequences matched fourteen known *Dhc.* clones as well PCE/TCE dechlorinating *Dhc.* spp. strains according to BLAST analysis. *Dhc. ethenogenes*, *Dhc.* sp. strain CBDB1, *Dhc.* sp. strain FL2 and bacterium DCEH2 matched the primer/probe sequences with a 100% fit (Table 3). The primer/probe sequences also matched several uncultured bacterium clones that reductively dechlorinate TCE (GenBank Accession #s: AF348755, AF427937, AF427912, AF427910, AF427908, AF427907). The primer/probe sequences were nonspecific to other known PCE/TCE dechlorinators unrelated to *Dhc. sp.*; the closest similarity was with *Dehalobacter restrictus*.

The *tceA* reductive dehalogenase gene was also analyzed by BLAST, and only matched the known *Dhc. ethenogenes tceA* reductase gene. The primer/probe sequences were checked against *Dehalospirillum multivorans pceA* reductase, and no sequence similarities were found for several *Desulfitobacterium* spp. dehalogenase gene sequences (results not shown). The optimized concentrations for all primer/probe sets used were as follows: forward primer, 300 nM; reverse primer, 300nm; probe 300 nm.

Tabel 1 - Primers and probes used in this study.

Primers and Probes	Sequence
Desulfuromonas spp.	
Forward 16S rDNA	5' GACATCCCGATCGCACCTTA 3'
Taqman 16S rDNA	5'FAM-AACATAGGGGTCAGTTCGGCTGGIT-TAMRA 3'
Reverse 16S rDNA	5' CCATGCAGCACCTGTCACC 3'
Dehalococcoides spp.	
Forward 16S rDNA	5' CTGGAGCTAATCCCCAAAGCT 3'
Taqman 16S rDNA	5' FAM-TCCTCAGTTCGGATTGCAGGCTGAA-TAMRA 3'
Reverse 16S rDNA	5' CAACTTCATGCAGGCGGG 3'
Forward *tceA*	5' ATCCAGATTATGACCCTGGTGAA 3'
Taqman *tceA*	5' FAM-TGGGCTATGGCGACCGCAGG-TAMRA 3'
Reverse *tceA*	5' GCGGCATATATTAGGGCATCTT 3'

Table 2 - *Desulfuromonas spp.* 16S rDNA primer/probe sets

Organism	5' - Froward primer - 3'	5' - Probe[ab] - 3'	5' - Reverse primer - 3'
	GACATCCCGATCGCACCTTA	AACATAGGGGTCAGTTCGGCTGGIT	CATGCAGCACCTGTCACC
Dsf. michiganensis str. BRS1	••••••••••••••••••••	•••••••••••••••••••••••••A•	••••••••••••••••••
Dsf. Michiganensis str. BB1	••••••••••••••••••••	•••••••••••••••••••••••••A•	••••••••••••••••••
Dsf. chloroethenica	••••••••••••••••••••	•••••••••••••••••••••••••N•	••••••••••••••••••
Dsf. acetoxidans	••••••••N••••T••••••	•••••TT•••N•••••-••••N•A•	••••••••••••••••••
Dsf. acetexigens	••••••••••••••••T••C	••••G••A•••••••••••••••A•	••••••••••••••••••
Dsf. succinoxidans	••••••••••••••••TC••	••••••••A••••••C•T••••••A•	••••••••••••••••••
Dsf. thiophila	••••••••••••••T••GA••	••••••TT•••••••••••••••A•	••••••••••••••••••
Dehalococcoides str. FL2	no significant similarity	no significant similarity	••••••••••••••••
Dehalospirillum multivorans	no significant similarity	••••••••••••	••••••••••••••
Dehalobacter restrictus	no significant similarity	no significant similarity	no significant similarity

[a]The adenosine was replaced with inosine in the probe in order to pick up the mismatch in *D. chloroethenica*

[b]Probe is 5' labeled with FAM and 3' labeled with TAMRA dyes

Table 3 - *Dehalococcoides spp.* 16S rDNA primer/probe sets

Organism	5' - Foraward primer -3'	5' - Probe[b] - 3'	Reverse primer
	CTGGAGCTAATCCCCAAAGCT	TCCTCAGTTCGGATTGCAGGCTGAA	CAACTTCATGCAGGCGGG
Dehalococcoides str. FL2	••••••••••••••••••••	••••••••••••••••••••••••••	••••••••••••••••••
Dehalococcoides ethenogenes	••••••••••••••••••••	••••••••••••••••••••••••••	••••••••••••••••••
Dehalococcoides CBDB1 clone	••••••••••••••••••••	••••••••••••••••••••••••••	••••••••••••••••••
Bacterium DCEH2	••••••••••••••••••••	••••••••••••••••••••••••••	••••••••••••••••••
Dehalococcoides clones[a]	••••••••••••••••••••	••••••••••••••••••••••••••	••••••••••••••••••
Dsf. michiganensis str. BRS1	no significant similarity	••••••••••••••	no significant similarity
Dehalospirillum multivorans	no significant similarity	GT••••••••••••••G••T•••C•	no significant similarity
Dehalobacter restrictus	no significant similarity	••••••••••••••••••••••	••••••••••••••

[a]*Dehalococcoides* sp. clones DHC-plk, DHC-pin, DHC-nftx, DHC-lnz, DHC-klf, DHC-kb1P, DHC-kb1C, DHC-kafb, DHC-dab, DHC-chd, DHC-bmtv, DHC-bmtp, DHC-bmtc, DHC-asd,

[b]Probe is 5' labeled with FAM and 3' labeled with TAMRA dyes

Table 4 - Specificity of RTm PCR for *Desulfuromonas* spp.

Species, strain or clone	Primer/probe set
Positive controls	16S rDNA[a]
Dsf. michaganensis strain BRS1	+
Dsf. michaganensis strain BB1	+
*S*train BRS1 16S rDNA clone	+
Negative controls	
Dsf. acetoxidans	+/-
Dsf. acetexigens	-
Dsf. thiophila	-
Dsf. succinoxidans	-
Dehalococcoides sp. strain FL2	-
Dehalobacter restrictus	-
Dehalospirillum multivorans	-
Escherichia coli	-

[a] +, logrithmic amplification detected; -, no amplification detected; +/-, minimal amplification detected

Table 5 - Specificity of RTm PCR for *Dehalococcoides* spp.

Species, strain or clone[a]	Primer/probe set	
Positive controls	16S rDNA[b]	*tceA*[b]
Dehalococcoides sp. strain FL2	+	+
Dhc. sp. strain FL2 16S rDNA clone	+	ND
Bacterium CBDB1 16S rDNA clone	+	ND
Dhc ethenogenes 16S rDNA clone	+	ND
Negative controls		
Dsf. michaganensis strain BRS1	-	-
Dsf. michaganensis strain BB1	-	-
Dehalobacter restrictus	-	-
Dehalospirillum multivorans	-	-
Escherichia coli	-	-

[a] *Dehalococcoides* clones were obtained from F. Löffler at the Georgia Institute of Technology, Atlanta, GA

[b] +, logrithmic amplification detected; -, no amplification detected; ND, amplification not performed

Specificity

DNA from a variety of pure culture strains and clones was used to test the specificity of the primer/probe sequence developed for *Dsf.* spp. (Table 4) and *Dhc.* spp. (Table 5). Pure cultures of *Dsf. michiganensis* strain BRS1 were used as positive controls for the *Dsf. spp* group primer/probe sets; a 16S rDNA clone of strain BRS1 was also used as further confirmation. Logarithmic amplification was detected for the samples mentioned, confirming specificity for the dechlorinating *Dsf. spp.* tested. Within the negative controls tested, only *Dsf. acetoxidans* yielded a slight amplification signal; the signal was weak in comparison to the dechlorinating *Dsf.* spp. tested and comparable to no-template controls. The primer/probe combination for *Dhc.* spp. amplified the strains tested. A logarithmic increase was found in the tested *Dhc.* strains and bacterial clones. PCE/TCE dechlorinating organisms not related to *Dhc.* spp. failed to produce an amplification signal.

Sensitivity

The sensitivity of RTm PCR for the 16S rDNA and *tceA* genes was tested using a dilution series of *Dsf. michiganensis* strain BRS1 and *Dhc.* spp. strain FL2 DNA. To ensure that all results are comparable, a threshold value of 0.2 was used. This allows for detection of the logarithmic fluorescent increase, while avoiding background signal from the no-template control. The no-template control would often release some residual signal, however such a signal was not

in the form of a logarithmic increase. Similar signals were observed in control reactions without polymerase suggesting probe degradation (10).

Standard curves were developed by relating C_t values to corresponding numbers of calculated gene copies for *Dsf. spp.* 16S rDNA and *Dhc.* spp. 16S rDNA and *tceA* genes (Figure 7). *Dsf. michiganensis* strain BRS1 showed a linear response over 5 orders of magnitude, ranging from 6.05 to 6.05 X 10^5 16S rDNA gene copies (r^2 = 0.995). A linear response was also observed for the 16S rDNA and *tceA* genes of *D* sp. strain FL2 over a range of four to five orders of magnitude, ranging from 73 to 7.3 x 10^5 and 7.3 X 10^4, respectively (r^2 = 1.00 for both regressions).

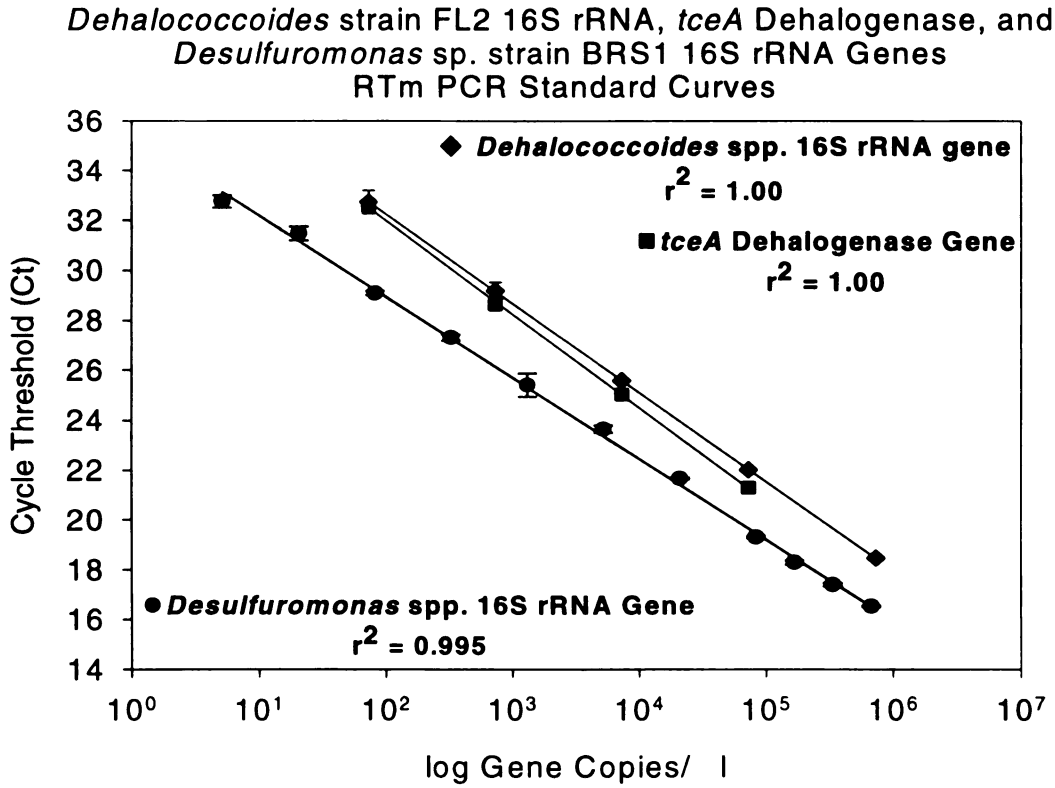

Figure 7. RTm PCR standard curve showing log Gene Copies vs. Cycle Threshold. Calculations based on the following assumptions: 1 gene copy per genome, and genome size of 1.5 Mb for strain FL2, and 2.5 Mb for BRS1.

Bachman core samples

The EFX reactor showed higher quantities of *Dhc.* spp. than *Dsf.* spp. The reactor, originally inoculated with Bachman enrichments and used to bioaugment the Bachman Road Site, contained 5.39 x 10^6 of *Dhc.* spp. and 1.99 x 10^2 of *Dsf.* spp. (Table 6).

Bioaugmented (test plot) dechlorinating populations tested for in soil cores were higher than the biostimulated (control plot) populations at all depth ranges tested 6 months after initialization of the field experiment (Table 6). There was a one to two order of magnitude difference in population between the biostimulated and bioaugmented plots; this is especially apparent in the 12-16' and 16-20' depth ranges sample for *Dhc.* sp. (Figures 8 and 9). Dechlorinating populations near the injection wells were higher by 0.5 – 1.0 orders of magnitude than near the extraction wells in both the bioaugmented and biostimulated plots. *Dhc.* spp. 16S rDNA gene copies were found in higher quantities than *Dsf.* spp. at all sites sampled, this difference in concentration was from 2-4 orders of magnitude depending on the site location. The detection limit for *Dhc.* spp. was 7.8 x 10^3 16S rDNA gene copies/g of aquifer material, and for *Dsf.* spp. 6.2 x 10^2 16S rDNA copies/g of aquifer material.

Table 6 - Quantitative Estimation on May 24 of *Dehalococcoides* and *Desulfuromonas* Populations in the Biostimulation Plot and Bioaugmentation Plot Using Real-Time PCR

		16S rRNA gene copies per g[a] aquifer material			
		Dehalococcoides spp.		*Desulfuromonas spp.*	
Plot	Depth (ft)	Injection[b]	Extraction[b]	Injection[b]	Extraction[b]
Bioaugmented	8-12	**3.8E+04 ± 7.1E+03**	NQ[d]	ND[c]	ND[c]
	12-16	**2.5E+06 ± 3.3E+05**	**3.4E+05 ± 5.9E+04**	NQ[d]	**1.2E+04 ± 1.3E+03**
	16-20	**1.5E+06 ± 1.6E+05**	**4.0E+05 ± 1.1E+05**	NQ[d]	NQ[d]
Biostimulated	8-12	**8.6E+03 ± 8.6E+02**	ND[c]	ND[c]	ND[c]
	12-16	**8.35E+04 ± 2.5E+04**	**3.4E+04 ± 9.3E+03**	**4.2E+03 ± 7.0E+02**	NQ[d]
	16-20	**7.7E+04 ± 1.6E+04**	**1.0E+04 ± 5.4E+03**	NQ[d]	NQ[d]

	Dehalococcoides spp.	*Desulfuromonas spp.*
Column Reactor	1.0E+06 ± -1.1E+05	1.6E+02 ± -1.0E+02

[a] *Dehalococcoides ethenogenes* contains a single rRNA operon (http://tigrblast.tigr.org/ufmg/). The rRNA coy number of *Desulfuromonas* spp. is not known. Triplicate samples of soil-extracted DNA were pooled; three PCR reactions were run for each sample point. [b] Aquifer solids were collected in the center of the plot (injection) and near the extraction wells respectively. [c]ND - not detectable by RTM PCR. [d]NQ - detectable but not quantifiable.

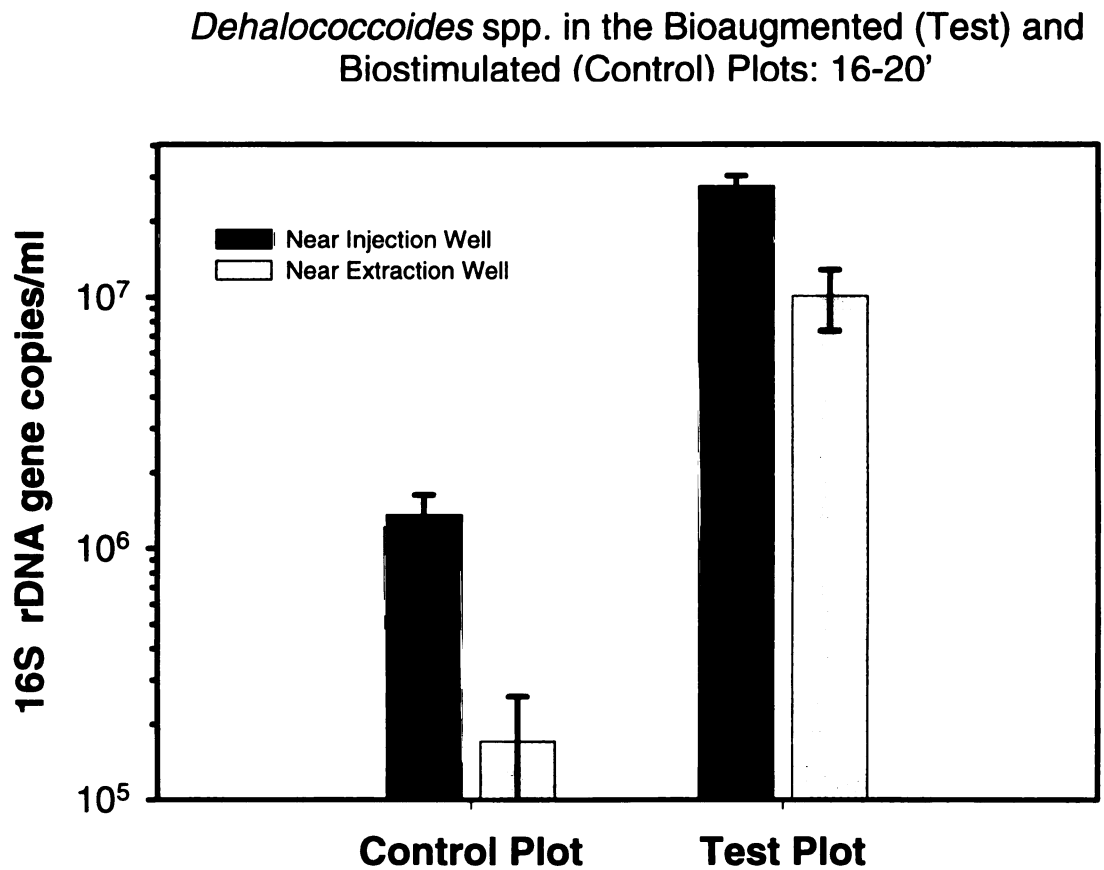

Figure 8. Plot of 16S gene copies/ml of extracted aquifer material at the depth range of 16-20'. Error bars represent triplicate RTm PCR reactions.

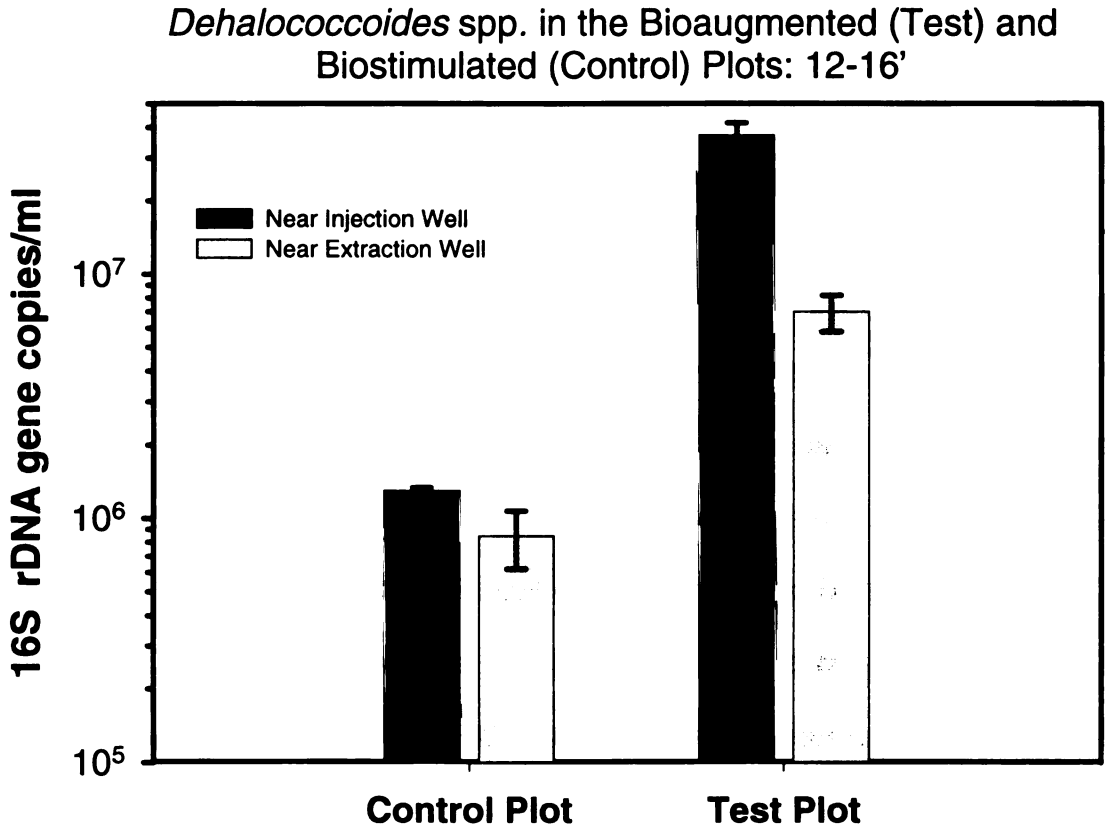

Figure 9. Plot of 16S gene copies/ml of extracted aquifer material at the depth range of 12-16'. Error bars represent triplicate RTm PCR reactions.

Discussion and Conclusions

RTm PCR was shown to be a specific and sensitive tool for the quantification of dechlorinating populations in a PCE contaminated environment. The design of primers specific to the selected dechlorinating groups was possible even when such groups contained a high number of phylogenetically related strains. The dechlorinating group *Dhc.,* for example, has over 25 different 16S rDNA clones, many of which matched the primers and probe designed for this study using the *Dhc.* sp. strain FL2 16S rDNA sequences. The Primer Express software as well as BLAST inquiries provided the knowledge necessary to design group specific primer and probes. It should be noted that use of the database only assures that the probes are specific or non-specific to groups within the database. Furthermore, this is not definitive information and the assumption that these specific matches will occur in the environment has to be made, as it is possible other microorganisms in soil could be targeted by the probes (37). In theory, PCR should be capable of detecting only one copy of the 16S rDNA gene; however even with RTm PCR, such precision becomes limited below 100 gene copies (10, 37). The sensitivity in soil is also much less than what is producible in liquid cultures, such as a reactor. This difference was expected as prior research found similar reductions in sensitivity between pure culture studies and soil (37). This loss of sensitivity was not a critical factor in this research since the dechlorinating organism populations within the contaminated environment were at a level detectable by RTm PCR. In order for PCE dechlorination by these organisms, especially *Dhc.* spp., to be effective the

number of organisms present should be well within the concentration window of RTm PCR (37). Many environmental variables exist that affect detection limits; i.e. the soil matrix, DNA extraction protocol used (37).

As Table 3 shows, a large number of *Dhc. spp.* sequences in the database were identical to the designed primers and probe. All strains with identical probe and primer sequences are capable of dechlorinating PCE to ethene. In addition, the primers designed for detection of *Dhc. spp.* amplified only 16S rDNA from *Dhc. spp.* clones and *Dhc.* like clones (Table 5) The *Dsf. spp.*, probe and primer sequences also matched only the PCE dechlorinating *Dsf. spp.* available for this research (Table 4). Aside from the slight amplification of *Dsf. acetoxidans*, which resembles background found in no-template controls, no other non-dechlorinating *Dsf. spp.*, or non-*Dsf.* PCE dechlorinators were amplified. With these above results, it was concluded that the primers and probes were highly specific for the groups they were designed to detect in the Bachman Road Site.

The Bachman Road Site was inoculated with an enrichment culture previously harvested from a scaled up, production bioreactor. The *Dhc. spp./ Dsf. spp.*, ratio within the Bachman core samples was very similar to that in the reactor inoculum samples. Non-quantifiable (NQ) but detectable results were approximately 1 X 10^2 rDNA copies/g of aquifer material (Table 6). When the bioreactors were sampled and amplified with RTm PCR, a distinct difference in *Dhc.* and *Dsf. spp.* population numbers was detected. A difference of almost 3-4 orders of magnitude was observed, with *Dhc.* being in the majority in the reactor.

This observation is further supported by the ability of the reactor populations to convert PCE to ethene at a high rate; *Dhc. spp.* is the only known bacterial group capable of converting PCE to ethene. As hypothesized, the above observations were also seen in the Bachman cores sampled. *Dhc. spp.* was found to be present in high numbers. As the population data suggests, the Bachman Road Site is capable of complete reduction of PCE to ethene (21). Further evidence from the site showed that the bioaugmented site was capable of removing PCE at a higher rate than the biostimulated site (21). A distinct difference in detectable dechlorinators within the plots was also seen. Population differences between the aquifer cores within a given plot are detectable. Cores taken near the injection wells where the inoculum and lactate was fed are roughly an order of magnitude higher than near the sample extraction wells down gradient.

The use of molecular tools, with their speed, accuracy, and efficiency can prove useful in the field of bioremediation and microbial ecology (14). As stated by Fennell et al., 2001, "molecular-probing (which could and should be extended in the future to include virtually all known dechlorinators and/or dehalogenases) can provide a relatively quick and facile method for investigating spatial distributions of dechlorinators on-site". With low detection limits RTm PCR appears promising for such purposes. The current practices of bioaugmentation and biostimulation in the field are being assessed (21, 44). There exist many differences between the remediation approaches: from cost and speed, to effectiveness and consistency. Studying the removal of pollutants between the remediation approaches provides one level of understanding. However, by

applying microbiological data on a quantitative level, through RTm PCR, the differences can be assessed even further.

CHAPTER 3

The Use of Real Time PCR (RTm PCR) to Monitor the Growth of the PCE Dechlorinating *Dehalococcoides spp.* During a Bioreactor Scale-up

Introduction

"Unfortunately, it is currently difficult or impossible to connect positive PCR results with actual population concentrations of dechlorinating bacteria, or to know whether they are highly active" (7). This while true of standard qualitative PCR is not the case with RTm PCR, as Chapter 2 and the recent work by Rodrigues et al. (2002) has demonstrated. RTm PCR was shown to be useful to quantify dechlorinators in the environment. Rodrigues et al. (2002) utilized RTm PCR to detect the PCB-degrading *Rhodococcus sp.* RHA1 strain in soil. The research showed that the detection of RHA1 in microcosms yielded similar quantitative results when compared to culturable plate counts (37). The anaerobic and sensitive growth requirements of PCE dechlorinators do not provide the opportunity for culturable plate counting methods to quantify these microbes. Hence, a molecular tool is needed to quantitatively assess these microbes.

Chapter 2 detailed the process of quantifying *Dhc. spp.*, and *Dsf. spp.* in a reactor and contaminated aquifer. That project, however, quantified the populations only at a single point in time. The ability to monitor a specific group of PCE dechlorinating organisms over time was missing. By monitoring organisms over time, a better understanding of the population dynamics can be

gained (37). In addition, monitoring at a quantitative level can assess population responses, to outside influences. Aside from monitoring population changes, the ability to estimate the dechlorination activity of those organisms would also prove useful. RTm PCR with its low detection limit, potential for a higher degree of specificity over that of traditional PCR, and high throughput ability was an ideal method by which to tackle the above issues (10, 14, 37).

EFX Systems, Lansing, MI maintains a 75 L reactor used for harvesting PCE-dechlorinating biomass. As Chapter 2 detailed, this reactor was shown to contain a high population of *Dhc. spp.* and dechlorinated PCE to ethene at a high rate. In a pilot test described in Chapter 2, an inoculum from this reactor was injected into a test plot at a PCE contaminated aquifer in Oscoda, MI. Due to the success of that study it was decided to repeat the process at another PCE contaminated aquifer in Schoolcraft, MI. The plan is to bioaugment the aquifer in a way similar to that used for the Bachman Road Site study. The main difference between the projects is the scale of the proposed inoculum; a much larger inoculum is planned for the Schoolcraft study. Due to the increased inoculum demand; a reactor scale-up project was initiated at EFX Systems. A 1250 L stainless steel reactor was designed at EFX Systems, Lansing, MI. The reactor would be started from an initial inoculum containing cultures from the 75 L reactor already described as well as water from the Schoolcraft Site; this reactor would be monitored for several months until PCE dechlorination activity was sufficiently stabilized and active. The scale-up project provided a unique opportunity by which to monitor a known dechlorinator ex-situ via RTm PCR.

The goal of this particular study was to monitor, over time, the growth of the PCE dechlorinating *Dhc. spp.* during a controlled reactor scale-up. *Dhc. spp.*, in particular, *Dhc. ethenogenes* "...remains the best target for probing to assess dechlorination potential", (7) and was thus a perfect candidate for this particular study. With the prior knowledge that *Dhc. spp.*, is present in high quantity in the 75 L reactor and is the only organism currently known to dechlorinated PCE to ethene, it was chosen for this study. The reactor also provided an ideal medium in which to test the monitoring ability of RTm PCR. Previous experiments have shown that more primer/probe sensitivity is obtained in liquid cultures than from soil (37). This study proposed to look at the population growth of *Dhc. spp.*, over a specified period during the reactor scale-up. The initial study examined the population size of *Dhc. spp*, as measured by the presence of the 16S rDNA and *tceA* dehalogenase genes. Both genes are estimated to be present on the genome in one copy. Experiments showed nearly identical standard curves for the two genes, when quantifying the same organism confirming that the genes likely exist in equal copies on the genome (Figure 7, Ch. 2). Detection of the t*ceA* gene, however, was slightly more sensitive than for the 16S rDNA gene. This difference in sensitivity could be accounted for by the location of the genes within the genome and corresponding structural differences that may decrease accessibility of the primers and probe to their target (37). With many organisms being uncultivable, molecular tools are often all that is available for monitoring and detecting their presence in the environment. As

shown in the Chapter 2, RTm PCR is a useful tool for detecting *Dhc. spp.*; can it be used for tracking this group over time?

Of even more importance would be monitoring the potential dechlorinating activity of *Dhc. spp.* in the reactor. Many methods are available for monitoring the removal of PCE. Gas chromatography (GC) monitors the presence of many known chlorinated compounds. The *tceA* gene is known to code for the TCE dehalogenase of *Dhc. ethenogenes.* This enzyme is implicated in the dechlorination process of PCE to ethene. As detailed in Chapter 2 the *tceA* gene probes were shown to be specific to the *Dhc.* group of dechlorinators. Furthermore, the *tceA* probes were capable of amplifying, with high specificity, a known *Dhc. sp.* strain FL2. The probes were also capable of detecting the dehalogenase gene in similar quantities to that of the 16S rDNA gene. The nearly identical results from amplification by two different gene probes further support the specificity of the *tceA* gene to *Dhc.* spp., both in pure and mixed culture. Since it should be expected that two different genes with identical standard curves would provide the same results in the field.

The aim of this study was also to isolate RNA from the reactor samples over time, and use reverse transcriptase RTm PCR to attempt the quantification of the *tceA* gene's mRNA during bioreactor operation. Knowing that mRNA levels correspond to their respective enzyme levels and activity, we should expect a trend to emerge corresponding to the addition of PCE and/or lactate and the growth of *Dhc.* spp. in the reactor.

It is hypothesized that: *Dhc. spp.* populations will increase over time in direct correlation to the addition of PCE and lactate in the reactor. *Dhc.* spp. will reach a population limit by which the available "space" for more organisms becomes limited. It is also hypothesized that *tceA* mRNA levels will show an effectual response to the additions of PCE and lactate over time. This response will follow a similar trend to the population of *Dhc. spp.* in the reactor.

Methods

Primer Design and RTm PCR

Primer/probe sequences for *Dhc. spp* were designed using Primer Express Software as detailed in Chapter 2. A series of calibration reactions, detailed in Chapter 2, were performed, to determine ideal concentrations of the forward and reverse primer, and probe. Plots and statistics were performed using Sigma Plot 2001.

Reactor Design

The reactor used for the scale-up was composed of 1250 L of water and is maintained by EFX systems, Lansing, MI. Added to the reactor was 100 L of inoculum stored from previous smaller reactors, as well as added sterilized-filtered Schoolcraft ground water stock. A nutrient solution, 1.25 L, and trace elements were added. The pH was adjusted to fall between 7.5-6.9 using H_3PO_4, and Na_2S. The reactor initially contained 5 ml of PCE and 1000 g of lactate. Before the first sample was taken the reactor was recycled for 2 h.

When PCE concentrations were depleted, as determined by GC, additional PCE and lactate amendments were made.

Reactor Sampling

N_2 purged serum bottles (160 ml) were used to collect 100 ml of reactor effluent. The effluent was divided roughly 60 ml/40 ml. Microbial biomass was harvested by centrifugation. The larger volume, 60 ml, was spun down and used for RNA extractions, the lesser volume, 40 ml, was spun down and used for the DNA extractions. There were a total of ten time points taken over a 42-day period. Pellets were stored under N_2 at –80° C until use.

Nucleotide Extraction

DNA and RNA were extracted from reactor effluent and pure cultures then harvested by centrifugation. DNA was isolated as detailed in Chapter 2. DNA from all time points was extracted at the same time.

RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA). A slight modification to the protocol was made in which the DNase treatment was performed twice for each sample extracted. In addition all equipment was wiped down with RNase inhibitors and RNase-free PCR tubes were utilized. The concentration of RNA was calculated using the absorbance at 260 nm. RNA from all samples taken at different time points was extracted at the same time.

RTm PCR of the Reactor Effluent Samples

RTm PCR was performed on the total DNA extracted from the effluent samples. Total DNA, 100 ng, of was added to each RTm PCR reaction, 30 µl, and each time point was performed in triplicate using the same stock DNA. The primer/probe sets for the 16S rDNA gene and *tceA* gene of *Dhc. spp.* were used. RTm PCR Ct values were converted to gene copies/ ml reactor effluent utilizing regression equations from standard curves developed from pure cultures of *Dhc.* sp. strain FL2.

Two-step reverse transcriptase RTm PCR was performed with the RNA samples. The first step, reverse transcription, was carried out in 25 µl reaction volume containing extracted mRNA, 0.03 - 0.46 µg, and the reaction components called for in the Perkin Elmer RTm PCR reverse transcriptase kit (PE Applied Biosystems, Foster City, CA). Reverse transcription was run at 48°C for 30 min with a 5 min hold at 95° C as recommended by the protocol. During reverse transcription fluorescent release is not detected. After reverse transcription, 25 µl of the transcription product was transferred to tubes for the RTm PCR step two. The reaction volume of step two was increased to 50 µl as opposed to 30 µl to increase the sensitivity of the procedure. Since the amount total extracted RNA varied between samples, data was corrected based on total RNA extracted. No templates as well as no reverse transcriptase controls were performed for each time-point. Data were corrected based on the slight signal observed without reverse transcriptase present. This correction took into account any residual DNA present in the extracted RNA sample that could not be removed by DNase

treatment. Since the total amount of residual DNA may have varied between the different time points, each time had an independent no-reverse transcriptase control. This way the residual DNA could be determined for each sample, and the correction would be sample specific. All RTm data points are the means of triplicate RTm reactions utilizing DNA or RNA from the same sample. All statistics and graphs were performed using Sigma Plot 2001.

Results

Primer/Probe design

Results for the primer/probe set for the 16S gene and *tceA* functional dehalogenase gene of *Dhc. ethenogenes* can be found in Chapter 2.

RTm PCR (Population dynamics)

RTm PCR, utilizing the 16S rDNA and *tceA* gene probes, successfully quantified *Dhc. spp.* over time in the reactor (Figure 10). Quantifiable results were found at all time points observed, during the 42-day test period. The population curves obtained utilizing either set of gene probes were nearly identical in trend and quantity. Quantifiable responses to PCE/lactate amendments are clearly visible along the population curve. Distinguishable increases in population size were detectable shortly after amendments were made. The intensity of these responses decreased over time as the total population within the reactor increased. Towards the last days of the study the

population increase leveled off and amendments had little affect on further population increase.

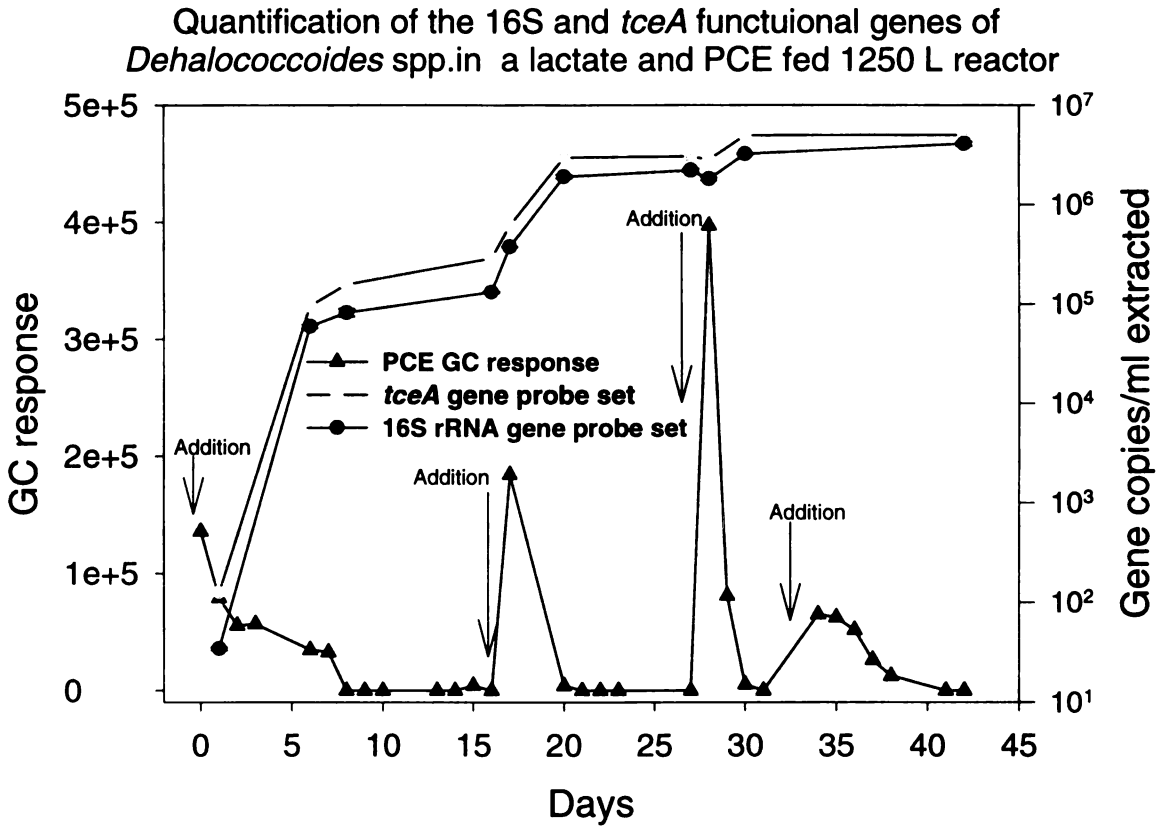

Figure 10. Line graph showing the relationship between the quantitative increase in 16S rDNA and *tceA* gene copies/ml of reactor effluent and the concentration of PCE in the reactor over a 42-day period. The arrows indicate PCE/lactate additions. The data point obscures error bars for gene copies/ml, each data point represents triplicate RTm PCR reactions.

RTm PCR (Quantification of *tceA* mRNA)

Two-step reverse transcriptase RTm PCR amplified extracted *tceA* gene mRNA from the samples taken (Figure 11). Samples were corrected for contaminating residual DNA as described in the Material and Methods. The quantity of *tceA* mRNA responded to the addition and subsequent metabolism of PCE and or lactate in the reactor. During sample points in which PCE concentration was low or absent in the reactor mRNA levels decreased. The degree at which the mRNA concentration responded to PCE/lactate amendments also corresponded to the increase in quantity of the *tceA* gene (Figure 12).

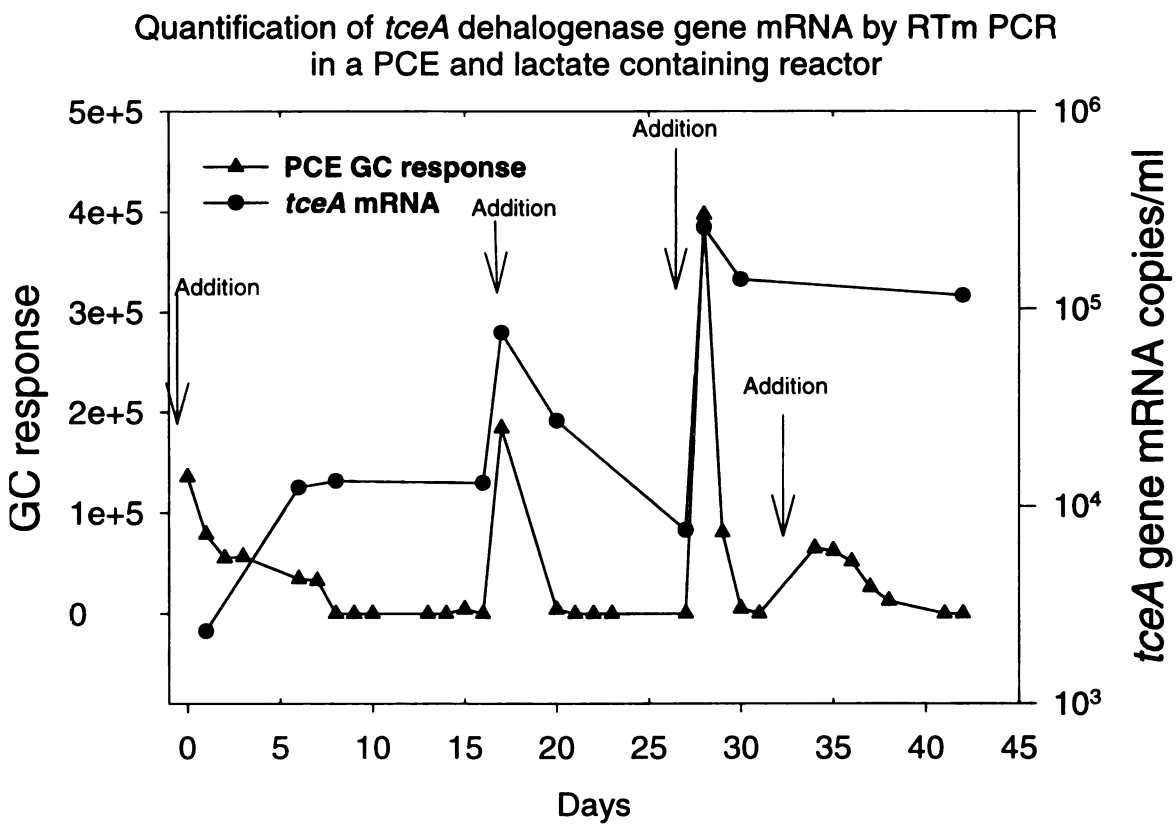

Figure 11. Graph showing the relationship between the corrected quantitative response of *tceA* gene mRNA copies/ml of reactor effluent and the concentration of PCE in the reactor over a 42-day period. The arrows indicate PCE/lactate additions. The data point obscures error bars for gene copies/ml, each data point represents triplicate RTm PCR reactions.

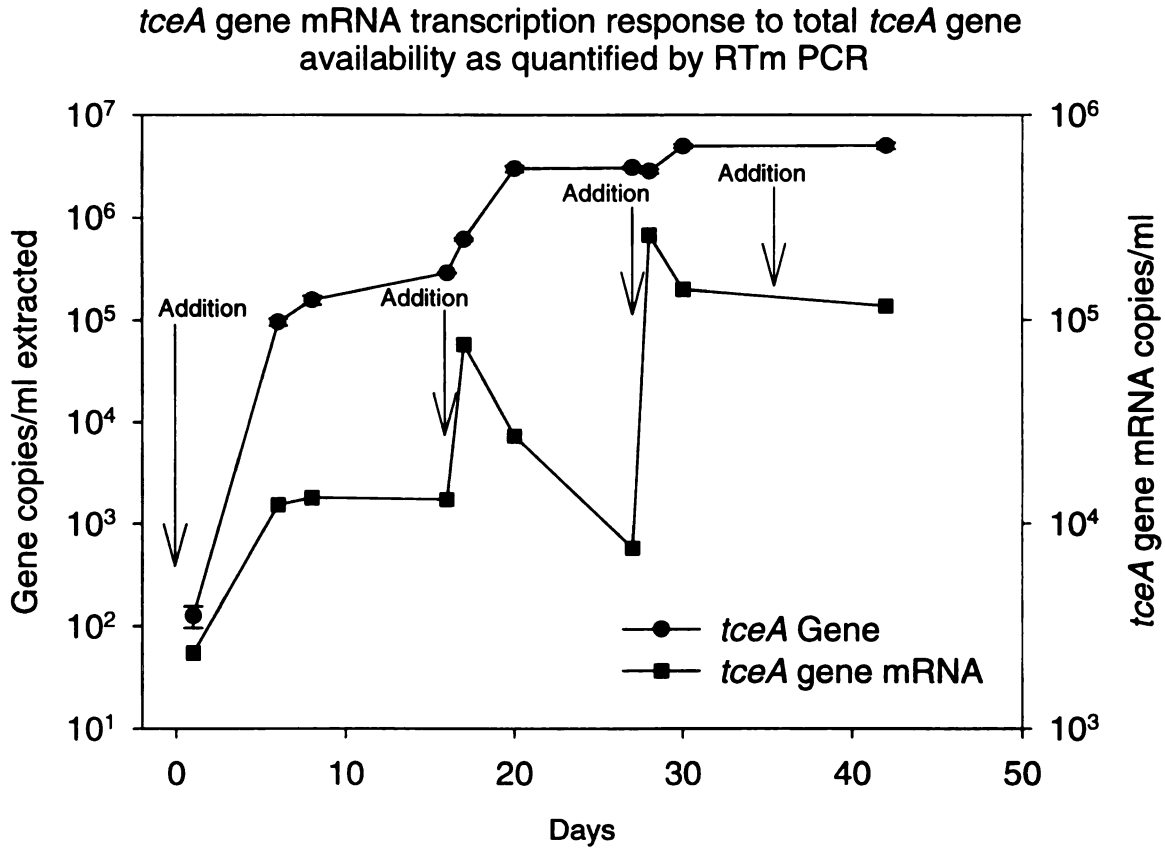

Figure 12. Graph showing the transcriptional response of *tceA* gene mRNA in comparison to the quantifiable *tceA* gene in a PCE/lactate containing reactor. The arrows indicate additions of PCE/lactate. The data point obscures error bars for gene copies/ml, each data point represents triplicate RTm PCR reactions.

Discussion and Conclusions

"Many of the molecular techniques currently used in microbial ecology lack a quantitative component." (14, 37). The ability to quantitatively monitor PCE-dechlorinating populations and assess their dechlorination activity over time should prove beneficial in the field of bioremediation. Rodrigues et al. (2002) utilized RTm PCR, to quantify an engineered PCB degrading *Rhodococcus sp.* in an aerobic soil microcosm (37). Working with an engineered aerobe capable of being cultivated on plates provided an additional measure by which to confirm the results obtained through RTm PCR.

The ability to avoid DNA contamination and assess probe specificity are just a few critical factors for RTm PCR (37). Contamination can be avoided if special measures are taken to assure extraneous DNA is not introduced into the PCR reaction, this contamination is an even greater concern when dealing with reverse transcriptase RTm PCR. The use of no-template controls assures that reaction components are free of contaminating DNA. In reverse transcriptase reactions, DNase treatments are essential to limit contaminating DNA. Even though dual DNase treatments were performed on the extracted RNA, small amounts of DNA were detected; this is a shortcoming of the highly sensitive nature of RTm PCR. The quantifying results obtained by RTm PCR made it possible to correct the *tceA* mRNA values.

As hypothesized the levels of mRNA coincided with the addition and metabolism of PCE and/or lactate in the reactor. This type of response is

expected, mRNA transcription is typically induced by the substrate for the enzyme. *Dsf. michiganensis'* PCE reductase gene in induced in the presence of PCE (22). Thus, the fact that PCE, causes an increase in the quantity of *tceA* mRNA is expected. What is compelling is that the degree at which the transcription occurs can also be correlated with the overall concentration of detectable *tceA* genes (Figure 12). With more available genomic *tceA* gene template, the concentration of transcribed mRNA would expectedly increase. This increase was shown to occur in the samples tested. One noted observation, however, was that the concentration of *tceA* mRNA was less than the concentration of detectable *tceA* gene. One possible rationale for this is that the growth response is due to the presence of lactate and not PCE. Since lactate was fed at the same time as PCE, it is not possible to discern what substrate the population is increasing in response too. It would be expected that if the response is due to the addition of PCE, and subsequent transcription of the *tceA* gene, we would expect higher concentrations of the *tceA* mRNA. This conclusion is further supported by the amount of PCE present in the reactor; it was not enough to support the amount of population growth seen.

The use of reverse transcriptase RTm PCR for monitoring the actual activity of dechlorinators in the environment is closer to becoming a feasible molecular tool for environmental microbiology. By being able to monitor the activity of dehalogenating organisms, we can begin to understand their response to added substrates and other variables (21). This would add another variable by which to assess bioremediation strategies. The next approach would be the

utilization of this technique in the field, when more efficient methods of mRNA extraction in soil become available.

When working with 16S rDNA genes special care has to be taken when selecting probes due to the conserved nature of 16S rDNA genes (37). More leeway in probe design is available when working with functional genes, such as the *tceA* dehalogenase gene. The probes designed for both the 16S rDNA gene and *tceA* functional gene were found to be highly specific for the PCE/TCE dechlorinating *Dehalococcoides* group. In addition, these gene probes were found to give similar RTm PCR fluorescent responses when presented with the same template DNA or cDNA. This consistency in fluorescent response was observed over the length of the 42-day experiment. The ability to monitor with two unique gene probes provides an additional degree of confidence. Unlike many aerobic dechlorinators, the anaerobic dechlorinators are often not plate countable; this is the case with *Dhc.* spp. By being able to cross verify results with two gene probes, a higher degree of certainty about observed results can be obtained. The question of specificity in one probe can be supported by observed specificity in another or vice versa.

RTm PCR was shown capable of quantifying the PCE dechlorinator, *Dhc.* spp, over time. In addition, the sensitivity of RTm PCR made it possible to detect minor changes in the population in response to outside variables. These changes could be seen during additions of PCE/lactate to reactor, as time progressed and the reactor became more populated with microorganism, the response to PCE/lactate additions became less distinguishable. However, even

changes less than an order of magnitude can be detected during the PCE/lactate additions. This shows that growth of *Dhc.* spp. is directly correlated with the addition and consumption of PCE and lactate. Furthermore, results from these experiments provide credible evidence to the capabilities of RTm PCR in monitoring *Dhc.* spp. By monitoring the population of dechlorinators, the success or failure of an experiment can be determined. By knowing the population status of dechlorinating organism, potential pitfalls can be avoided. RTm PCR has shown itself a useful tool in the field of bioremediation. Not only has RTm PCR successfully monitored *Dhc.* populations over time but it was also shown to monitor mRNA level for a functional gene as well.

APPENDICES

Appendix 1. Mineral Salts Medium for BB1 and BRS1

Mineral Salts Medium for BB1 and BRS1

Salts	1 x [g/L]	100 x stock [g/L]
NaCl	1.0	100.0
$MgCl_2$ x 6 H_2O	0.5	50.0
KH_2PO_4	0.2	20.0
NH_4Cl	0.3	30.0
KCl	0.3	30.0
$CaCl_2$ x 2 H_2O	0.015	1.5

To prepare medium	1 L
100 x salts	10 ml
Trace element solution	1 ml
Se/Wo solution	1 ml
Resazurin (0.1% solution)	1 ml
H_2O bidest.	987 ml

Boil, cool down to room temperature under flushing with N_2/CO_2 (80/20)

Add 0.2 mM L-cysteine 0.035 g/L
Add 0.2 mM Na_2S x 9 H_2O 0.048 g/L
Add 30 mM $NaHCO_3$ 2.52 g/L

Adjust pH to 7.2 - 7.3 with CO_2 (to lower the pH with CO_2 is less time consuming)

Dispense medium (**final** vol. = 100 ml), close the bottles with black rubber stoppers

Autoclave when medium turns clear.

Electron donors (acetate or lactate) and acceptor (PCE) can be added prior to heat sterilization.

Strain BRS is tolerant against sulfide and no inhibition was seen with up to 1 mM Na_2S.

Trace element solution:
Per liter: HCl (25% solution, w/w), 10 ml; $FeCl_2 \times 4\ H_2O$, 1.5 g; $CoCl_2 \times 6\ H_2O$, 0.19 g; $MnCl_2 \times 4\ H_2O$, 0.1 g; $ZnCl_2$, 70 mg; H_3BO_3, 6 mg; $Na_2MoO_4 \times 2\ H_2O$, 36 mg; $NiCl_2 \times 6\ H_2O$, 24 mg; $CuCl_2 \times 2\ H_2O$, 2 mg

Se/Wo solution:
Per liter: 6 mg $Na_2SeO_3 \times 5\ H_2O$, 8 mg $Na_2WO_4 \times 2\ H_2O$ and 0.5 g NaOH

Wolin Vitamins

Wolin, F. A., M. J. Wolin, and R. S. Wolfe. 1963. Formation of methane by bacterial extracts. J. Biol. Chem. **238:**2882-2886.

Vitamins	1000 x [mg/L]	Final conc.
biotin	20 mg/L	0.02 mg/L
folic acid	20 mg/L	0.02 mg/L
pyridoxine hydrochloride	100 mg/L	0.1 mg/L
riboflavin	50 mg/L	0.05 mg/L
thiamine	50 mg/L	0.05 mg/L
nicotinic acid	50 mg/L	0.05 mg/L
pantothenic acid	50 mg/L	0.05 mg/L
vitamin B_{12}	1 mg/L	0.001 mg/L
p-aminobenzoic acid	50 mg/L	0.05 mg/L
thioctic acid	50 mg/L	0.05 mg/L

Adjust pH to ~7.5 with 10 M NaOH (takes some time)

Aliquot in 20 ml portions, freeze, store in dark place (light sensitive)
Prepare a 200 x working stock solution, filtersterilize

PCE → *cis*-DCE pure culture (*Desulfuromonas* sp. strain BB1)

Add	Per 100 ml	Final aqueous conc.
Acetate (1 M stock)	0.25 ml	2.5 mM
Wolin vitamins 200 x	0.5 ml	1 x
PCE (25 µl per ml HD)	1 ml	0.092 mM
Inoculum	2 ml	2%

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