Elucidating the microbial community within a trichloroethene contaminated competent bedrock aquifer located at Pease International Tradeport, Portsmouth, NH

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Elucidating the microbial community within a trichloroethene contaminated competent bedrock aquifer located at Pease International Tradeport, Portsmouth, NH

Abstract
A trichloroethene (TCE)-contaminated competent bedrock aquifer exists at Pease International Tradeport in Portsmouth, NH. Characterization by the Bedrock Bioremediation Center (BBC) at the University of New Hampshire showed active in situ anaerobic degradation of TCE. Formation water from discrete, hydraulically connected fractures was extracted to characterize and enumerate the prevalent TCE biodegradation processes using Fluorescent in situ hybridization (FISH). Eubacteria (90%), Archaea (5%), and sulfate reducing bacteria within Desulfobacteriaceae (1--26%) were present at all depths (13--30 meters). Two probes targeting the anaerobic TCE-dechlorinating genus, Dehalococcoides (4--10%) were developed. FISH in combination with Flow Cytometry was employed to automate the analysis; however it proved difficult to differentiate microorganisms from background interference. This baseline understanding of community structure is essential for determining if a system is poised to support in situ bioremediation, and aids in monitoring shifts in the microbial population when bioremediation efforts are applied.

Keywords
Biology, Microbiology

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ELUCIDATING THE MICROBIAL COMMUNITY WITHIN A TRICHLOROETHENE CONTAMINATED COMPETENT BEDROCK AQUIFER LOCATED AT PEASE INTERNATIONAL TRADEPORT, PORTSMOUTH, NH

BY

Michelle M. Majko
B. S., Rutgers University, 2002

THESIS

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This thesis has been examined and approved.

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August 13, 2010
Date
DEDICATION

This thesis is dedicated to my mother who has always reminded me to follow my dreams and that if you apply yourself, anything is possible. It is also dedicated to my father who in reading to me every night from the encyclopedia when I was little, inspired my interest in science, art, nature, and travel.
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TABLE OF CONTENTS

DEDICATION iii
ACKNOWLEDGEMENTS iv
LIST OF TABLES vii
LIST OF FIGURES viii
ABSTRACT ix

1.0 INTRODUCTION 1
1.1 Trichloroethene Contamination at the former Pease Air Force Base 1
1.2 Microbial Degradation of Chlorinated Solvents 1
1.3 Natural Attenuation of Chlorinated Ethenes at Fractured Bedrock Sites 3
1.4 Approaches of Examining Microbial Diversity 4
1.5 The use of FISH and Flow Cytometry to Study Microbial Biodiversity 9
1.6 Thesis Objectives 11

2.0 MATERIALS AND METHODS 12
2.1 Groundwater Collection 12
2.2 Total Direct Counts 13
2.3 Fluorescent in situ Hybridization 14
2.4 Dehalococcoides 16S rRNA Probe Development 17
2.5 Probe Evaluation 21
2.6 Checking for Non-specific Binding 23
2.7 FISH for Flow Cytometry 23
2.8 Flow Cytometry 24
3.0 RESULTS AND DISCUSSION 25

3.1 Site Characterization 25

3.2 Groundwater Chemistry 25

3.3 FISH Analysis of Groundwater 28

3.4 FISH-FCM Analysis of Groundwater 34

3.5 Analysis of FISH-FCM using dual-probes 36

4.0 CONCLUSION 42

LIST OF REFERENCES 44
### LIST OF TABLES

Table 1: Oligonucleotide Probes | 16
---|---
Table 2: Chemical Analysis of BBC Groundwater Samples | 27
Table 3: Total Direct Counts of DAPI-stained Groundwater from BBC wells | 28
Table 4: FISH-FCM Example Run | 34
Table 5: Comparison of Data Obtained from Single-Probed Samples Compared to Dual-Probed Samples | 41
LIST OF FIGURES

Figure 1: *E. coli* Probe Accessibility Table  
Figure 2: *Thermomicrobium roseum* 16s rRNA Secondary Structure  
Figure 3: DHC Probe Evaluation Slides  
Figure 4: Aerial view of plume migration from 1998 to 2002  
Figure 5: Morphologies of Microbial Populations of BBC Groundwater  
Figure 6: *Eubacteria and Archaea* Example Pictomicrograph  
Figure 7: Abundance of *Eubacteria, Archaea*, and Sulfate-reducing Bacteria as determined by FISH  
Figure 8: Abundance of Dehalorespiring Bacteria as determined by FISH  
Figure 9: TCE, DCE, VC, and Ethene Concentrations  
Figure 10 FISH-FCM Testing of probes Univ1390, EUB338, and ARCH915 on the Bacteria *E. coli*  
Figure 11 FISH-FCM *E. coli* probed with Univ1390-OG and EUB338-Cy5. Data analyzed separately using the plot diagram to view probe conferred fluorescence from one channel.  
Figure 12: Autofluorescence caused by debris in BBC Groundwater samples
ABSTRACT

ELUCIDATING THE MICROBIAL COMMUNITY WITHIN A TRICHLOROETHENE CONTAMINATED COMPETENT BEDROCK AQUIFER LOCATED AT PEASE INTERNATIONAL TRADEPORT, PORTSMOUTH, NH

By
Michelle M. Majko

University of New Hampshire, September, 2010

A trichloroethene (TCE)-contaminated competent bedrock aquifer exists at Pease International Tradeport in Portsmouth, NH. Characterization by the Bedrock Bioremediation Center (BBC) at the University of New Hampshire showed active in situ anaerobic degradation of TCE. Formation water from discrete, hydraulically connected fractures was extracted to characterize and enumerate the prevalent TCE biodegradation processes using Fluorescent in situ hybridization (FISH). Eubacteria (90%), Archaea (5%), and sulfate reducing bacteria within Desulfobacteriaceae (1-26%) were present at all depths (13-30 meters). Two probes targeting the anaerobic TCE-dechlorinating genus, Dehalococcoides (4-10%) were developed. FISH in combination with Flow Cytometry was employed to automate the analysis; however it proved difficult to differentiate microorganisms from background interference. This baseline understanding of community structure is essential for determining if a system is poised to support in situ bioremediation, and aids in monitoring shifts in the microbial population when bioremediation efforts are applied.
1.0 INTRODUCTION

1.1 Trichloroethene Contamination at the former Pease Air Force Base

Degreasing operations of aircraft electronics took place over a 10 year period beginning in 1956 at Building 113 on Site 32 at the Pease International Tradeport (formerly Pease Air Base) in Portsmouth, NH. A 1,200 gallon concrete tank buried beneath the northeastern corner of the building served as a reservoir for degreasing wastes, primarily Trichloroethene (TCE). The tank contained an overflow discharge pipe that released contaminants directly into the overburden soil, weathered and competent bedrock. In 1990, about 400 tons of contaminated upper sand and clay was removed. A pilot pump and treat system was established to remediate groundwater in the lower sand layer and weathered bedrock from 1991 to 1995. The bedrock portion of the site was deemed to be a Technical Impracticability zone as TCE is a dense non-aqueous phase liquid (DNAPL) which by definition, does not dissolve in water and is more dense than water, thus sinking within the fractures of the bedrock system. Sheet piles were installed in the overburden in 1996, and groundwater pumping within the sheet pile commenced in 1997. Boreholes were drilled into the bedrock within the contaminant plume by the Bedrock Bioremediation Center at the University of New Hampshire to commence characterizing the site.

1.2 Microbial Degradation of Chlorinated Solvents

Tetrachloroethene (PCE) and TCE can be naturally degraded into lesser chlorinated compounds by microorganisms intrinsic to the environment. Three categories of metabolic processes exist for the biodegradation of chlorinated ethenes. These processes are reductive dechlorination, cometabolism, and direct oxidation.
Reductive dechlorination is an anaerobic process in which chlorinated ethenes are used as electron acceptors instead of electron donors because they are a poor source of electrons due to the chlorine-carbon bonds (Wiedemeier et al., 1998). Dechlorination of PCE and TCE to dichloroethene (DCE) occurs under mildly reducing conditions such as NO$_3^-$ or Fe$_3^+$. Dechlorination of DCE to vinyl chloride (VC) and VC to ethene requires stronger reducing conditions of SO$_4^{2-}$ or methanogenesis (Vogel et al., 1987). In addition, PCE and TCE are more readily degraded than DCE or VC due to the higher oxidized carbon atoms in these molecules. Only one organism, Dehalococcoides ethenogenes 195 is capable of complete degradation of PCE to non-toxic ethene (Maymo-Gatell, et al., 1997). For this reason, its presence and abundance within the groundwater at the former Pease Air Force base is of considerable interest.

Cometabolism is an aerobic process in which chlorinated ethenes are oxidized by metabolic enzymes, however, no energy is gained. Studies have revealed that this process is carried out by methanotrophic bacteria (Wilson et al., 1985), methane monooxygenase enzymes (Henry et al., 1994), ammonia monooxygenase enzymes (Rasche et al., 1991) and toluene dioxygenase enzymes (Heald et al., 1994). Lastly, direct oxidation is an aerobic or mildly anaerobic (iron reducing) process in which less chlorinated ethenes are used as electron donors (McCarty, et al., 1994).

TCE and its daughter compounds pose risk to the environment and human health where their concentration exceeds groundwater quality standards. Vinyl chloride is a known human carcinogen and typically accumulates in the environment due to the fact more energy is required in removing a single chlorine atom than with a fully chlorinated molecule such as PCE or TCE. Due to the health risks, their persistence in the environment, and difficult removal, in situ chlorinated ethene degradation is an important process at contaminated sites.
Chemical characteristics and tools to study the microbial community structure and abundance are very important in predicting which processes are dominant in the degradation of TCE and daughter compounds. With initial chemical characteristics at Site 32 showing evidence of reducing conditions and full decomposition of TCE to ethene, it was hypothesized that reductive dechlorination was potentially taking place. It was therefore a priority to determine whether Dehalococcoides sp. were present and how abundant in comparison they are in other chloroethene-contaminated bedrock aquifers.

1.3 Natural Attenuation of Chlorinated Ethenes at Fractured Bedrock Sites

Several studies exist that have shown that in situ microorganisms are capable of degrading chlorinated ethenes in fractured bedrock by a variety of metabolic processes. One of the largest contaminated sites is located at the Idaho National Engineering and Environmental Laboratory – Test Area North (INEEL-TAN) site where a 6 km TCE plume exists in a deep fractured basalt aquifer. Based on field and laboratory data, TCE degradation was dominated by aerobic cometabolism by indigenous methanotrophs, propanotrophs, and phenol-oxidizers (Barnes et al., 2005).

At a TCE-contaminated fractured dolomite aquifer in upstate New York, in situ reductive dechlorination was exhibited. Further analysis by amplified ribosomal DNA restriction indicated the presence of a low diversity, sulfur-transforming community outside the plume and a high diversity community including Dehalococcoides ethenogenes-type microorganisms inside the plume (Hohnstock-Ashe et al., 2001).

In a TCE-contaminated fractured shale bedrock aquifer in Oak Ridge, TN, there was evidence of anaerobic biodegradation of highly chlorinated compounds. Enrichment cultures revealed the presence of methanotrophs, iron-reducing bacteria, and sulfate-reducing bacteria. The presence of methanotrophs suggests that aerobic
biodegradation by cometabolism of TCE with methane may also be occurring (Lenczewski et al., 2002).

Within a karst aquifer contaminated with TCE in Tennessee, geochemical analysis showed sulfate-reducing conditions were prevalent. RNA-hybridization techniques identified sulfate-reducers, methanotrophs, and ammonia oxidizers. These studies exemplify that there are multiple routes of in situ chloroethene degradation, depending on the chemical composition and influence of intrinsic microorganisms. Similarities seen between each of the sites included complex hydrology which may have influenced the range of oxidizing-reducing conditions seen throughout. Most sites showed that reductive dechlorination existed, but in addition cometabolism was also taking place. Unfortunately, these studies did not focus on determining the abundance of key microorganisms and rather only reported the diversity and chemical composition of the site.

The use of naturally occurring, abundant, and metabolically active microorganisms to degrade contaminants in groundwater and sorbed to fracture surfaces may offer the best alternative for cost effective and efficient remediation. Other remedial alternatives used in unconsolidated sediments (e.g., pump and treat) may not be as successful in bedrock because of the complex and variable hydraulics of the fractures. It is important to know what types of organisms are present, and the characteristics of the environment that they are in, to help understand if remediation is taking place or if there are limiting factors impacting the rate of biodegradation.

1.4 Approaches of Examining Microbial Diversity

There are three general approaches currently used to study subsurface microbial populations at contaminated sites; traditional culture-based methods, molecular-based
methods and laboratory microcosms. Application of these methods provides information on the diversity and function of microbial populations and communities.

Traditionally, assessing microbial diversity involved cultivating microorganisms using enrichment cultures in the laboratory. This technique when coupled with molecular techniques aids in identifying microorganisms in a community, but cannot provide an estimate of abundance. The method involves duplicating the resources and conditions of the environment in which the organisms grow, typically in a liquid culture. There are some limitations when applying enrichment cultures in studying microbial diversity. Only a small fraction of the microorganisms in nature have been cultivated (Ward et al., 1992), therefore the culture may not be truly representative of the microorganisms present. Major discrepancies have been noted (summarized by Amann et al., 1995) between the percentage of culturable Bacteria with total cell counts from different habitats. One of the reasons for this difference may be accredited to the difficulty in replicating the conditions in which many Eubacteria and Archaea grow, little is known about the growth requirements for many microorganisms (Amann et al., 1995). Another reason may be attributed to certain organisms which are interdependent on others for growth, for example endosymbiotic bacteria of worms and mollusks (Fisher et al., 1990), or in some cases the most fit organism for the particular nutritional and incubation conditions may outcompete other members of the community. Enrichment cultures provide information on types of organisms present, but may not yield an accurate picture, therefore it is important to use molecular approaches to gain more information on microbial communities.

Various molecular methods have been developed to identify and study microorganisms, overcoming problems associated with non-culturable bacteria. These methods include numerous DNA- and RNA-based techniques. There is an advantage to using 16S rRNA analysis in studying microbial diversity. Microorganisms contain one
copy of genomic DNA which can contain up to approximately 14 copies of rDNA, whereas there are multiple ribosomes which can contain up to 1000 copies of rRNA. The small subunit is commonly chosen for phylogenetic studies based on its high copy number and highly conserved sequence which can be used to discern between the three domains of life, *Eubacteria*, *Archaea*, and *Eukarya* (Woese et al., 1990), allowing for broad selection within a diverse community. In addition 16S rRNA gene sequences contain hypervariable regions that can be used to identify organisms to the genus and species level. Sequence variation in rRNA has prompted the development of unique probes and primers which are designed to complement sequences on the 16S rRNA gene and are used to identify both culturable and non-culturable organisms (Amann et al., 1995). The gene is easy to sequence, and a large database is available for sequence alignment and identification (Cole et al., 2005).

In one approach using the 16S rRNA, the PCR-Cloning-Sequencing technique, organisms are harbored from their natural environment and DNA is extracted using standardized techniques (Akkermans et al., 1998). The extracted DNA is fragmented (enzyme digest or PCR) resulting in a mixture of products characteristic of the different rRNAs of the community. The separate fragments are each cloned into a vector for subsequent transformation into a host organism, typically *E. coli*. Once a clonal library has been established, 16S rRNA sequence analysis of resulting clones can be compared against a database to infer what organisms comprise the microbial community. This technique offers identification of organisms within a community, however it does not provide insight into abundance.

Another common approach used to analyze microbial diversity is Denaturing Gradient Gel Electrophoresis (DGGE). Community profiling using DGGE of PCR-amplified rDNA fragments was first demonstrated on a microbial mat and bacterial biofilms (Muyzer et al., 1993). The principle of the method is based on the fact that
double-stranded DNA dissociates at high temperatures (in the case of Temperature Gradient Gel Electrophoresis), or under the influence of denaturing chemicals as a function of different G+C content (Fischer et al., 1979 and 1980). During electrophoresis, DNA fragments of the same length with different base-pair sequences are resolved within a polyacrylamide gel containing linearly increasing gradient of denaturants. As the DNA helix migrates to the positive pole it begins to dissociate and looses mobility due to the change of the helical structure. At a certain position in the denaturing gradient gel, the transition of a helical to a partially melted molecule causes its migration to halt (Fischer et al., 1979 and 1980). DNA fragments with different sequences will migrate differentially generating a unique fingerprint of the community. This technique can be used to estimate the richness and abundance of the predominant members of a microbial community. In addition Individual DGGE bands can be excised from the gel and sequenced for identify and for phylogenetic studies.

Another technique used in community analysis, Dot-Blot Hybridization, can be used for the identification of DNA and RNA species. In 16S rRNA analysis, it was pioneered as a tool for a ground-breaking study monitoring the population changes in the rumens of cattle (Stahl et al., 1988). In this method, non-amplified denatured rRNA representative of the population is used, instead of amplified rRNA as in the approaches described above. The extracted rRNA mixture is applied to a membrane and probed with universal and specific oligonucleotide probes. The relative abundance can be calculated by dividing the amount of specific probe bound to a sample by the amount of hybridized universal probe. Probes are typically radioactively labeled, however non-radioactive methods of labeling are currently available (DIG labeled probes). Abundances of microorganisms are expressed as the fraction of the total rRNA in the sample determined by the hybridization of the universal probe, therefore only the relative
abundance of a population can be measured and cannot be converted into actual cell numbers using this technique.

Whole-Cell Hybridization, differs from other molecular techniques in that signature 16S rRNA sequences are detected within intact cells rather than extracting nucleic acids for further analysis. There are a broad range of phylogenetic probes available allowing for analysis of diverse communities. There are several detection methods, including radioactively labeled oligonucleotides (Giovanoni et al., 1988), fluorescently labeled oligonucleotides (DeLong, et. al., 1989), or using an immunofluorescence approach (Bohlool et al., 1980). Whole-Cell Hybridization allows visualization of cell morphology and enumeration of abundance. Limitations which are associated with this technique include variability in cell permeability; a limited low-end detection limit of $10^4$ cell/ml; background fluorescence from debris inherent with environmental samples; and the limitation of using of only two to three probes in combination on one sample due to the range of epi-fluorescent microscope filter sets.

Lastly, microcosms are frequently used in studying the behavior of ecosystems by simulating in situ conditions of an ecosystem. They are very useful in the prediction of processes occurring in a system that is not easily characterized in situ. Work has been performed at the BBC Site 32 to measure rates of anaerobic reductive dechlorination of TCE (Druschel et al., 2009). This method when coupled with molecular techniques can help ascertain what organisms are present in a system and an estimate of their abundance when an extrapolation calculation is applied.

Although 16s rRNA based studies have fostered the ability to study microbial diversity, there are inherent limitations to many of the techniques. Particularly with environmental samples, populations may be misrepresented due to the physical and chemical nature of the environment they inhabit. For example, adherence of cells to their environmental matrix (Jacobsen et al., 1992), the ability of inhibitors extracted along
with DNA such as humic acid from soil which impacts PCR amplification (Tsai, et al., 1992). In addition to the environmental influences, the cells themselves can impact a community fingerprint. For example, resistance to cell lysis (Feinstein, et al., 2009), and extraction of non-representative extracellular DNA which adhered to cells from lysed or non-viable cells. Biases have also been identified in creation of clonal libraries. A study by Rainey et al., 1994 uncovered that while using the same PCR product, different cloning techniques yielded different libraries. This variance might be attributed to PCR artifacts or contaminating DNA introduced from reagents used during the extraction process.

1.5 The use of FISH and Flow Cytometry to Study Microbial Biodiversity

FISH using rRNA-targeted oligonucleotide probes applied to fixed whole cells has been frequently used as a direct and quick tool for analyzing the diversity of environmental samples (Amann et al., 2001, Amann et al., 1990, Burggraf et al., 1994, and Manz et al., 1992). Populations can be characterized by applying probes to varying levels of phylogeny. Specific species can be distinguished by oligonucleotides complementary to variable regions of the rRNA molecule (Stahl et al., 1988). By selecting more conserved regions, probes can be developed to target specific genera or higher taxons (e.g. Eubacteria, Archaea, and Eukaryotes). Probes with different fluorochrome labels can be used in combination on one sample (multiple probing). In addition to multiple probing, the sample can also be stained with a general DNA stain such as DAPI to determine total cell counts. The fraction of the sample that is bound by the probes can be compared to total cells, providing a useful tool for enumeration of a population. FISH has also been employed to estimate the cellular activity based on fluorescence intensity which is proportional to the cellular rRNA content and metabolic activity (Amann et al., 1990 and Poulsen et al., 1993).
Environmental samples characterized using FISH are typically visualized using epi-fluorescence microscopy (FISH-Microscopy). One of the common occurrences in analysis of environmental samples is the limit of detection. A study showed that the sensitivity of FISH for detection of bacteria may range between $4 \times 10^5$ and $1 \times 10^6$ CFU/ml (Hogardt, et al., 2000). Similarly, in a study involving bacteria in cultures of seawater sediment, the minimum detection limit was $2.8 \times 10^5$ cells/ml, which was the concentration where only 1 cell was detected in 9 fields of view (Bruns et al., 1998).

Enumeration by epi-fluorescence microscopy provides valuable information on the morphologic characteristics of the cell. However, there are some limitations. it is labor intensive, it does not provide high sample throughput which is a necessity in environmental investigations, and there is a lower limit of detection around $2 \times 10^5$ cells/ml. To overcome these limitations, the combination of FISH with Flow Cytometry (FISH-FCM) automates the analysis of diverse microbial populations (Joachimsthal et al., 2004, Kempf et al., 2005, Lange et al., 1997, Lipoglavsek et al., 2004, Rigottier-Gois et al., 2003, Wallner et al., 1995 and 1997, and Zoetendal et al., 2002). Detection rates are on the order of $10^3$ cells per second and are detected based on light scatter, autofluorescence or various types of fluorescence staining (for an overview see refs Porter, et al., 1996; Shapiro, et al., 1995; Givan, et al., 2001).

Most environmental population studies using FISH-FCM have been performed in the aquatic environment (Casamayor et al., 2002, Eilers et al., 2000, Fuchs et al., 2000, Gerdts et al., 2005, Groben et al., 2005, Joachimsthal et al., 2004, Mou et al., 2005, Pernthaler et al., 2001, Sekar et al., 2004, Simon et al., 1995, and Worden et al., 2000). Major advances were made in the understanding of the structure and function of marine microorganisms using these methods. Populations could be monitored to detect changes in abundance due to grazing (Barcina, et al., 1997), due to contamination in the marine environment (Cid, et al., 1995), and due to UV radiation (Mostajir, et al., 1999).
Many studies have also focused on measuring the distribution and abundance of marine picoplankton using Flow Cytometry (Li, et al., 1988, Chisholm, et al., 1988, and Robertson, et al, 1989). FISH-FCM is particularly efficient for conducting population studies involving frequent measurements made over a time period. The automation and rapid detection rate of Flow Cytometry has greatly enabled the advancement of microbial ecology.

1.6 Thesis Objectives

The objectives of this research were to characterize key organisms involved in TCE degradation from formation water extracted from discrete, hydraulically connected fractures. As the presence and abundance were of interest, the best suited technique was Fluorescent in situ Hybridization (FISH) using 16S rRNA probes. *Eubacteria* and *Archaea* abundance was determined. Due to high levels of sulfate observed in initial groundwater characterization, sulfate-reducing bacteria were also enumerated. Two new 16S rRNA FISH probes targeting *Dehalococcoides sp.* were developed and tested on a pure culture of *Dehalococcoides ethenogenes* strain 195 and a PCE-butyrate consortium. The two probes were also used to analyze groundwater samples to determine the prevalence of *Dehalococcoides sp.* within the TCE plume. Lastly, a FISH-FCM protocol was developed to evaluate whether formation water could be rapidly enumerated for *Eubacteria, Archaea*, sulfate-reducing bacteria, and *Dehalococcoides sp.* These results were compared to FISH-Microscopy counts and to DAPI total cell counts to determine if the two methods yielded similar abundances.
2.0 MATERIALS AND METHODS

2.1 Groundwater Collection

Groundwater samples were collected from discrete fractures within each of the test boreholes using a packer in which an interval was temporarily isolated by means of inflatable packers. The sample was drawn from the zone located between the packers. Using the packer, tests were conducted in the wells at Site 32 to determine the hydraulic conductivity and connectivity of the specific fractures in the bedrock. Four locations were selected based on geophysical evaluation that they are hydraulically connected, BBC 1 at 13 meters, BBC 1 at 15 meters, BBC3 at 27 meters, and BBC5 at 30 meters.

The sampling of each fracture was meant to obtain representative water, and attendant water quality from them. The concept of sampling the fracture was to remove the influence of the wellbore water and obtain the formation water. The initial water pumped from the sampling zone was sampled. Thereafter, a water sample was taken after every one to five liters of water which was pumped from the well. In between samples, pH, conductivity (Ω), and temperature (T) were monitored in a flow cell, and the values recorded. When the monitoring indicated that the real time parameters (pH, Ω, and T) have stabilized, two complete sets of water samples were collected for analyses.

Samples were collected in 1L sterile glass bottles and delivered in a cooler with ice packs to the analytical laboratory on the same day of collection. The groundwater samples from discrete fractures from the boreholes were sampled for TCE, DCE, VC, acetone, methane, ethane, ethene, NPOC, NH₄⁺, PO₄, Br⁻, Cl⁻, SO₄²⁻, S²⁻, NO₃⁻, Fe²⁺, Cu, Total Fe, Mn, K, DO, alkalinity, conductivity, pH, and temperature. Samples reserved for
FISH were immediately preserved with 10% buffered neutral formalin (VWR, West Chester, PA) at a final concentration of 1% and stored at 4°C.

2.2 Total Direct Counts

Total cells counts were determined by microscopic counts of 4',6'-diamidino-2-phenylindole (DAPI, 200 ng/µl; Molecular Probes, Eugene, OR) stained cells. Cells were stained by adding 100 µl of DAPI to 5 ml of sample (final concentration 20 ng/µl) and incubated in a dark refrigerator for 20 min at 4°C. The sample was then filtered onto a black Isopore™ Membrane Filter (diameter 25 mm, pore size 0.2 µm, type GTBP; Millipore, Billerica, MA) and rinsed with 2 ml of phosphate-buffered saline (PBS; 0.13 M NaCl, 7 mM Na2HPO4, and 3 mM NaH2PO4 [pH 7.2]). Filters were transferred with sterile forceps to a microscope slide. The cover slip was dabbed with immersion oil and placed on top of the filter. Slides were examined at 1,000X magnification using a Zeiss Axioskop 2 Plus epi-fluorescence microscope (Zeiss, Thornwood, NY) equipped with a 50 W mercury high pressure bulb and specific filter sets (DAPI: Zeiss 01; Cy3: Zeiss 20; FITC/Oregon Green: Zeiss 10). Photomicrographs were taken with a Zeiss AxioCam MRm digital camera. Exposure times were 0.2 sec. for DAPI stained cells. Ten distinct fields of view were photographed and processed in triplicate, resulting in a total of 30 photomicrographs for an individual groundwater sample. This regime was more stringent than other publications, where at most 20 different randomly selected fields were analyzed per sample (Aulenta, et al., 2004), and in another study at lest 1,000 DAPI-stained cells were counted in > 10 fields of view (Sekar et al., 2003). The following formula was used to determine the total number of cells which would then be used later to calculate the percent of probed cells from the total population:
cells per ml = \( \frac{\text{average } \# \text{ cells counted} \times \text{area of the filter (mm}\text{^2})}{\text{area of field of view (mm}\text{^2}) \times \text{volume of sample filtered (ml)}} \)

2.3 Fluorescent In Situ Hybridization

Groundwater samples were concentrated to at least \(10^5 - 10^8\) cells/ml by centrifugation of 30 ml of groundwater to 1 ml using a Beckman J2-M1 centrifuge equipped with a JA-20 rotor (Beckman, Fullerton, CA) for 10 min at 10,000 rpm. Cells were then washed twice with PBS and resuspended to a final volume of 1 ml in PBS. Cells were then stained with DAPI (20 ng/µl) and incubated in a dark refrigerator for 20 min at 4°C and viewed under an epi-fluorescent microscope to offer assurance that a statistically significant amount of cells were affixed to the slide for further comparison of probed cells to DAPI stained cells. Following DAPI staining, 10 µl of each sample was added to a six-well, Teflon-coated slide (Erie Scientific Co., Portsmouth, NH) and air dried. Cells were dehydrated onto the slide by an ethanol gradient. They were placed in a Petri dish that contained 50% EtOH and submerged for 3 min. The EtOH was removed and slides were submerged in 75% then 100% for 3 min each. After the slides were dried, 10 µl of hybridization buffer containing probe (0.9 M NaCl, 20 mM Tris-HCl [pH 7.2], 0.1% SDS, and 5 ng/µl FISH probe) was spotted on to the well. When more than one probe was used, 10 µl of hybridization buffer containing probe (30 ng/µl for Dehalococcoides probes and 5 ng/µl for all other probes) was added to the well. The prepared slide was then placed inside a glass Petri dish on top of a Kimwipe saturated with hybridization buffer to keep the inside of the Petri dish humid. The Petri dish was sealed around the edges with Parafilm (VWR), placed into a ziplock bag, then weighted down to the bottom of the water bath by a beaker filled with water. To prevent light from entering the water bath which would subsequently cause the fluorescent label on the
probe to fade, the transparent lid was covered by a black cloth. Slides were hybridized for 4 hrs at 46°C. Following hybridization, each well of the slide was washed with 10 µl of wash buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.2] and 0.1% SDS; prewarmed to 48°C). The slides were placed back into the Petri dish, resealed, and allowed to post-hybridize for 20 min at 48°C. After post-hybridization, slides were carefully rinsed with molecular grade water and air dried for approximately 15 min in the dark. Once dry, 6 small drops of DABCO, an anti-bleaching agent (0.1635 g of 1,4-diazabicyclo-[2,2,2]-octane, 5 ml of H₂O; Sigma, St. Louis, MO), were added to a coverslip to fill each slide well. The coverslip was placed onto the slide (DABCO side down) and the edges of the coverslip were sealed using warm paraffin wax (50% petroleum, 50% paraffin): Suggestion: apply wax by flaming a bent metal rod and dipping into jar of paraffin wax then running the rod along the edge of the cover slip). Slides were observed with an epi-fluorescent microscope and accompanying filter sets. Exposure times for FITC and Cy3 filters were set to 1.2 sec. Photomicrographs of the same field of view were taken for each probe and DAPI in the order of FITC, Cy3, and DAPI, to prevent photo-bleaching of the probes by the DAPI filter. For each FISH run, both positive and negative controls were used with each probe as described in Table 1. EUB338 targets most Eubacteria. NON338 is a control probe used to detect false positives, which is complementary to EUB338. ARCH195 targets Archaea. SRB385Db targets some Sulfate-Reducing Bacteria (SRB) of the δ-Proteobacteria and many non-SRB. Dhe1279t targets some Dehalococcoides species.
**Table 1: Oligonucleotide Probes**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence 5’ – 3’</th>
<th>Reference</th>
<th>(+) control</th>
<th>(−) control</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338</td>
<td>GCT GCC TCC CGT AGG AGT</td>
<td>Amann, et. al., 1990</td>
<td>E. coli</td>
<td>Thermococcus sp.</td>
</tr>
<tr>
<td>ARCH915</td>
<td>GTG CTC CCC CGC CAA TTC CT</td>
<td>Stahl, et. al., 1991</td>
<td>Thermococcus sp.</td>
<td>E. coli</td>
</tr>
<tr>
<td>SRB385Db</td>
<td>CGG CGT TGC TGC GTC AGG</td>
<td>Rabus, et. al., 1999</td>
<td>Desulfovibrio escamblense</td>
<td>E. coli</td>
</tr>
<tr>
<td>NON338</td>
<td>ACT CCT ACG GGA GGC AGC</td>
<td>Wallner, et. al., 1993</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Dhe1259t</td>
<td>AGC TCC AGT TCA CAC TGT TG</td>
<td>Yang, et. al., 2003</td>
<td>D. ethenogenes 195, PCE-butyrate culture</td>
<td>E. coli</td>
</tr>
<tr>
<td>DHC412</td>
<td>CAG GTA CCG TCA TTA TTC</td>
<td>This study</td>
<td>D. ethenogenes 195, PCE-butyrate culture</td>
<td>E. coli</td>
</tr>
<tr>
<td>DHC775</td>
<td>CCT ATA CCT AGT GTC CAT</td>
<td>This study</td>
<td>D. ethenogenes 195, PCE-butyrate culture</td>
<td>E. coli</td>
</tr>
<tr>
<td>DHC1165</td>
<td>AAC CTA TTG TTC TGT CCA TTG</td>
<td>This study</td>
<td>D. ethenogenes 195, PCE-butyrate culture</td>
<td>E. coli</td>
</tr>
</tbody>
</table>
2.4 *Dehalococcoides* 16S rRNA Probe Development.

Three 16S rRNA-targeted oligonucleotide FISH probes for the specific detection of *Dehalococcoides* species were developed. The design of the probes was based on the 16S rRNA gene sequence alignment of six previously described species found at chloroethene contaminated sites; *D. ethenogenes* strain 195 (Maymo-Gatell, et al., 1997), *Dehalococcoides* sp. BAV1 (He, et al., 2005), FL2 (He, et al., 2003), DCEH2 (Yang, et al., 2003), CBDB1 (Adrian, et al., 2000) and VS (Cuples, et al., 2003). The sequences were retrieved from RDP-II (Maidak, et al., 1997) and GenBank (Altschul, et al., 1997) and were aligned using Clustal W (Thompson, et al., 1994). Probes were selected based on zero mismatches to the six reference strains and centralized mismatches to control strains (*Escherichia coli* and *Thermomicrobium roseum*) using IDT DNA Primer Quest online software (http://scitools.idtdna.com/Primerquest/). Probes were also restricted to a melting temperature of 46°C, and in length were no longer than 21 basepairs or shorter than 18 basepairs. The resulting probes were first screened to ensure sequence specificity by confirming they only targeted organisms within the *Dehalococcoides* genus, using PROBE_MATCH from RDP-II and BLAST from GenBank. If the probe matched with an organism other than in the *Dehalococcoides* genus, it was rejected.

In the second screening step, the accessibility of the probes with regards to the 2° structure of the molecule was evaluated by comparing the 16S rRNA targeting position of the probes with the *E. coli* probe accessibility table developed by Behrens, et al., 2003 (Figure 1). Probe conferred fluorescence can be limited by the three dimensional structure of the ribosome as noted by Amman, et al., when a newly developed probe failed although binding of the general Bacterial probe EUB338, a positive control, was uninhibited. In the first systematic study of the accessibility of *E. coli* 16S rRNA by Fuchs, et al., 1998 it was demonstrated that even a small shift in the
target region can decrease cell fluorescence as much as 80%. It was therefore a critical step in probe development to target the most accessible regions of the secondary structure based off of the mapping study. *Dehalococcoides ethenogenes* 195 is predicted to have a secondary structure more comparable to *Thermomicrobium roseum* (Figure 2) than that of *E. coli* (Cannone, *et al.*, 2002), therefore, the secondary structure of *T. roseum* was used as a template.

The three newly developed probes in Table 1 were abbreviated DHC after *Dehalococcoides* followed by the nucleotide position that the 3' end of the probe hybridizes to according to *Dehalococcoides ethenogenes* 195 positions.
**Figure 1:** *E. coli* Probe Accessibility Table. Distribution of relative fluorescence hybridization intensities of 176 oligonucleotide probes targeting the 16S rRNA of *E. coli*. The different colors indicate different brightness classes, as explained in the key. (Behrens, et al., 2003)
Secondary Structure: small subunit ribosomal RNA

Thermomicrobium roseum
(M34115)
1. Bacteria 2. Green non-sulfur bacteria
3. Chloroflexaceae group 4. Thermomicrobium
Nov, 1999

Figure 2: Thermomicrobium roseum 16s rRNA Secondary Structure. (Cannone, et. al., 2002)
2.5 Probe Evaluation

The newly developed DHC probes and the recently published *Dehalococcoides* probe Dhe1259t (Yang, et. al., 2003) were tested on two cultures: fixed cells of a pure culture of *Dehalococcoides ethenogenes* 195 (donated by Steve Zinder, Cornell University) and a mixed culture growing on PCE and butyrate also containing *Dehalococcoides ethenogenes* 195 (donated by Donna Fennell, Rutgers University). Each of the probes were also tested on non-target organisms including *E. coli* C3000 (UNH culture collection) and *Thermococcus* sp. (donated by Costantino Vetriani, Rutgers University). The newly designed probes were tested over a range of formamide concentrations (0, 15, and 30%) at a set hybridization and wash temperature to determine optimal stringency. The optimal stringency for each probe was taken as the highest formamide concentration before specific hybridization was lost. Seven slides were prepared in total, 3 for each of the two target organisms and one control slide as described in Figure 3. Samples were concentrated to $10^5$-$10^6$ cells/ml and spotted onto the wells of the microscope slide. The same procedure for FISH was followed. Hybridization buffer (0.9 M NaCl, 20 mM Tris HCl, 0.1% SDS) containing 5 ng/ml probe was prepared. Probe concentrations for the DHC and Dhe1259t probes were set at 30 ng/µl as described in Yang et. al., 2003. Slides were hybridized at 46°C for 4 hrs. Following hybridization the cells were rinsed with post-hybridization wash buffer (20 mM Tris-HCl, 0.1% SDS) prewarmed to 48°C containing the appropriate NaCl concentration according to the formamide concentration in the hybridization buffer (0% formamide – 0.9M NaCl; 15% formamide – 0.318M NaCl; 30% formamide – 0.102 M NaCl).
DNA stain/Probe-Fluorochrome/Organism
1. DAPI/DHC412-OG, EUB338-Cy3/D. ethenogenes 195
2. DAPI/DHC758-OG, EUB338-Cy3/D. ethenogenes 195
3. DAPI/DHC1165-OG, EUB338-Cy3/D. ethenogenes 195
4. DAPI/Dhe1258t, EUB338-Cy3/D. ethenogenes 195
5. DAPI/All DHC probes, EUB338-Cy3/D. ethenogenes 195
6. DAPI/All DHC probes, EUB338-Cy3/E. coli

Three identical slides were prepared for 0, 15, and 30% formamide concentrations.

DNA stain/Probe-Fluorochrome/Organism
1. DAPI/DHC412-OG, EUB338-Cy3/PCE-butyrate consortia
2. DAPI/DHC758-OG, EUB338-Cy3/ PCE-butyrate consortia
3. DAPI/DHC1165-OG, EUB338-Cy3/PCE-butyrate consortia
4. DAPI/Dhe1258t, EUB338-Cy3/PCE-butyrate consortia
5. DAPI/All DHC probes, EUB338-Cy3/PCE-butyrate consortia
6. DAPI/All DHC probes, ARCH915-Cy3/Thermococcus sp.

Three identical slides were prepared for 0, 15, and 30% formamide concentrations.

Control Slide
DNA stain/Probe-Fluorochrome/Organism
1. none/none/PCE-butyrate consortia
2. DAPI/none/PCE-butyrate consortia
3. DAPI/NON338-Cy3/PCE-butyrate consortia
4. none/none/E. coli
5. DAPI/none/E. coli
6. DAPI/NON338/E. coli

This slide was only prepared once since the formamide concentration of NON338 (0%) was already determined.

Figure 3: DHC Probe Evaluation Slides
2.6 Checking for Non-specific Binding

Cultures of *E. coli* and *Thermococcus* sp. were probed with NON338, Univ1390, EUB338, SRB385, and ARCH915-Cy5 to test for non-specific binding. If this occurred, it could be controlled by varying the percent of formamide in the probe buffer. Currently, the additional formamide to control non-specific binding was not necessary for these probes.

2.7 FISH for Flow Cytometry

Samples analyzed by Flow Cytometry were prepared in liquid suspension rather than on a microscope slide. The following is an account of the changes made to the FISH protocol. Samples were concentrated as described for FISH except after the final wash step the supernatant was removed leaving approximately 10 µl remaining. The pellet was re-suspended in 90 µl of hybridization buffer containing probe and 30% formamide, and incubated in a 46°C water bath for 4 hrs. Following hybridization, the samples were centrifuged for 10 min at 10,000 rpm, the supernatant was removed and the pellet was re-suspended in the same wash buffer used for FISH. The tubes were placed back into the water bath and incubated at 48°C for 20 min. After post-hybridization the samples were then prepared for the Flow Cytometer. Cells were centrifuged for 10 min at 10,000 rpm, and re-suspended in 1 ml of 50% EtOH in PBS. The samples were stored at 4°C until the following day. In order to determine the percent of the sample that ran through the Flow Cytometer, which was used to standardize the data, 10 µl of Flow-Check FITC Maxi-Brite 1.0 x 10^7 polystyrene beads (Polysciences, Warrington, PA) were added to each sample. The stock tube of beads was vortexed each time they were added to a new sample.
2.8 Flow Cytometry

Analyses by Flow Cytometry were completed with a FACSCalibur Flow
Cytometer (Becton-Dickinson, Franklin Lakes, NJ) equipped with Argon (488 nm) and
Red Diode (635 nm) lasers and optical filters for the measurement of forward light
scatter, 90° (side) light scatter, and blue, red, and green fluorescence. Data file
management was performed with a Mac G4 host computer using FACStation software.
Graphics and population analysis were performed with WinMDI 2.8 available as public
domain software from The Scripps Research Institute (http://facs.scripps.edu).
3.0 RESULTS AND DISCUSSION

3.1 Site Characterization

A contaminant plume migrated through weathered and competent bedrock extending downward and laterally approximately 0.5 kilometers beyond the identified source area (Figure 4). The bedrock is comprised of metasandstone of the Silurian and Ordovician Kittery Formation interbedded with Jurassic diabase dikes.

![Diagram showing plume migration from 1998 to 2002](image)

*Figure 4: Aerial view of plume migration from 1998 to 2002*

3.2 Groundwater Chemistry

Packer waters ranged in temperature from 22.9 to 50.5°C, were alkaline (131–180 mg/L as CaCO₃, pH 8.2 to 9.6), mildly reducing (Eh of –208 to 160 mV (Eighmy, et al., 2007), DO of 0.7 to 2.5 mg/L [5.0 mg L⁻¹ was noted in BBC1-49.5ft which was
probably an anomaly). Sulfate was the dominant anion in the packer sample water (63–150 mg/L), however, no sulfide was detected, indicating that sulfate-reduction was not occurring. Fe (II) was measureable (0.1 mg/L) and Fe (III) (0.02 to 0.3 mg/L) (Eighmy, et al., 2007) indicating the system is capable of iron (III) reduction. Chemical analysis of the four samples used for analysis by FISH are depicted in Table 2.

H₂ was present in a number of the BBC wells with a range of 2.2 to 7.3 nM (Eighmy et al., 2007). Hydrogen profiles are indicative of dominant terminal electron accepting processes (TEAP) of anaerobic microbial metabolism as described by Chapelle et al., 1995, 1996. Different terminal electron accepting processes have characteristic H₂ steady state concentrations in solution that reflect dominant metabolic redox reactions. Nitrate reduction exhibits H₂ concentrations below 0.1 nM, Iron (III) reduction has a characteristic range of 0.2 to 0.8 nM, sulfate-reduction between 1 and 4 nM, and methanogenesis between 5 to 15 mM. Although initial chemical composition of the groundwater appeared to favor sulfate-reducing conditions, using the hydrogen profile guidelines it appears the system at site 32 is more poised for methanogenesis.

Although levels of sulfate were more than enough to support sulfate-reduction, it was not likely the dominant TEAP because the metabolic by-product, sulfide, was not detected. Levels of methane were (150 to 520 mg L⁻¹), suggesting methanogenesis was occurring. Methanogenic conditions often prevail in contaminant plumes after all other electron acceptors (O₂, NO₃, Fe³⁺, and SO₄) have been used up by other members of the subsurface community.

In terms of contaminants, cis-1,2 DCE was by far the dominant chlorinated compound (73 to 430 mg L⁻¹). Some trans-1,2 DCE was observed but at low concentrations (9 to 170 mg L⁻¹). TCE concentrations were moderate (10 to 330 mg L⁻¹). VC concentrations were also low (4 to 31 mg L⁻¹). Ethene was also detected (2 to 32 mg L⁻¹). These data suggest that biodegradation of TCE had occurred, resulting in some of
the TCE being completely dechlorinated. However, much of the TCE was only partially dechlorinated, and therefore DCE persisted.

### Table 2: Chemical Analysis of BBC Groundwater Samples.

<table>
<thead>
<tr>
<th>Well</th>
<th>BBC1</th>
<th>BBC1</th>
<th>BBC3</th>
<th>BBC5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth (m)</td>
<td>12.2 - 13.7</td>
<td>14.3 - 15.8</td>
<td>25.9 - 27.4</td>
<td>29.1 - 30.6</td>
</tr>
<tr>
<td>Date (mm/dd/yy)</td>
<td>06/17/02</td>
<td>06/13/02</td>
<td>06/26/02</td>
<td>06/24/02</td>
</tr>
<tr>
<td>TCE</td>
<td>0.330</td>
<td>0.200</td>
<td>0.010</td>
<td>0.043</td>
</tr>
<tr>
<td>trans-1,2-DCE</td>
<td>0.170</td>
<td>0.110</td>
<td>0.009</td>
<td>0.045</td>
</tr>
<tr>
<td>cis-1,2-DCE</td>
<td>0.430</td>
<td>0.330</td>
<td>0.073</td>
<td>0.320</td>
</tr>
<tr>
<td>VC</td>
<td>0.031</td>
<td>0.014</td>
<td>0.004</td>
<td>0.014</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.160</td>
<td>0.670</td>
<td>0.030</td>
<td>0.120</td>
</tr>
<tr>
<td>Methane</td>
<td>0.520</td>
<td>0.150</td>
<td>0.170</td>
<td>0.260</td>
</tr>
<tr>
<td>Ethane</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ethene</td>
<td>0.032</td>
<td>0.010</td>
<td>0.002</td>
<td>0.011</td>
</tr>
<tr>
<td>NPOC (non-purgeable organic carbon)</td>
<td>1.97</td>
<td>2.30</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>0.054</td>
<td>NA</td>
<td>0.062</td>
<td>NA</td>
</tr>
<tr>
<td>PO₄⁻</td>
<td>ND</td>
<td>0.1</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>Br⁻</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.2</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>17</td>
<td>16</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>74</td>
<td>63</td>
<td>150</td>
<td>110</td>
</tr>
<tr>
<td>S²⁻</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Fe₂⁺</td>
<td>0.15</td>
<td>0.15</td>
<td>&lt;0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Cu</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>Total Fe</td>
<td>NA</td>
<td>0.38</td>
<td>NA</td>
<td>0.09</td>
</tr>
<tr>
<td>Mn</td>
<td>0.16</td>
<td>2.00</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>K</td>
<td>6.0</td>
<td>6.0</td>
<td>4.8</td>
<td>6.8</td>
</tr>
<tr>
<td>DO</td>
<td>0.7</td>
<td>5.0</td>
<td>1.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Alkalinity as CaCO₃</td>
<td>177</td>
<td>180</td>
<td>145</td>
<td>131</td>
</tr>
<tr>
<td>Conductivity (mS cm⁻¹)</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>pH</td>
<td>8.2</td>
<td>9.1</td>
<td>8.9</td>
<td>9.6</td>
</tr>
<tr>
<td>Temp (°C)</td>
<td>22.9</td>
<td>23.7</td>
<td>24.6</td>
<td>10.3</td>
</tr>
</tbody>
</table>

All concentrations are represented in units of mg L⁻¹ unless otherwise noted. Abbreviations: TCE, trichloroethylene; DCE, dichloroethene; VC, vinyl chloride; NPOC, non-purgeable organic carbon; DO, dissolved oxygen. ND: not detected. NA: not analyzed.

With site characteristics exhibiting mildly reducing conditions and high sulfate concentrations, the presence and abundance of sulfate-reducing bacteria was of interest and therefore a previously published phylogenetic probe was applied to groundwater samples. In addition, daughter compounds of TCE were present as well as levels of
ethene indicating that TCE was being completely degraded. Since *Dehalococcoides* species are key microorganisms involved in chloroethene degradation, it was important to determine if they were present and abundant. Two probes were developed and tested in this study to detect and quantify this key player in TCE removal.

### 3.3 FISH Analysis of Groundwater

Direct cell counts were performed to examine the microbial abundance and morphologic diversity. Abundance was estimated by DAPI staining and direct counting by epi-fluorescence microscopy and ranged from $1.90 \times 10^5$ and $8.61 \times 10^5$ cells mL$^{-1}$ (Table 3). These results were comparable to that of a TCE-contaminated fractured bedrock in NJ which contained between $10^5$ – $10^6$ cells/gram of core material (Bradley et al., 2009) and to that of a TCE-contaminated fractured dolomite aquifer which contained $2.7 \times 10^5$ cells/ml (Hohnstock-Ashe et al., 2001).

**Table 3: Total Direct Counts of DAPI-stained Groundwater from BBC wells**

<table>
<thead>
<tr>
<th>Well # - depth (ft) – date</th>
<th>Cells/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBC1-42.5-061702</td>
<td>$1.90 \times 10^5$</td>
</tr>
<tr>
<td>BBC1-49.5-061302</td>
<td>$6.24 \times 10^5$</td>
</tr>
<tr>
<td>BBC3-61.5-070202</td>
<td>$1.06 \times 10^5$</td>
</tr>
<tr>
<td>BBC3-87.5-062602</td>
<td>$8.61 \times 10^5$</td>
</tr>
<tr>
<td>BBC5-97.9-062402</td>
<td>$6.15 \times 10^5$</td>
</tr>
<tr>
<td>BBC6-115.5-050903</td>
<td>$2.19 \times 10^5$</td>
</tr>
<tr>
<td>BBC6-152.5-061003</td>
<td>$4.32 \times 10^5$</td>
</tr>
<tr>
<td>BBC6-174.5-072403</td>
<td>$5.18 \times 10^5$</td>
</tr>
<tr>
<td>BBC6-197.5-072403</td>
<td>$4.88 \times 10^5$</td>
</tr>
</tbody>
</table>

The morphology was dominated by short rods including several dividing cells, but also present were cocci, vibrios, and an occasional long thin rod around 5 µm long.
(Figure 5). *Eubacteria* and *Archaea* were detected in all 4 of the groundwater samples (Figure 6). *Eubacteria* identified by probe EUB338 were dominant, representing between 93 - 96% of total cells. Another 4 - 7% were affiliated with the domain *Archaea* (including some methanogens) as identified by the probe ARCH915 (Figure 7).

![Morphologies of Microbial Populations of BBC Groundwater](image)

**Figure 5: Morphologies of Microbial Populations of BBC Groundwater**

Sulfate Reducing Bacteria were of interest given that the concentration of \( \text{SO}_4^{2-} \) was relatively high in the groundwater. The SRB385Db probe (targeting the \( \delta \)-Proteobacteria and some non-sulfate-reducing bacteria) identified an abundance ranging between 1 - 28% and were found to be most abundant in BBC3 and BBC5 where levels of \( \text{SO}_4^{2-} \) was the highest (Figure 7). Although SRB were detected by FISH, sulfide was not detected in the groundwater. SRB mediate the formation of sulfide as a result of respiration processes that require \( \text{SO}_4^{2-} \) as a terminal electron acceptor (Brock et. al.,
1992). It was thought that sulfide precipitated out of the water forming pyrite (FeS$_2$), which was uniformly distributed throughout the host rock, however, speciation by X-Ray Photoelectron Spectroscopy of the microfracture surfaces did not detect any sulfide. The system was likely limited by organic carbon bioavailability. The location of the sulfide sink was ultimately uncertain and it was therefore unknown whether the SRB were metabolically active and if sulfate-reduction was truly occurring (Eighmy, et. al., 2007).

*Figure 6: Eubacteria and Archaea Example Pictomicrograph.* Same field of view for BBC5 - 30 meters groundwater sample DAPI stained (A) and probed with EUB338-OG (B) and ARCH915-Cy3 (C).
Figure 7: Abundance of Eubacteria, Archaea, and Sulfate-reducing Bacteria as determined by FISH. Groundwater samples collected from BBC1 (13m) on 06/17/02, BBC1 (15m) on 06/13/02, BBC3 (27m) on 06/26/02, and BBC5 (30m) on 07/20/02. Error bars represent one standard error.

Figure 8: Abundance of Dehalorespiring Bacteria as determined by FISH. Dehalococcoides were found in all wells and comprised between 4 to 10% of the total cells as determined by DAPI. Error bars represent one standard error.

The presence of TCE and its daughter products provided the necessary evidence that natural attenuation was taking place at the BBC site. Dehalococcoides sp. were present within all wells studied ranging from 4 to 10% of total cells (Figure 8). This abundance is higher than that found at a TCE-contaminated fractured bedrock site in NJ which exhibited 0.2 – 2.0% Dehalococcoides sp. (Bradley et al., 2009). The percentage of Dehalococcoides sp. appeared to be greater nearer the shallower depths of the
boreholes and more abundant with a greater concentration of *Eubacteria*. This may indicate that a co-dependence exists between *Dehalococcoides* and other microbial species. No particular correlation could be made between the abundance of *Dehalococcoides* and TCE, DCE, or VC concentrations (Figure 9). Some of the TCE was completely dechlorinated to ethene, while most was only partially dechlorinated, resulting in the persistence of DCE. The degradation of the contaminants was most likely limited by the low amounts of dissolved organic carbon in the system. A major trend which can be noted about the chemistry between wells, is that a higher amount of TCE and breakdown products is found in well BBC1 located within the plume, compared to those located on the edge (BBC3 and BBC5) (Figure 4). It is also interesting to note that the concentration of *trans*-1,2-DCE, *cis*-1,2-DCE, VC, and ethene were all higher in BBC5 than in BBC3. BBC5 is located closer to the source of contamination originating from Building 113.

![Figure 9: TCE, DCE, VC, and Ethene concentrations.](image)

Few studies have examined chloroethene degradation in fractured rock, and there has not been any data published on abundance of key dechlorinating bacteria as determined by FISH. Typically in this type of system, degradation occurs under anaerobic conditions, however aerobic microenvironments can exist. Lenczewski et. al.
have demonstrated TCE degradation in fractured shale bedrock under iron and sulfate-
reducing conditions. Ratios of TCE and daughter products were similar to those of the
BBC site. An increase in daughter products with increasing distance from the source of
contamination was noted, and the rate limiting step was similarly the conversion of cis-
DCE to ethene. The groundwater chemistry alluded to sulfate and iron-reduction with
detectable levels of S$^{2-}$ and Fe(II). Levels of SO$_4^{2-}$ were much lower compared to the
BBC site (6.26-1.99 mg/L vs. 63-150 mg/L) and Fe(II) was higher in the study than the
BBC site (1.59-8.56 mg/L vs. 0.1-0.15 mg/L).

In a study conducted on a PCE and benzene contaminated aquifer below an
industrial landfill, sulfate-reduction was imposed as a part of an in situ remediation
strategy and stimulated by nutrient amendments (Beeman, et al., 2002). SO$_4^{2-}$
concentrations ranged from 240 to 86 mg/L during the anaerobic degradation process,
similar to concentrations at the BBC site, and all PCE and daughter products were
dechlorinated to ethane and ethene. Once the addition of SO$_4^{2-}$ was stopped the
concentration dropped and a conversion to methanogenic conditions took place as
levels increased to 0.75 mg/L, 40 times the baseline concentration.

These data coupled to the chemical composition of the site, provides evidence
that key players in TCE degradation are present. However, cis-DCE and VC are
persistent and may require stronger reducing conditions of methanogenesis. The slow
conversion may be limited by the non-detectable amount of carbon. Lines of evidence
such as contaminant concentration, redox potential and microbial community analysis all
play an important role in predicting what is occurring at a contaminated site and how it
can be improved. The analysis of formation water by FISH demonstrated that this
technique is a rapid tool in community analysis and can be utilized to infer abundance of
at the domain and species level.
3.4 FISH-FCM Analysis of Groundwater

FISH was coupled with Flow Cytometry in effort to cut down on time analyzing results using microscopy. Analysis including positive and negative controls is outlined in Table 4.

Table 4: FISH-FCM Example Run

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Probe 1</th>
<th>Probe 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. E. coli</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>2. E. coli</td>
<td>Univ1390-OG</td>
<td>None</td>
</tr>
<tr>
<td>3. E. coli</td>
<td>EUB338-Cy5</td>
<td>None</td>
</tr>
<tr>
<td>4. E. coli</td>
<td>EUB338-OG</td>
<td>None</td>
</tr>
<tr>
<td>5. E. coli</td>
<td>Univ1390-OG</td>
<td>EUB338-Cy5</td>
</tr>
<tr>
<td>6. E. coli</td>
<td>ARCH915-Cy5</td>
<td>None</td>
</tr>
<tr>
<td>7. E. coli</td>
<td>Univ1390-OG</td>
<td>ARCH915-Cy5</td>
</tr>
<tr>
<td>8. E. coli</td>
<td>EUB338-OG</td>
<td>ARCH915-Cy5</td>
</tr>
<tr>
<td>9. Thermococcus sp.</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>10. Thermococcus sp.</td>
<td>Univ1390-OG</td>
<td>None</td>
</tr>
<tr>
<td>11. Thermococcus sp.</td>
<td>ARCH915-Cy5</td>
<td>None</td>
</tr>
<tr>
<td>12. Thermococcus sp.</td>
<td>Univ1390-OG</td>
<td>ARCH915-Cy5</td>
</tr>
<tr>
<td>13. Thermococcus sp.</td>
<td>EUB338-Cy5</td>
<td>None</td>
</tr>
<tr>
<td>14. Thermococcus sp.</td>
<td>EUB338-OG</td>
<td>None</td>
</tr>
<tr>
<td>15. Thermococcus sp.</td>
<td>Univ1390-OG</td>
<td>EUB338-Cy5</td>
</tr>
<tr>
<td>16. Thermococcus sp.</td>
<td>ARCH915-Cy5</td>
<td>EUB338-OG</td>
</tr>
<tr>
<td>17. Sample</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>18. Sample</td>
<td>Univ1390-OG</td>
<td>None</td>
</tr>
<tr>
<td>19. Sample</td>
<td>EUB338-Cy5</td>
<td>None</td>
</tr>
<tr>
<td>20. Sample</td>
<td>EUB338-OG</td>
<td>None</td>
</tr>
<tr>
<td>21. Sample</td>
<td>Univ1390-OG</td>
<td>EUB338-Cy5</td>
</tr>
<tr>
<td>22. Sample</td>
<td>ARCH915-Cy5</td>
<td>None</td>
</tr>
<tr>
<td>23. Sample</td>
<td>Univ1390-OG</td>
<td>ARCH915-Cy5</td>
</tr>
<tr>
<td>24. Sample</td>
<td>EUB338-OG</td>
<td>ARCH915-Cy5</td>
</tr>
</tbody>
</table>

After running a set of samples and controls, density plots of the resulting particles (or events) detected by Flow Cytometry were obtained from WinMDI 2.8 software to graphically represent and analyze these data. The first step in evaluating a population involved resolving the probed population of cells from debris and non-probed events on
the plotted axis corresponding to the filters specific to the probes used in the assay. As an example, *E. coli* un-probed cells were plotted with the x axis set to FL1 (green fluorescence) and the y axis set to FL4 (far red fluorescence) since the fluorescent probes Univ1390-OG and EUB338-Cy5 were detected with these filters. Next, the quadrant tool was set to subtract the lower left quadrant on the plot that was representative of non-fluorescing events and was applied throughout the analysis of the same sample (Figure 10A). After the non-probed events were identified, the data produced from a probed population was examined.

To obtain an event count for probed-bound cells, located in the quadrant corresponding to the filter/probe combination utilized, the gate tool was applied which allows the user to select those events for automated counting (Figure 10D). Enumeration of a population was based on the ratio of probed cells to an internal bead standard (Figures 10C and E) added to the sample (del Giorgio et al., 1996) using the following formula to compute the bacterial concentration of the sample:

\[
\text{number of cells per ml} = \frac{\text{cell count from Flow Cytometry} \times \text{volume of beads added} \times \text{original bead concentration/ml}}{\text{bead count from Flow Cytometry} \times \text{volume of cells added}}
\]

Oregon Green standardization beads fall into the high end of green fluorescence and are larger than Bacteria, and therefore fall conveniently in the far lower right corner (Figure 10E).

The probe-bound population in this example was *E. coli* stained with Univ1390-OG was plotted against FL1 vs. FL4 (Figure 10B). Cells and beads were enumerated by defining a “region” around the population fluorescing from the Univ1390-OG probe which was designated “R1”, and the population of beads “R2” (Figure 10C). The WinMDI program then allows selection of each of the populations individually where an automated count is performed (Figures 10D-E). The total number of cells was then
calculated by dividing the number of probed cells by the proportion of sample that was run through the Flow Cytometer. The proportion of sample was calculated by dividing the number of beads by the total number of particles counted by Flow Cytometry. In this example 100000 total particles were counted and 1068 beads were detected. The proportion of sample that was analyzed was 0.01068. The total number of cells which bound with the Univ1390-OG probe was $3.18 \times 10^6$ cells/ml. These steps were followed for each individual sample that was probed using a single probe.

The total number of *Eubacteria* within the same population of *E. coli* was determined using the probe EUB338-Cy5 and was plotted against FL1 vs. FL4 (Figures 10F-H). Using gating and selecting regions it was estimated that $3.22 \times 10^6$ cells/ml were *Eubacteria*. The relative abundance of EUB338-Cy5 probed cells compared to total cells probed with Univ1390-OG was 101%. Using two discrete samples to analyze the relative abundance introduces some statistical error. To alleviate this problem, both Univ1390-OG and EUB338-Cy5 were applied to a single sample of *E. coli* and analyzed on a FL1 vs. FL4 grid (Figures 10 I-J). This is referred to as dual-probing.

3.5 Analysis of FISH-FCM using dual-probes

Next it was determined if two probes on one sample yielded comparable results to only using one probe. To test this, *E. coli* was probed with Univ1390-OG and EUB338-Cy5 (Figures 10 I-J). The positive population which fluoresced from both probes occurred in the upper right quadrant (Figure 10 I). The concentration of cells which were positive for both probes was $3.05 \times 10^6$ cells/ml.

Although cells are dual-probed, using the software, each probe population can be viewed separately by evaluating the results using a filter targeting one of the probes and a filter targeting that doesn't target either of the two probes. Theoretically the results should be the same since the plot is the only parameter being changed. Using this type
of analysis, it was determined that $3.04 \times 10^6$ cells/ml bound with the Univ1390-OG probe separately (Figures 11B-D) and $3.02 \times 10^6$ cells/ml bound with the EUB338-Cy5 probe separately (Figures 11D-F). It would be expected that both results should equal that of the # of cells/ml of dually probed cells which was $3.05 \times 10^6$ cells/ml (Table 9). The results were very close. This concentration compared to the estimated concentration of cells stained by both Univ1390 and EUB338 was less than 1%. It was determined that for the analysis of groundwater samples, dual probing would be utilized.

Initial optimization of the protocol using control strains of E. coli and Thermococcus sp. as noted in Figures 10 and 11, and Table 8, it appeared that Flow Cytometry proved to be a useful tool in rapid enumeration. However, when subjected to groundwater from the Site 32 boreholes, analysis was nearly impossible. In most cases, the samples contained particles and debris which autofluoresced (Figure 12). Therefore, FISH-FCM was not used for this study to analyze BBC groundwater samples and results were based off of epi-fluorescence microscopic counts.
Figure 10A-L: FISH-FCM Testing of probes Univ1390, EUB338, and ARCH915 on the Bacteria E. coli
Figure 10A-L (continued): FISH-FCM Testing of probes Univ1390, EUB338, and ARCH915 on the Bacteria E. coli
Figure 11A-F: FISH-FCM E. coli probed with Univ1390-OG and EUB338-Cy5. Data analyzed separately using the plot diagram to view probe conferred fluorescence from one channel.
Table 5: Comparison of Data Obtained from Single-Probed Samples Compared to Dual-Probed Samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Probes</th>
<th>x-axis / fluorochrome</th>
<th>y-axis / fluorochrome</th>
<th># cells</th>
<th># beads</th>
<th>cells/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>FL1 / OG</td>
<td>FL4 / Cy5</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Univ1390-OG</td>
<td>FL1 / OG</td>
<td>FL4 / Cy5</td>
<td>33964</td>
<td>1068</td>
<td>3.18 x 10^6 Univ</td>
<td></td>
</tr>
<tr>
<td>EUB-Cy5</td>
<td>FL1 / OG</td>
<td>FL4 / Cy5</td>
<td>42163</td>
<td>1309</td>
<td>3.22 x 10^6 EUB</td>
<td></td>
</tr>
<tr>
<td>Univ1390-OG + EUB-Cy5</td>
<td>FL1 / OG</td>
<td>FL4 / Cy5</td>
<td>30707</td>
<td>1007</td>
<td>3.05 x 10^6 both Univ + EUB</td>
<td></td>
</tr>
<tr>
<td>Univ1390-OG + EUB-Cy5</td>
<td>FL2 / none</td>
<td>FL1 / OG</td>
<td>30491</td>
<td>1004</td>
<td>3.04 x 10^6 Univ</td>
<td></td>
</tr>
<tr>
<td>Univ1390-OG + EUB-Cy5</td>
<td>FL2 / none</td>
<td>FL4 / Cy5</td>
<td>30362</td>
<td>1004</td>
<td>3.02 x 10^6 EUB</td>
<td></td>
</tr>
</tbody>
</table>

Figure 12: Autofluorescence caused by debris in BBC Groundwater samples
4.0 CONCLUSION

This research interlinks to a larger study conducted by the Bedrock Bioremediation Center, which examined different aspects of the microbial diversity at Site 32 by means of Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), Denaturing Gradient Gel Electrophoresis (DGGE), and Real-Time PCR (RT-PCR). The microbiology of microfracture surfaces of the bedrock was investigated using Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). Biopatches of attached microbes were found on microfracture surfaces in small depressions, cracks or crevices. The microorganisms were predominantly rod-shaped similar to that of the groundwater samples processed for FISH. Diverse morphologies such as spirilla, and stalked bacteria were noted on TEM micrographs of soft calcite surface precipitate. Samples from microfractures were amplified with specific primer sets, detecting the presence of both Eubacteria and Archaea (which includes methanogens). Positive results were also observed for Dehalococcoides sp., sulfate-reducing bacteria, and iron reducing bacteria (specifically the Geobacteraceae). Denaturing gradient gel electrophoresis community profiles from PCR-amplified 16S rDNA showed between 7 and 27 bands, indicating significant population diversity of the microfracture surfaces. For further information on these studies reference Eighmy et al., 2006 and 2007.

There are several studies where Dehalococcoides sp. were enumerated in pure cultures, enrichment cultures, and in lab-scale bioreactors (Yang et al., 2003; Aulenta, et al., 2004), yet knowledge about the abundance of this genus in the environment is limited. A novel approach was taken in this study, where the abundance of key microorganisms within a TCE-contaminated bedrock aquifer, particularly
Dehalococcoides sp. were enumerated using FISH. The success of this technique and newly developed 16S rRNA probe to detect and enumerate Dehalococcoides sp. in formation water from a TCE-contaminated bedrock aquifer presents an opportunity for monitoring in situ bioremediation. This method combined with chemical analysis, can be applied pre- and post-treatment of a site to monitor and characterize the microbial processes and aid in determining an appropriate remediation strategy.
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