FINAL REPORT

Impacts of Sampling and Handling Procedures on DNA- and RNA-based Microbial Characterization and Quantification of Groundwater and Saturated Soil

SERDP Project ER-1560

July 2012

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2. FRONT MATTER

Table of Contents

1. COVER PAGE i

2. FRONT MATTER ii

Table of Contents ii

List of Tables iv

List of Figures v

List of Acronyms viii

Keywords ix

Acknowledgements ix

3. ABSTRACT 0

4. OBJECTIVE 3

5. BACKGROUND 4

6. MATERIALS AND METHODS 8

6.1. Effect of DNA Extraction Method and Sample Mass on Quantification of *Dehalococcoides* spp. Dechlorination Genes and Microbial Community Structure 8

6.1.1. Construction of Nucleic Acid Standards 8

6.1.2. Effect on Quantification of *Dehalococcoides* spp. Declorination Genes 9

6.1.3. Effect on Microbial Community Structure 11

6.2. Effect of Storage and Handling Conditions on Quantitative Gene Detection and Microbial Community Structure 13

6.2.1. Site Selection and Sampling 13

6.2.2. Groundwater Storage Experiment 14

7. RESULTS AND ACCOMPLISHMENTS 16

7.1. Effect of DNA Extraction Method and Sample Mass on Quantification of *Dehalococcoides* spp. Dechlorination Genes and Microbial Community Structure …….. 16

7.1.1. PCR, Cloning, and DNA Standards ……. 16

7.1.2. Quantitative PCR Assays Tested on Soil and Comparison of Two Commercial DNA Extraction Kits……... 20

7.1.3. Effect of DNA Extraction and Sample Mass on Quantification of *Dehalococcoides* spp. Dechlorination Genes ……..21

7.1.4. Effect of Sample Mass and Extraction Method on Microbial Community Structure ……... 24

7.1.5. Effect of Cell Separation During DNA Extraction …… 28

7.1.5.1 Effect on Yield and Gene Detection by Q-PCR …….. 28

7.1.5.2. Effect on Microbial Community Profiles ……...29

7.1.6. Summary of Results for Effects of Sample Size and Extraction Method …….32

7.2. Effect of Storage and Handling Conditions on Quantification of *Dehalococcoides* in Groundwater and Bacterial Community Structure ……. 32

7.2.1. Effect of Storage on Quantitative Gene Detection ……... 32

7.2.2. Effect of Storage on Bacterial Community Structure ……... 35

8. CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH/IMPLEMENTATION 40

9. LITERATURE CITED 42

10. APPENDICES 48

List of Technical Publications ……. 48

List of Tables

Table 6.1.1.1. *Taq*Man primers and probes used to quantify *Dehalococcoides* 16S, total Bacteria 16S and RDase genes ……..... 9

Table 6.2.1. Geochemical and biological data from the Lumberton site. Three recovery wells (RW) and five monitoring wells (MW) were sampled. DNA was extracted from groundwater using the $Al_2(SO_4)_3$ method and *Dehalococcoides* (Dhc) levels were measured by qPCR. Gas chromatography was used to measure levels of pechloroethene (PCE), trichloroethene (TCE), cis-dichloroethene (c-DCE), vinyl chloride (VC), ethene, chloride, and sulfate. Dissolved oxygen (DO) and total organic carbon (TOC) were measured by colorimetric methods …….13

Table 6.2.2. Particle size analysis of a continuous soil core collected from the Lumberton site..14

Table 7.1.2.1 Total Bacteria 16S rRNA (Bac), *Dehalococcoides* spp. 16S rRNA (Dhc), and VC reductase genes *bvcA* and *vcrA* detected by qPCR using two different DNA extraction methods. Copy numbers are per gram of aquifer sediment……...20

Table 7.1.4.1. Similarity Percentage Analysis (SIMPER) of T-RFLP data from sediment inoculated with KB-1 and *E. coli tce*A cultures and extracted using four methods: Qiagen (Q), Mo Bio Powersoil (M), FastDNA (F), and $Al_2(SO_4)_3$ (A) and three sample masses (0.25, 1.0 and 10.0 g). The second column shows the percent of KB-1 T-RFs detected in the inoculum that were also captured by each extraction method. Dissimilarity Within Methods shows the percent dissimilarity of the T-RFLP profiles within replicates of each method (Note: M 0.25 and Q 10 have only two replicates). Dissimilarity Between Methods describes dissimilarity between the T-RFLP profiles of paired methods…… 27

Table 7.1.4.2. Analysis of Similarity (ANOSIM) of bacterial T-RFLP data from sediment inoculated with KB-1 and *E. coli tce*A cultures. The R statistic for each pairwise comparison shows the dissimilarity between methods. The higher the R value the greater the replicate similarity within the method versus that between methods. Only pairwise comparisons that are statistically different in T-RFLP profiles at the 0.05 significance level are presented: Qiagen (Q), Mo Bio Powersoil (M), FastDNA (F), and the $Al_2(SO_4)_3$ (A) method and three sample masses (0.25, 1.0 and 10.0 g)…….. 28

Table 7.2.1. t-test comparisons of gene copy numbers detected in groundwater and stored under a range of conditions. Groundwater was stored either filtered or unfiltered and at either 4º C or 25 º C for 48 hours. The control was filtered within 3 hours of collection and DNA was immediately extracted. Probability values are for unpaired t-tests between control and treatments and within treatments. Dashes indicate comparisons where the differences between the means were not significantly different. P-values of < 0.05 indicate that the means were significantly different at the 95% confidence interval…….…. 35

Table 8. Advantages and disadvantages of DNA extraction methods used in this study…….. 41

List of Figures

Figure 7.1.1.1. PCR amplification of 16S rRNA genes from KB-1 and SDC-9 using a *Dehalococcoides* – specific primer (DHC16S9F) and a universal Bacterial primer (BACT 1492R). Lane 1: DNA marker. Lanes 3-4: KB-1 template. Lanes 6-7: SDC-9 template. Lane 8: negative control…… 16

Figure 7.1.1.2. PCR amplification of *tce*A gene from SDC-9 using TCD RDase primers 797F and 2490R. Lane 1: DNA marker. Lanes 4-5: SDC-9 template. Lanes 1, 3, 6: negative control. ……16

Figure 7.1.1.3. PCR of *vcr*A RDase gene, amplified using primers vcrABF and vcrABR. Lane 1: DNA marker. Lanes 3-4: KB-1 template. Lanes 6-7: SDC-9 template. Lane 9: negative control. ……16

Figure 7.1.1.4. Nested PCR of *bvc*A RDase gene, amplified using degenerate primers RRF2 and B1R and primers bvcAF and bvcAR. Lane 1: DNA marker. Lanes 4-5 and 9-10: KB-1 template. Lanes 6 -7 and 11-12: SDC-9 template. Lane 13: negative control……...…. 16

Figure 7.1.1.5. *Taq*man standard curve for Bacterial 16S rRNA gene (log copy number on x-axis). Target concentrations were linear between 4.8 x 10^2 and 4.8 x 10^7 copies/reaction although still detectable below this range. All reactions were performed in triplicate…… 17

Figure 7.1.1.6. *Taq*man standard curve for *Dehalococcoides* 16S rRNA gene. Target concentrations were linear between 4.8 x 10^3 and 4.8 x 10^8 copies/reaction although still detectable below this range. All reactions were performed in triplicate…….. 18

Figure 7.1.1.7. *Taq*man standard curve for vinyl reductase gene *bvcA*. Target concentrations were linear between 2.1 x 10^2 and 2.1 x 10^8 copies/reaction. All reactions were performed in triplicate…… 18

Figure 7.1.1.8. *Taq*man standard curve for vinyl reductase gene *vcrA*. Target concentrations were linear between 4.7 x 10^2 and 4.7 x 10^7 copies/reaction. All reactions were performed in triplicate…… 19

Figure 7.1.1.9. *Taq*man standard curve for vinyl reductase gene *tceA* (log copy number on x-axis). Target concentrations were linear between 4.7 x 10^2 and 4.7 x 10^7 copies/reaction. All reactions were done in triplicate…….. 19

Figure 7.1.3.1. DNA yields from four extraction methods for three sample masses and from the KB-1 inoculum. DNA yields were determined from the inoculum added and are expressed as the amount of KB-1 DNA added per g of sediment. Error bars denote SD, n = 3 extracted samples. ……21

Figure 7.1.3.2. Sample DNA spectra from sediment inoculated with KB-1 and *E. coli tce*A cultures and extracted (0.25 g) using four methods: Qiagen, Mo Bio Powersoil, FastDNA, and

the $Al_2(SO_4)_3$ method. Due to high contamination at the 230 nm wavelength, FastDNA spectra were plotted on the secondary Y-axis……... 22

Figure 7.1.3.3. Concentrations of *Dehalococcoides* 16S rRNA genes in cells/g of sediment and in the KB-1 inoculum. For the inoculum, cell numbers were normalized to the amount inoculated per g of sediment. Bars with same letters denote no statistical differences. All other comparisons were significant at the 0.05 level. Error bars denote SD, n = 3 extracted samples with 3 Q-PCR reactions per sample……. 23

Figure 7.1.3.4. Concentrations of the *tceA* gene in cells/g of sediment and in the *E. coli tce*A inoculum. For the inoculum and viable plate counts, cell numbers were normalized to the amount inoculated per g of sediment. Bars with same letters denote no statistical differences. All other comparisons were significant at the 0.05 level. Error bars denote SD, n = 3 extracted samples with 3 Q-PCR reactions per sample…… 23

Figure 7.1.3.5. Recovery of added *Dehalococcoides* in soil…….. 24

Figure 7.1.4.1. Shannon-Weiner Diversity Indices of various sample mass/extraction methods for Bacteria…… 25

Figure 7.1.4.2. Multi dimensional scaling plot of the Bray-Curtis coefficient of bacterial T-RFLP matrices from sediment inoculated with KB-1 and *E. coli tce*A cultures and extracted using four methods: Qiagen, Mo Bio Powersoil, FastDNA, and the $Al_2(SO_4)_3$ method and three sample masses (0.25, 1.0 and 10.0 g). Qiagen 1 and 10 g samples were omitted due to PCR inhibition. KB-1 and *E. coli tce*A, and FastDNA are displayed with dropdown lines to show three dimensional scatter. The soil matrix (*) is the uninoculated soil……... 25

Figure 7.1.4.3. Shannon-Weiner Diversity Index for various sample sizes/extraction method combinations for Archaea ……... 26

Figure 7.1.4.4. Bray-Curtis Similarity of various sample mass/extraction methods for Archaea …… 26

Figure 7.1.5.1. Quantitative PCR detection of *Dehalococcoides* 16S rRNA and *tce*A genes in DNA extracted from soil using the $Al_2(SO_4)_3$ method directly and with the addition of two cell separation steps: vortexing and sonication. Bars with same letters denote no statistical differences. All other comparisons were significant at the 0.05 level. Error bars denote SD, n = 3 extracted samples with 3 Q-PCR reactions per sample……. 28

Figure 7.1.5.2. Multi dimensional scaling plot using the Bray-Curtis coefficient showing the effect of cell separation steps on bacterial T-RFLP similarity matrices. The direct $Al_2(SO_4)_3$ extraction was compared to $Al_2(SO_4)_3$ extraction with additional vortexing or sonication steps on soil inoculated with the initial KB-1 and *E. coli tce*A cultures and on uninoculated soil…... 29

Figure 7.1.5.3. Bray-Curtis Similarity for Archaea, effect of cell separation steps…… 29

Figure 7.2.1.1. Effect of groundwater storage temperature and condition on qPCR detection of 16S genes targeting total bacteria and *Dehalococcoides spp*. Groundwater was stored either filtered (Fl) or unfiltered (W) at either 4 ºC or 25 ºC for 48 hours. Unfiltered groundwater was also stored at 4 ºC for 14 days (W 4C 14d). The control was filtered within 3 hours of collection and DNA was immediately extracted……... 33

Figure 7.2.1.2. Effect of groundwater storage temperature and condition on qPCR detection of the dechlorinating functional genes *tce*A, *vcr*AB, and *bvc*A. Groundwater was stored either filtered (Fl) or unfiltered (W) at either 4 º C or 25 º C for 48 hours. Unfiltered groundwater was also stored at 4 º C for 14 days (W 4C 14d). The control was filtered within 3 hours of collection and DNA was immediately extracted……... 34

Figure 7.2.2.1. Dominant (>1%) Bacterial TRF profile based on *Hha*I digestion of 16S rRNA gene of filtered groundwater samples under varying storage conditions……. 36

Figure 7.2.2.2. Dominant (>1%) Bacterial TRF profile based on *Hha*I digestion of 16S rRNA gene of stock groundwater samples under varying storage conditions……. 37

Figure 7.2.2.3. Bray-Curtis dendogram showing similarity of T-RFLP patterns of bacterial 16S rDNA extracted from groundwater samples under varying storage conditions……... 37

Figure 7.2.2.4. Relative abundance of inferred bacteria based on various T-RFs obtained from *in silico* restriction enzyme digests (using *Hha*I, *Msp*I, and *Rsa*I) from groundwater samples stored under varying conditions……. 39

List of Acronyms

ANOSIM	Analysis of Similarity
BLAST	Basic Local Alignment Search Technique
CAP	Community Analysis Package
cDCE	cis-Dichloroethene
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
MBT	Molecular Biological Technique
MW	Monitoring Well
NCBI	National Center for Biotechnological Information
PAT	Phylogenetic Analysis Tool
PCE	Perchloroethene
PCR	Polymerase Chain Reaction
Q-PCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RW	Recovery Well
SIMPER	Similarity Percentage
T-RF	Terminal Restriction Fragment
T-RFLP	Terminal Restriction Fragment Length Polymorphism
TCE	Trichloroethene
VC	Vinyl Chloride

Keywords

DNA, Quantitative PCR, Molecular Biological Techniques, Dehalococcoides, Microbial Community Structure, Sampling, Handling, Storage, T-RFLP

Acknowledgements

The Principal Investigators would like to thank the postdoctoral investigator and graduate and undergraduate students who worked on this project: Kristin Hicks, Mark Jason So, Nikia Smith, and Michael Carpenter (dec.). We would like to thank the SERDP Program Manager, Dr. Andrea Leeson, and the SERDP staff for their support, assistance, and patience.

3. ABSTRACT

Objectives

To accurately evaluate the performance of groundwater bioremediation processes, methods that can quantify the populations and the in-situ activity of relevant groups of microorganisms are needed. Molecular biological techniques that rely on DNA extraction and PCR amplification are widely used to determine microbial community structure and the concentrations of specific organisms or genes in environmental samples. A study was conducted to determine an optimal sample handling and processing strategy that will yield accurate results for real-time PCR and Terminal Restriction Fragment Length Polymorphism in soil and aquifer sediments.

Technical Approach

The effect of DNA extraction methods and sample mass on the observed microbial community, the detection of the 16S rRNA genes for Bacteria and *Dehalococcoides spp.*, and the *tce*A gene that codes for trichloroethylene (TCE) reductase found in some *Dehalococcoides* strains, was assessed. In addition, the effects of storage temperature, time, and condition on quantitative *Dehalococcoides* 16S rRNA and *tce*A genes, and two VC reductase functional genes (*vcr*A and *bvc*) in both groundwater and soil samples were investigated. Homogenized aquifer sediment was inoculated with a mixed *Dehalococcoides* consortium (KB-1) and an *E. coli* clone containing the *tce*A gene. DNA was extracted from three sample masses using three commercial DNA extraction methods. A new laboratory method for DNA extraction was also developed. In addition, two indirect methods that use cell separation steps prior to DNA extraction were assessed. Quantitative-PCR was used to measure concentrations of the 16S rRNA gene of *Dehalococcoides* spp and the *tce*A gene. T-RFLP was used to characterize the bacterial and archaeal community profiles.

Results

Sample mass did not affect Q-PCR results for DNA extracted using the lab method but gave inconsistent results when the kits were used. T-RFLP profiles were less sensitive to extraction method than Q-PCR results, but the lab method resulted in more consistent reproducibility and community similarity matrices. The storage and temperature experiment showed that the storage condition had a major impact on numbers of total bacteria and the bacterial community structure. Storage of samples at 25 ºC for two days and storage at 4°C for 14 days resulted in significant differences in detected concentrations of *Dehalococcoides* and Bacteria 16S rDNA and *tce*A, *bvc*, and *vcr*A genes. These storage conditions also resulted in significant shifts in the bacterial community structure as revealed by T-RFLP. None of the commercially available kits were deemed suitable for quantitative analyses. The T-RFLP analysis also showed that some commercial kits resulted in strong biases with respect to microbial community structure. The lab method was successful in eliminating the effect of extracellular DNA, with recoveries of less than 0.1% of added extracellular DNA even at high levels of addition (> 10^9 copies added per sample). Below is a table of advantages and disadvantages of each DNA extraction method.

DNA Extraction Method	Advantages	Disadvantages
Mo Bio Power Soil	• Kits available for several sample sizes • Most rapid DNA extraction method (<20 minutes per sample) • Involves minimal reagent preparation • At higher sample sizes, produces bacterial community profile similar with most of the DNA extraction methods used	• Very small DNA yield regardless of sample size used • Produces DNA extracts with high impurity
FastDNA Spin Kit for Soil	• Relatively high DNA yield • Relatively rapid DNA extraction method (<30 minutes per sample) • Involves minimal reagent preparation	• Sample size non-scalable (sample size limited to only 0.25g) • Produces DNA extracts with high impurity • Yielded dissimilar bacterial community profiles compared to other extraction methods used
QiaAMP DNA Blood Kit	• Kits can be customized for several sample sizes • Involves minimal reagent preparation	• DNA extracted with high impurity • Slow DNA extraction (<40 minutes per sample) • High PCR inhibition at larger sample sizes resulting to failed downstream PCR processes
Lab Method	• Scalable to desired sample size • Produces highest DNA yield compared to tested commercial kits • Produces high DNA purity • Produces bacterial community profile similar with most of the DNA extraction methods regardless of sample size used • Produces highest QPCR results for spiked samples • Can remove extracellular DNA	• Relatively more labor-intensive method • Involves several reagent preparations • Slowest extraction method (<50 minutes per sample)

Benefits

Results of initial experiments led to the development of a DNA extraction method. The new method, which utilizes aluminum sulfate to immobilize PCR-inhibiting compounds prior to cell lysis, gave superior results compared to the three commercial kits with respect to DNA yield, DNA purity, and gene detection. In addition, the lab method resulted in more consistent Q-PCR results, and more consistent microbial community analysis. The lab method also has the

potential of eliminating the effect of inactive (extracellular) DNA. This method may be of value to researchers performing molecular biological techniques at DoD sites. The study also showed that storage conditions will affect microbial community structure analysis. Careful assessment of DNA extraction methods and sample storage conditions are needed in bioremediation studies to allow comparisons of results in the research community.

4. OBJECTIVE

The main objective of research conducted under ER-1560 is to determine the effects of sampling and handling on the results of DNA- and RNA-based techniques. The specific objectives were: (1) To determine the effects of various nucleic acid extraction conditions on microbial diversity (Bacteria and Archaea), and levels of *Dehalococcoides* spp. 16S rRNA and vinyl chloride reductive dehalogenase genes; (2) To determine the effects of various sampling and storage conditions on microbial diversity and levels of *Dehalococcoides* spp. 16S rRNA genes and vinyl chloride reductase genes; and (3) To determine an optimal sample handling and processing strategy that will yield accurate results for real-time PCR and T-RFLP of groundwater and sediment samples.

5. BACKGROUND

In recent years, the use of molecular biological techniques (MBTs) has allowed microbial ecologists and environmental engineers to determine microbial community structures in environmental samples without the limitations of traditional approaches such as conventional morphological analyses (staining and microscopy), and culture-based techniques. These new methods are based on the detection and quantification of target molecules that are unique for specific microbial populations, such as lipids and membrane components, proteins, and nucleic acids. Of these, nucleic acid based techniques have received the broadest acceptance. They rely on the identification of signature sequences present in the DNA of a population (e.g., ribosomal RNA [rRNA] genes or metabolic genes). The most common approaches used in bioremediation research are those based on the polymerase chain reaction (PCR) amplification of 16S rRNA genes. In general, these techniques can be used to detect the presence of specific organisms (e.g., detection of 16S rRNA genes specific to target organisms), or characterize the microbial community (e.g., generation of clone libraries, denaturing gradient gel electrophoresis [DGGE], and Terminal Restriction Fragment Length Polymorphism [T-RFLP]). More recently, PCR has been used in a quantitative format, using real-time PCR technology (e.g., Taqman or SYBR Green approaches). Non-PCR-based approaches include direct hybridization with oligonucleotide probes (e.g., Fluorescence *In Situ* Hybridization and quantitative slot-blot hybridization). The choice of MBT depends on the purpose of the study- whether it is detection or quantification of a specific microbial group or species, or determining the different microbial groups present in an environmental sample.

The versatility of MBTs has led to a resurgence in research on the use of these tools for monitoring biological systems, including groundwater bioremediation. Because of its sensitivity compared to direct hybridization/probing, PCR is increasingly used to analyze groundwater samples and soil samples from contaminated sites. However, PCR, like all molecular techniques, has limitations. For example, the known biases in the amplification process (collectively known as PCR bias) include, among others: selective DNA extraction, differential primer annealing, and variable amplification (Altwegg, 1995; Witzingerode *et al*., 1997; Ludwig and Schleifer, 2000). Webster *et al*. (2003) found that template DNA from extremely low biomass sediment samples was susceptible to PCR bias and random amplification. In complex and spatially heterogeneous environments such as groundwater aquifers, it can be problematic to derive ecologically meaningful microbial data from very small amounts of samples (e.g., in the order of grams of soil) (Chandler, 1998). While the latter issue is not specific to MBTs, the problem is magnified by the relatively small amounts of samples needed to perform PCR. A question of specific interest is the relative merit of sampling groundwater versus the associated saturated soil. To date, this issue has received scant attention.

The issues of sampling are compounded by the lack of standards for sample processing. The Expert Panel Workshop on Research and Development Needs for the Environmental Remediation Application of Molecular Biological Tools (August 2005) noted the lack of understanding of the effects of sample processing and handling. Presumably, when collecting environmental samples for DNA- or RNA-based molecular characterization, immediate analysis or immediate freezing for subsequent analysis is ideal for determining the microbial community structure at the time of sampling. When samples are not analyzed immediately, changes in environmental factors may shift species- or group-specific rRNA, rRNA gene, or metabolic gene

levels and skew the results of molecular microbial analysis (Keith *et al*., 2005). These concerns have not been comprehensively studied. In general, groundwater researchers collect soil samples, cap and seal the containers and ship the samples to the laboratory on ice (Fennell *et al*., 2001; Hendrickson *et al*., 2002; Major *et al*., 2002; Sung *et al*., 2006). Similarly, groundwater samples are collected in sterile containers, kept anaerobic, and shipped on ice (Hendrickson *et al*., 2002; Major *et al*., 2002; Macbeth *et al*., 2004). Alternatively, groundwater is filtered and frozen immediately (Hohnstock-Ashe *et al*., 2001; Lendvay *et al*., 2003). While transportation times are not routinely reported, it is realistic to assume that this may take hours if not days. When samples are used as inocula for column studies (e.g., for reductive dechlorination), then greater attention is placed on maintaining anaerobic conditions. Once in the laboratory, it is common practice to open the containers in an anaerobic hood and homogenize before sub-sampling for DNA/RNA extraction and analysis.

The effects of shipping conditions and length of shipping/storage time of groundwater and aquifer soil samples have not been studied. Haldeman *et al*. (1995) found that storage of deep subsurface samples (400 m below the surface) caused an increase in the number of microorganisms and a decrease in microbial diversity. The shift was attributed to bacterial growth and resuscitation of dormant microorganisms. Rochelle *et al*. (1994) reported that immediate freezing of sediment samples was necessary to prevent shifts in microbial diversity. For example, aerobic conditions promoted the growth of beta and gamma proteobacteria, while anaerobic conditions resulted in mostly alpha-proteobacterial sequences. Sediment samples taken anaerobically and frozen within 2 hours showed the most diversity.

The use of molecular biology methods to understand biological processes assumes that the DNA extracted is representative of the DNA in the sample and of sufficient quality for downstream analysis such as PCR. Interpretation of subsequent data and its extrapolation to the broader field of research assumes that this first step, the extraction of total DNA, produces an accurate representation of the actual microbial community. Previous research has ably demonstrated that the choice of DNA extraction method has a profound effect on downstream results. DNA extraction methods vary in their cell dispersion, cell lysis and DNA recovery efficiencies, as well as in the yield and purity of the extracted DNA (Inceoglu et al., 2010; Krsek and Wellington, 1999; Klerks et al., 2006; Martin-Laurent et al., 2001; Miller et al., 1999; Thakuria et al., 2008). DNA purity is of special concern for environmental samples because humic substances and other co-extracted soil compounds sorb extracted DNA (Tsai and Olson, 1992; Young et al., 1993), interfere with membrane hybridizations (Alm et al., 2000) and restriction enzyme digestion of DNA (Tebbe and Vahjen, 1993), and inhibit PCR reactions (Fitzpatrick et al., 2010; Klerks et al., 2006; Miller et al., 1999; Sagova-Mareckova et al., 2008; Tebbe and Vahjen, 1993; Whitehouse and Hottel, 2007; Wilson, 1997). Interference of soil extracts and of the matrix itself (e.g., sand, clay, compost, activated carbon) to the derived DNA quantity and quality is often severe and difficult to pre-determine. Indirect DNA purification steps, wherein cells are separated from the matrix prior to DNA extraction, are often used to counteract matrix interference.

Various protocols for the extraction of DNA and RNA exist, and there is no single standard method. In general, DNA extraction from soils and sediments can be direct or indirect. Direct methods use cell lysis within the sample matrix, and subsequent isolation of DNA from the matrix and cell debris (first used by Ogram et al., 1988), while indirect methods involve the

separation of cells from the environmental matrix and subsequent extraction of DNA (first used by Holben et al., 1988). Direct extraction is perhaps more commonly used, and is typically performed using physical (bead beating, sonication, freeze-thawing), chemical (use of detergents), or a combination of both. Direct methods have been shown to result in higher yield of DNA (Steffan *et al*., 1988), although cell separation results in greater purity (Gabor *et al*., 2003).

Most studies using MBTs focus on DNA yield; whether or not the DNA extraction leads to bias (i.e., less sequence diversity determined by downstream PCR analysis) is usually not addressed. The assumption is that a higher DNA yield results in a larger and more diversified pool of PCR templates, and hence higher diversity. Indeed, some researchers have shown that direct extraction of DNA is better than cell separation at assessing bacterial diversity (Martin-Laurent *et al*., 2001; Marco-Luna *et al*., 2006). However, other research disputes these findings, and find no correlation between yield and bacterial diversity (Stach *et al*., 2001; Gabor *et al*., 2003; Lyautey *et al*., 2005). Ranjard *et al*. (2003) showed no effect of DNA yield on Bacterial community structure, but showed differences in fungal community structures. Previous work in our lab show that surprisingly, indirect methods of cell extraction (hand squeezing) result in HIGHER Bacterial and Archaeal diversity in municipal solid waste samples (Staley *et al*., 2011). This ongoing debate on the best DNA extraction procedure is extremely important for groundwater research, and yet to date, this has not been addressed.

In the last decade, molecular biology techniques have been used in field sites and laboratory studies to monitor and analyze bioremediation. Many of these studies specifically targeted *Dehalococcoides*. Hohnstock-Ashe *et al*. (2001) used Amplified Ribosomal DNA Restriction Analysis (ARDRA) to show the presence of *D. ethenogenes* in TCE-contaminated fractured dolomite aquifer. Löffler *et al*. (2000) designed specific primer pairs for *Desulfuromonas* and *Dehalococcoides*, and showed that sediments from three rivers yielded positive signals for *Desulfuromonas* BB1 strain and *Dehalococcoides*. The detection limit of a nested PCR approach (using bacterial primers followed by specific primers) was found to be 1 x 10^3 cells added per gram (wet weight). Fennell *et al*. (2001) combined microcosm studies, PCR analysis, and site data to show the heterogeneous distribution of dechlorination activity and *Dehalococcoides* at a TCE-contaminated site. Hendrickson *et al*. (2002) used a *Dehalococcoides*-specific PCR assay to sample 24 chloroethene-dechlorinating sites throughout North America and Europe. They showed that *Dehalococcoides* sequences were present in 21 of the sites; all these sites demonstrated complete dechlorination from PCE to ethene. On the other hand, in the three sites that *Dehalococcoides* sequences were not detected, dechlorination appeared to stop at 1,2-cis DCE. At sites where dechlorination had stalled at cis-DCE, bioaugmentation with the Pinellas inoculum led to complete dechlorination to ethene and the establishment of *Dehalococcoides* (Ellis *et al*., 2000; Major *et al*., 2002; Lendvay *et al*., 2003).

Other work involved phylogenetic analyses of the microbial communities in groundwater and soil. These studies often used PCR amplification of *Dehalococcoides* rRNA genes, followed by cloning/sequencing, denaturing gradient gel electrophoresis (DGGE), or T-RFLP (Richardson *et al*., 2002; Dennis *et al*., 2003; Macbeth *et al*., 2004; Freeborn *et al*., 2005; Grostern and Edwards, 2006; Rahm *et al*., 2006). PCR was performed using primers designed for one or more strains of *Dehalococcoides* (Löffler *et al*., 2000; Fennell *et al*., 2001; Richardson *et al*., 2002; Dennis *et al*., 2003; Fennell *et al*., 2004). Other studies designed and used probes directly

targeting 16S rRNA (Yang and Zeyer, 2003; Freeborn *et al*., 2005). Quantitative real-time PCR has been used to quantify *Dehalococcoides* (He *et al*., 2005; Grostern and Edwards, 2006; Rahm *et al*., 2006). However, it is becoming increasingly clear that 16S rRNA sequences are not enough to resolve strain differences. For example, Dehalococoides strains 195 and WL have 100% similarity, while strains BAV1, FL2, and CBDB1 share 99.9% similarity. Thus, researchers have started to look at reductive dehalogenase genes as targets for differentiating among strains and assessing bioremediation. These genes include *vcrA*, encoding a VC reductive dehalogenase in strain VS (Muller *et al*., 2004), and *bvcA*, encoding a VC reductive dehalogenase in strain BAV1 (Krajmalnik-Brown *et al*., 2004). Waller *et al*. (2005) designed degenerate primers for KB-1 reductive dehalogenase genes, and showed the existence of a pool of RDH genes, with each strain of Dehaloccoides having a different complement of genes. It seems apparent that understanding the presence and function of dehalogenase genes is the next step in understanding and monitoring bioremediation, and that MBTs will continue to focus on detecting and quantifying these genes.

The sampling, storage, and processing issues associated with the use of MBTs in bioremediation research need to be addressed systematically and comprehensively. In particular, improved sampling and processing procedures for use at groundwater remediation sites are needed. This will result in better understanding of the effectiveness and accuracy of MBTs in determining levels of relevant microbial populations and assessing the microbial community structure (diversity, richness, evenness, and other measures of diversity). The expected overall result is a set of sampling and processing procedures that would allow MBTs to be accurate, efficient, and effective as tools for assessing bioremediation of contaminated sites.

6. MATERIALS AND METHODS

6.1. Effect of DNA Extraction Method and Sample Mass on Quantification of *Dehalococcoides* spp. Dechlorination Genes and Microbial Community Structure

6.1.1. Construction of Nucleic Acid Standards

Samples and DNA Extraction

Two Dehalococcoides-containing mixed cultures were obtained. KB-1 is a commercial dechlorinating culture composed of *Dehalococcoides spp.* and was provided from an active chemostat culture courtesy of SiRem, Inc (Guelph, Ont., Canada). SDC-9 is a commercial mixed culture provided by Shaw Environmental, Inc (Lawrenceville, NJ). From these cultures, DNA was extracted using the MoBio UltraClean extraction kit (Mo Bio Laboratories, Carlsbad, CA).

Quantitative PCR

To generate standard curves for Q-PCR, DNA was extracted from the KB-1 culture using the Mo Bio Ultraclean™ Microbial DNA isolation Kit and target amplicons were created by PCR amplification of the isolated DNA. For the Bacteria 16S (Bac) and *Dehalococcoide*s 16S (Dhc) standards, a 1.5 Mb amplicon was obtained using a *Dehalococcoide*s specific forward primer (16S9F) and a universal bacterial reverse primer (1492R) (Holmes et al., 2006, Magnuson et al., 2000). For the *tce*A standard, a 1.7 Mb fragment was amplified using primers (797F; 2490R) specific to the *tce*A reductase functional gene (Magnuson et al., 2000). For the *vcr*A standard, a 1.5 Mb fragment was amplified using primers vcrABF and vcrABR (Duhamel et al., 2004) and for the *bvc* A 800 bp gene was amplified using a nested PCR approach with degenerate primers RRF2 and B1R followed by primers *bvc*AF and *bvc*AR (Dennis et al., 2003).

PCR conditions were: an initial 94°C denaturation (12 min); 30 cycles of 94°C (60 s), 50°C (45 s), and 72°C (120 s); and a final extension step at 72° C (12 min). Purified PCR fragments were cloned using the Invitrogen TopoTA cloning kit with One Shot Top10 Chemically Competent Cells, or Invitrogen TA Cloning Kit (Carlsbad, CA). The amplicons were ligated into a pCR®2.1 vector. The vectors were then used to transform INVαF' *E. coli* cells. The transformed *E. coli* were grown on LB plates with X-gal (Promega, Madison, WI). White colonies were then isolated and grown in LB media with 100μg/mL ampicillin. Plasmids were extracted from successful clones using the Qiagen QIAprep®Spin Miniprep kit and sequenced using M13F and M13R primers (MWG DNA Sequencing Services,) and quantified spectrophotometrically using a Nanodrop ND-1000. Sequences were manually aligned and identified in GenBank using NCBI BLAST.

Standard curves were created for *Taq*man primer-probe sets (Integrated DNA Technologies, Coralville, IA) summarized in Table 1. Primer and probe concentrations were optimized and melt curves were generated to ensure correct PCR reaction conditions. For environmental samples, a minimum of two reactions and three ten-fold dilutions were run on a Bio-Rad iQ5 Real Time PCR Detection System.

Table 6.1.1.1. *Taq*Man primers and probes used to quantify *Dehalococcoides* 16S, total Bacteria 16S and RDase genes.

Target Gene	Primer/Probe	Reference
Universal Bacteria 16S (Bac)	1055F, 1392R, 1115T	(Harms et al., 2003; Lane, 1991; Ritalahti et al., 2006)
Dehalococcoides 16S (Dhc)	1200F, 1271R, 1240T	(He et al., 2003)
*tce*A RDase	1270F, 1336R, 1294T	(Johnson et al., 2005)
*vcr*A RDase	1022F , 1093R, 1042T	(Ritalahti et al., 2006)
*bvc*A RDase	925F, 1017R, 977T	(Ritalahti et al., 2006)

Each 25 µl reaction contained 12.5 µl iQ Supermix (Bio-Rad Laboratories, Hercules, CA), 100 nM probe, and 300 nM primers (except Bac reactions which used 900 nM reverse primer) and 5µl DNA. PCR conditions were as described in the literature for the specific primer-probe set except Bac reactions which were: an initial 95°C denaturation (3 min); 40 cycles of 95°C (15 s), 52°C (30 s), and 72°C (90 s); and a final extension step at 72° C (10 min). A minimum of three reactions and three ten-fold dilutions were run for each sample on a Bio-Rad iQ5 Real Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). For each Q-PCR run, a negative (no template) control was used to test for false positives or contamination. The presence of nonspecific products or primer-dimers was confirmed by observation of a single melting peak in a melting curve analysis using the iCycler iQ5 optical system software v1.0. Only standard curves with regression coefficients $R^2 > 0.98$, and amplification efficiencies $E > 0.80$, were used. Gene copy numbers were converted to cells on a 1:1 basis for the *Dehalococcoide*s 16S gene (20, 54) and 500:1 for the *E. coli tce*A clone (www.invitrogen.com). Total bacteria were presented as 16S gene copy numbers. Tests of significance were performed as paired Student's t-tests in SigmaPlot 10.0 software (San Jose, CA).

6.1.2. Effect on Quantification of *Dehalococcoides* spp. Dechlorination Genes

Sample Collection

Sediment cores were collected using a Geoprobe (Salina, KS) from the 1.5 - 2.2 m depth of an aquifer in Elkton, MD. The site is an active rocket manufacturing site that has not been previously microbially characterized. Previous observations show that rapid degradation of 1,1,1-trichloroethane to 1,1-dichloroethane to chloroethane is occurring at this site. Particle size analysis was performed by the hydrometer method (Dept of Soil Science, NC State University). The Elkton sediment was a loamy sand composed of 83 % sand, 12 % silt and 5% clay. Cores were air dried and sieved to remove coarse material (2.3 mm; 8 mesh) and then homogenized by triple sieving.

Soil Sample Inoculation

Prior to DNA extraction, soil was inoculated with two cultures: KB-1 and *E. coli tceA*. KB-1 is a commercial dechlorinating culture composed of *Dehalococcoides spp.* and was provided from an active chemostat culture courtesy of SiRem, Inc (Guelph, Ont., Canada). The *E. coli tceA* inoculum was a clone containing the *tce*A gene, which was also used to create the

plasmid standard curve used in the *tce*A Q-PCR reactions. The culture was grown overnight in LB media with ampicillin and harvested in log phase. Both cultures were washed twice in sodium phosphate buffer, combined and inoculated to 100 g of homogenized soil at a moisture content equal to the pre-determined water-holding capacity of the soil. The inocula were homogenized into the soil by vortexing for 5 min. The inoculated soil was split into 0.25, 1 or 10 g samples in triplicate. Aliquots of each inocula were reserved and DNA was extracted in triplicate from each culture using four extraction methods. For the *E. coli tceA* inoculum, viable plate counts were performed in duplicate by diluting 40 μl of the inocula in 0.9% NaCl, plating on LB medium with ampicillin and incubating at 37 °C for 48 h.

DNA Extraction Methods

Three of the commercial kits most frequently used in the literature on dechlorinating bacteria were tested: Mo Bio Power Soil (Mo Bio Laboratories, Carlsbad, CA), FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH), and QiaAMP DNA Blood Kit (Qiagen, Inc. Valencia, CA). The lab-developed method utilizing an $Al_2(SO_4)_3$ pre-treatment was as follows: Humic substances were precipitated prior to cell lysis by addition of 400 μl of a low pH aluminum sulfate solution (100 mM $Al_2(SO_4)_3$ and 100 mM NaH_2PO_4; pH 6.0) (Dong et al., 2006) to each 0.25 g sample. For larger samples, solution volumes were scaled accordingly. The soil suspension was vortexed briefly, the pH was measured using an Accumet pH microelectrode (Fisher Scientific), and adjusted down to pH 6.0 with HCl as needed. To each sample was added: 0.25 g sterile, baked (200 °C; 3 h) 100 μm zirconium beads and 400 μl lysis solution (100 mM NaCl, 500 mM Tris, 10% w:v sodium dodecyl sulfate, 1% sodium pyrophosphate; pH 9). The pH of each sample was measured again and raised to 9.0-9.5 with NaOH if necessary. Cells were lysed by bead beating (BioSpec Products, Inc. Bartlesville, OK) for 1 min at maximum speed. Tubes were centrifuged (5 min; 16,100 X g) to pellet cell debris. The transferred supernatant was mixed with a 0.5 volume of 7.5 M ammonium acetate and incubated on ice for 10 min. Tubes were centrifuged (5min; 16,100 X g) and supernatant was transferred. DNA was precipitated with 1 volume of 100% isopropanol (10 min), centrifuged (10 min; 16,100 X g), resuspended in 1 ml 70% ethanol, and centrifuged again (3 min; 16,100 X g). Air-dried pellets were dissolved in TE buffer (pH 8.0).

For the Mo Bio method and per manufacturer's instruction, the Mo Bio PowerSoil was used for the 0.25 g samples and the Mo Bio PowerMax Soil for 1 and 10 g samples. The FastDNA kit is currently recommended for samples less than 1 g and so this method was only used for the 0.25 g samples. At the time of testing, there was no Qiagen kit designed for soil so a modified protocol provided by the manufacturer using the QiaAMP DNA Blood kit was used.

The effect of cell separation steps on recovery of target genes was tested using two cell separation pre-extraction methods and a direct extraction. In the first, sediment was vortexed in sodium phosphate buffer, strained through 70 μm cheesecloth and the strained solution was centrifuged to pellet detached cells (Staley et al., 2011). DNA was then extracted from the resulting pellet using the $Al_2(SO_4)_3$ method. In the second method, sediment was sonicated (30 s) in a solution of deionized water and 1% sodium pyrophosphate. The supernatant solution was filtered through a 0.22 μm membrane filter and DNA was extracted from the filter using the $Al_2(SO_4)_3$ method. Direct extractions were performed using the $Al_2(SO_4)_3$ method. Extractions were performed on triplicate 1 g samples.

Percent Recovery of Dhc genes from Vegetative Dehalococcoides Cells and tceA Genes from Extracellular E. coli tceA DNA

Soil was spiked with the KB-1 culture at a range of fractions: 0% (no addition), 5, 10, 25, 50 and 100% of the starting cell concentration. To estimate the overall extraction efficiency and to correct for variation due to extraction error, the same volume of *E. coli tceA* culture was added to each sample. The gene concentrations recovered post-extraction were normalized to those detected by Q-PCR in the inoculum culture. In the extracellular DNA experiment, DNA extracted from a pure culture of the *E. coli tce*A clone was added to sediment and directly extracted (triplicate samples of 0.25 g) using the $Al_2(SO_4)_3$ method. Four 100-fold DNA dilutions ranging from 3×10^5 to 3×10^{11} gene copies per sample were added.

6.1.3. Effect on Microbial Community Structure

T-RFLP

16S rRNA genes were amplified from each sample replicate using 6-carboxyfluoroscein (FAM)- labeled primers Bac-8F (AGAGTTTGATCCTGGCTCAG) forward and Bac-1492R (GGTTACCTTGTTACGACT) reverse for Bacteria (Klappenbach et al., 2000) and 6-carboxyfluoroscein (FAM)- labeled primers Arc-109F (ACKGCTCAGTAACACGT) forward and Arc-915R (GTGCTCCCCCGCCAATTCCT) reverse for Archaea (Banning et al., 2005). The PCR reaction was carried out in 50 µl volumes consisting of 2× FailSafe PCR PreMix F buffer (Epicentre Technologies, Madison, Wis.), 0.875 unit of FailSafe PCR enzyme mix and 1ng to 5ng of DNA. Thermocycler conditions were: an initial 95°C denaturation (3 min); 30 cycles of 95°C (60 s), 56°C (60 s), and 72°C (90 s); and a final extension step at 72° C (10 min). PCR products were visualized on a 1.0% agarose gel with ethidium bromide staining, purified using Wizard PCR Preps DNA Purification System (Promega, Madison, Wis.) and quantified on a Nanodrop 1000 Spectrophotometer. Purified PCR products (350 ng) for one replicate from each sample were digested in 20 ul reactions using *Hha*I, *Rsa*I and *Msp*I for Bacteria and *Alu*I, *Taq*I and *Bfa*I for Archaea according to manufacturer directions (New England Biolabs, Ipswich, Mass.). The restriction enzyme which produced the highest number of T-RFs for Bacteria (*Hha*I) and Archaea (*Alu*I) was then used on all three replicates for each sample. After digestion, unwanted nucleotides were removed using the Qiagen Nucleotide removal kit (Qiagen, Valencia, Calif.). Purified digested samples were then sent to the NCSU Genomics Sciences Laboratory (GSL) where the terminal restriction fragments (T-RFs) were separated via capillary electrophoresis via a 3730xl DNA Analyzer (Applied Biosystems) using MapMarker size standards labeled with X-Rhodamine (BioVentures, Murfreesboro, Tenn.).

T-RFLP Data Processing and Statistical Analysis

T-RFLP electropherograms were examined using Peak Scanner software (version 1, Applied Biosystems, Foster City, CA) and a peak threshold of 15. T-RF peaks were analyzed using the automated alignment and binning method provided by R program version 2.8.1 and data for entry into R program (R Development Core Team, 2008) was prepared using the binary version of ActivePerl (www.activestate.com/Products/ActivePerl). Peak areas less than 1% of total area were eliminated and remaining peak areas re-calculated. The data sets for each replicate were then imported into Community Analysis Package (CAP) software (Henderson and Seaby, 2007).

T-RFLP community profiles were analyzed by non-metric Multi-dimensional scaling (MDS) using the Bray-Curtis coefficient. MDS plots are typically in two or three dimensions depending on the dimension that provides the lowest stress function or 'goodness of fit' (Rees at al., 2004) with a stress function less that 0.1 indicating the most accurate ordination representation (Clarke, 1993). A PCA starting position within the CAP software was used to find the minimum stress function (Kruskal, 1964; Kruskal and Wish, 1977) and MDS plots were displayed in either two or three dimensions depending on which configuration produced the lowest stress function. Statistical similarity relationships between methods were determined using Analysis of Similarity (ANOSIM) (Clarke, 1998; Clarke 1993). Percent similarity and the contribution of each terminal fragment to the observed similarity (or dissimilarity) between and within groups in pairwise comparisons was determined using SIMPER (Clarke and Warwick, 2001; Rees et al., 2004; Wolsing and Prieme, 2004). In this study, the "within group" function of the algorithm was used to test the range in variation between presumably homogeneous replicates. The "between group" analysis provided percent dissimilarity between each method.

6.2. Effect of Storage and Handling Conditions on Quantitative Gene Detection and Microbial Community Structure

6.2.1. Site Selection and Sampling

In contrast to the sample size and extraction method experiments, this experiment was conducted in the field and molecular assays were used to detect native dechlorinating bacteria and gene levels. The experiment was performed at a chlorinated solvent-contaminated site in Lumberton, NC. Groundwater was collected from multiple recovery and monitoring wells at the Lumberton site. Analysis by gas chromatography identified areas of the site with significant concentrations of perchloroethylene and trichloroethylene as well as biological dechlorination daughter products, vinyl chloride and ethene. In addition, DNA was extracted from the groundwater and *Dehalococcoides* 16S rRNA genes were detected in concentrations up to 10^6 cells/ml. Relevant geochemical and biological data are presented in Table 6.2.1.

In an exploratory soil test of the Lumberton site, three continuous 2 inch cores were collected with a Geoprobe from the anaerobic, 12-32 ft depth of three locations where both *Dehalococcoides* 16S rRNA genes and ethene were detected in the groundwater. The locations selected were proximate to MW-1, MW-15 and RW-2. Exploratory coring near MW-1 and MW-15 found shallower water tables and more oxic conditions than the area near RW-2. The aquifer material in the RW-2 zone was selected for the storage experiments. The aquifer material in this zone contained both sandy and silty fractions overlaying an impermeable clay layer at 25 ft. Particle size analysis was performed at all depths and is reported in Table 6.2.3.

Table 6.2.1. Geochemical and biological data from the Lumberton site. Three recovery wells (RW) and five monitoring wells (MW) were sampled. DNA was extracted from groundwater using the $Al_2(SO_4)_3$ method and *Dehalococcoides* (Dhc) levels were measured by qPCR. Gas chromatography was used to measure levels of pechloroethene (PCE), trichloroethene (TCE), cis-dischloroethene (c-DCE), vinyl chloride (VC), ethene, chloride, and sulfate. Dissolved oxygen (DO) and total organic carbon (TOC) were measured by colorimetric methods.

	Screen ft bgs	Dhc cells/ml	DO mg/L	PCE ug/L	TCE ug/L	c-DCE ug/L	VC ug/L	Ethene ug/L	Methane ug/L	Chloride mg/L	Sulfate mg/L	TOC mg/L
RW-1	10-20	ND	0.3-0.4	1191	1585	4146	214	24	4499	11.4	20.8	7
RW-2	10-20	2.5e 6	0.4-0.6	641	1877	10784	920	159	4198	33.8	25.9	12
RW-3	10-20	1.1e 5	1	2	11	1668	218	32	2108	14.4	16.3	8
MW-11	8-18	1.3e 5	>1	2303	884	559	17	9	4968	2.5	< 0.5	10
MW-15	28-32	8.6e 5	>1	3	2	161	117	37	6658	3.8	20.4	12
MW-16	28-32	1.3e 5	>1	1	1	750	31	1	14101	3.6	12.9	13
MW-1	8.6-32	ND	>1	2953	762	331	11	7	5331	7.3	< 0.5	3
MW-2	8-22	ND	1	20	25	846	133	30	6194	9.5	53.6	3

Table 6.2.2. Particle size analysis of a continuous soil core collected from the Lumberton site.

	Sand	Silt	Clay	
	2.00-.05mm	.05-.002mm	<.002mm	USDA
Depth (ft)	%	%	%	Class.
12	98.6	0.6	0.8	sand
13	98.7	1.3	0.0	sand
14	98.9	1.1	0.0	sand
15	96.9	2.3	0.8	sand
16	84.8	5.3	9.9	loamy sand
17	85.0	5.2	9.8	loamy sand
18	88.0	4.7	7.3	loamy sand
19	86.6	4.7	8.7	loamy sand
20	86.4	6.3	7.3	loamy sand
21	86.0	5.7	8.4	loamy sand
22	89.4	3.9	6.6	sand
23	89.4	2.3	8.3	sand
24	87.7	9.0	3.3	sand
25a	89.5	6.9	3.6	sand
25b	34.9	45.8	19.3	loam
26	56.9	34.7	8.4	sandy loam
27	54.2	37.6	8.2	sandy loam
28	56.9	36.8	6.3	sandy loam
29	66.4	28.2	5.4	sandy loam

6.2.2. Groundwater Storage Experiment

Groundwater was collected from RW-2 into 12 sterile 1-quart mason jars, stored on ice and immediately returned to the lab (within 2 hours). Groundwater was mixed in a sterile 15 L carbuoy and returned to the original mason jars. As a control, 1.5 L of groundwater was immediately filtered onto 6 membrane filters (250 ml each) and frozen at -80º C. To simulate customary shipping times, the remaining groundwater was stored on ice for 24 hours. At 24 hours, groundwater was either filtered (250 ml) onto a 0.22 um membrane filter or left unfiltered. Filters or unfiltered groundwater was stored at either room temperature or 4º C for 2 days. In addition, unfiltered groundwater was stored at 4º C for 14 days. At the specified time, unfiltered groundwater was collected on membrane filters and DNA (3) and RNA (3) extractions were performed for each of the 8 treatments.

Nucleic Acid Extraction

DNA was extracted from soil (0.25 g) or groundwater filters using the $Al_2(SO_3)_4$ method that has been previously described. RNA was extracted by the following method: Samples were vortexed at medium speed for 1 minute with 0.5 g baked, zirconium beads, 250 ul of 50 mM sodium acetate/10 mM EDTA buffer (pH 5.1), 50 ul of 20% SDS and 1 ml phenol. Samples

were lysed by bead beating for 2 minutes, incubating at 60°C for 10 minutes and bead beating again for 2 minutes. Samples were centrifuged at 5,000 rpm for 5 min and the supernatant was transferred to a clean tube and placed on ice. The solid phase was rinsed with 200 μL pH 5.1 buffer, vortexed briefly, centrifuged and the supernatant was pooled with the first supernatant. The solution was centrifuged at 14,000 rpm for 6 minutes and the top aqueous phase was transferred to a clean tube. One volume of buffer was added to the spent phenol solution, vortexed for 10 seconds and centrifuged for 6 minutes at 14,000 rpm. The top aqueous layer was pooled with the first aqueous solution. One volume of phenol: chloroform: isoamyl alcohol was added to the solution, vortexed for 10 s, centrifuged at 14,000 rpm for 6 min, and transferred to a new tube. This step was then repeated using one volume of chloroform: isoamyl alcohol. Nucleic acids were precipitated by addition of 0.5 volume of 7.5 M NH_4Ac and 2 volumes of cold absolute ethanol. After incubation at -20°C for 1 hr, samples were centrifuged at 14,000 rpm for 30 min. The supernatant was removed and the RNA pellet was washed with 2 ml of cold 70% ethanol. The solution was centrifuged at 14,000 rpm for 10 minutes and the ethanol decanted. RNA was re-suspended in 100 uL of sterile, RNase-free water and treated with with DNase (Qiagen, Inc.)

Molecular Methods

Q-PCR assays targeting the universal bacterial 16S rRNA gene, *Dehalococcoides* 16S rRNA gene and the *tce*A, *vcr*AB, and *bvc*A genes were performed on DNA extracted from groundwater,a s previously described. T-RLFP using 16S rRNA gene for the Bacteria was performed as previously described.

7. RESULTS AND ACCOMPLISHMENTS

7.1. Effect of DNA Extraction Method and Sample Mass on Quantification of *Dehalococcoides* spp. Dechlorination Genes and Microbial Community Structure

7.1.1. PCR, Cloning and Sequencing of DNA Standards

DNA was extracted from the SDC-9 and KB-1 cultures using the Mo Bio Ultraclean™ Microbial DNA Isolation Kit. Target amplicons were created by PCR amplification of the isolated DNA (Figures 7.1.1.1- 4).

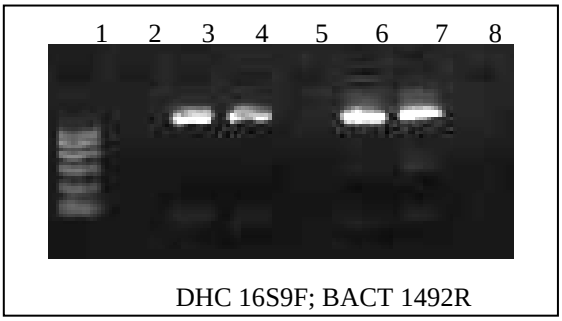

Figure 7.1.1.1. PCR amplification of 16S rRNA genes from KB-1 and SDC-9 using a *Dehalococcoides* – specific primer (DHC16S9F) and a universal Bacterial primer (BACT 1492R). Lane 1: DNA marker. Lanes 3-4: KB-1 template. Lanes 6-7: SDC-9 template. Lane 8: negative control.

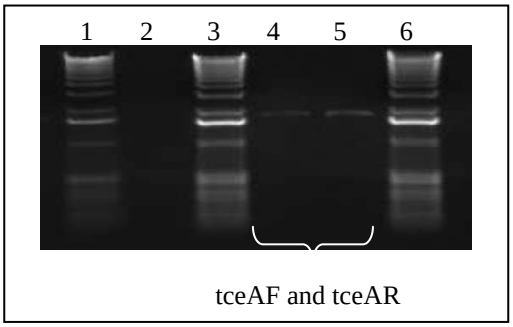

Figure 7.1.1.2. PCR amplification of *tce*A gene from SDC-9 using TCD RDase primers 797F and 2490R. Lane 1: DNA marker. Lanes 4-5: SDC-9 template. Lanes 1, 3, 6: negative control.

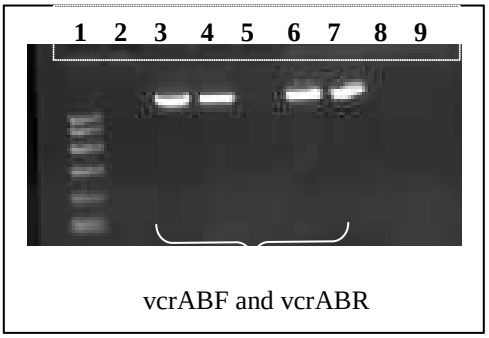

Figure 7.1.1.3. PCR of *vcr*A RDase gene, amplified using primers vcrABF and vcrABR. Lane 1: DNA marker. Lanes 3-4: KB-1 template. Lanes 6-7: SDC-9 template. Lane 9: negative control.

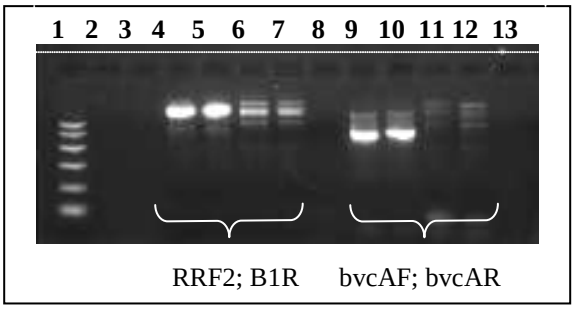

Figure 7.1.1.4. Nested PCR of *bvc*A RDase gene, amplified using degenerate primers RRF2 and B1R and primers bvcAF and bvcAR. Lane 1: DNA marker. Lanes 4-5 and 9-10: KB-1 template. Lanes 6 -7 and 11-12: SDC-9 template. Lane 13: negative control.

Sequences were 100% identical to the 16S region of *D. ethenogenes* 195 and to the *tce*A , *vcr*A, and *bvc*A genes respectively and had no mismatches in the target sites of qPCR primers and Taqman probes.

Quantitative PCR assays targeting total Bacteria (Bac), *Dehalococcoides* species (Dhc), the TCE reductase (*tce*A) and two VC reductase functional genes (*vcr*A and *bvc*) identified in different *Dehalococcoides* species were developed. Plasmid DNA was serially diluted and Q-PCR standard curves were generated for the four assays (Figures 7.1.1.5 – 7.1.1.9). The standard curves show linearity over 5 to 7 orders of magnitude of gene copy number.

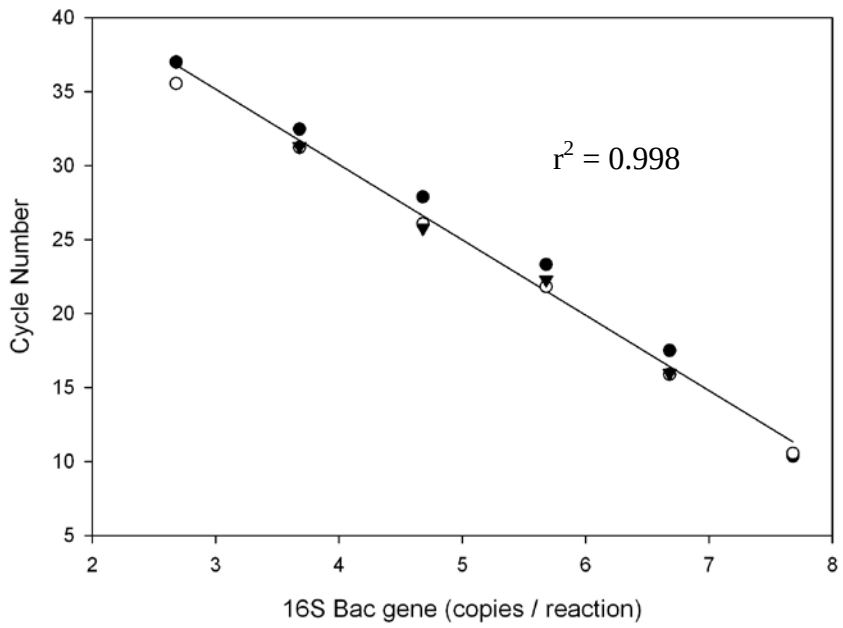

Figure 7.1.1.5. *Taq*man standard curve for Bacterial 16S rRNA gene (log copy number on x-axis). Target concentrations were linear between 4.8 x 10^2 and 4.8 x 10^7 copies/reaction although still detectable below this range. All reactions were performed in triplicate.

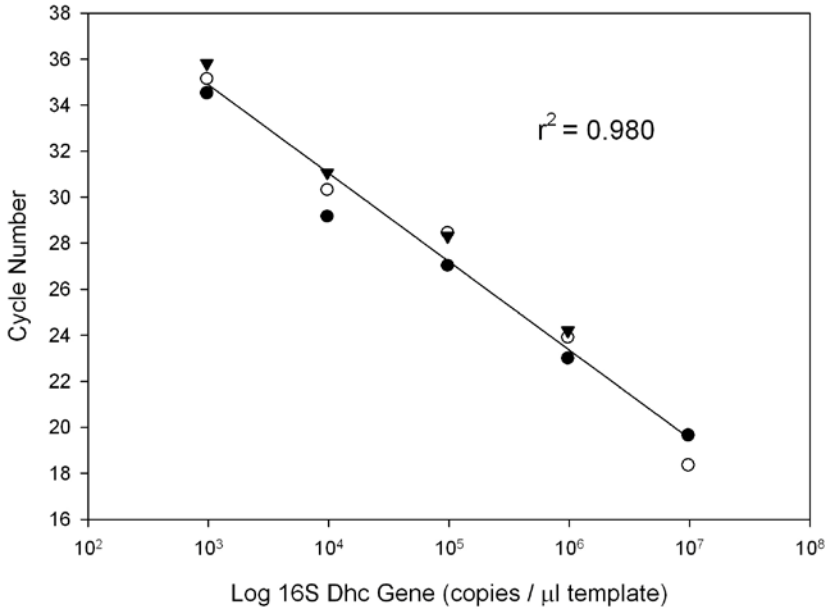

Figure 7.1.1.6. *Taq*man standard curve for *Dehalococcoides* 16S rRNA gene. Target concentrations were linear between 4.8 x 10^3 and 4.8 x 10^8 copies/reaction although still detectable below this range. All reactions were performed in triplicate.

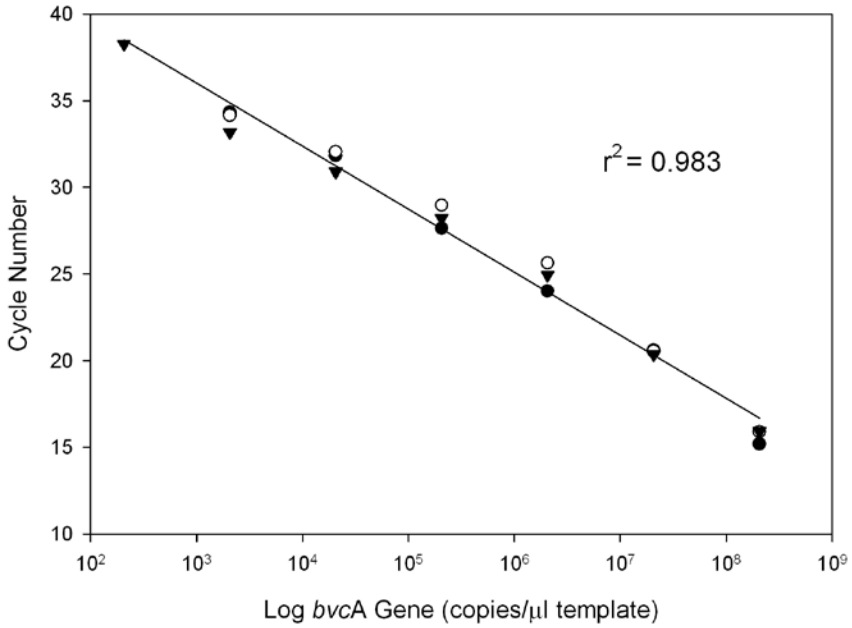

Figure 7.1.1.7. *Taq*man standard curve for vinyl reductase gene *bvcA*. Target concentrations were linear between 2.1 x 10^2 and 2.1 x 10^8 copies/reaction. All reactions were performed in triplicate.

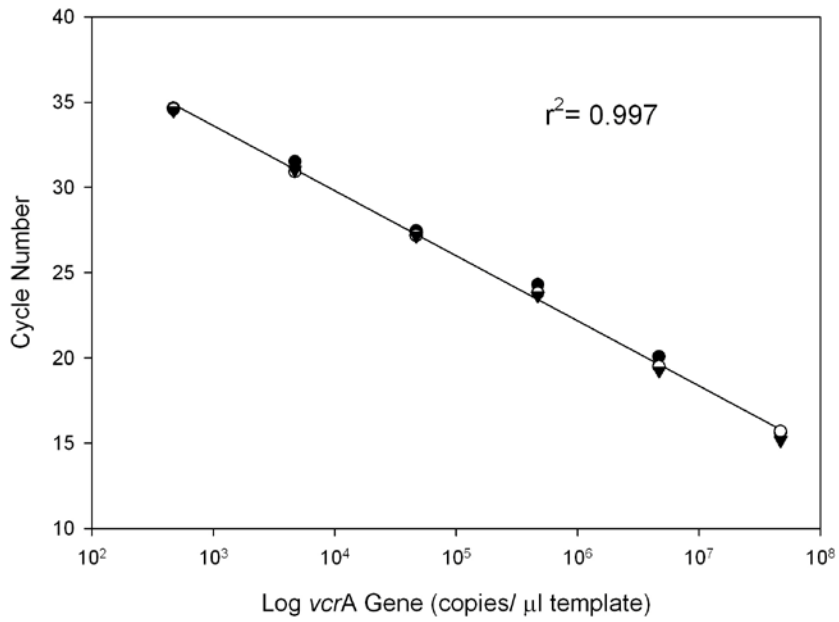

Figure 7.1.1.8. *Taq*man standard curve for vinyl reductase gene *vcrA*. Target concentrations were linear between 4.7 x 10^2 and 4.7 x 10^7 copies/reaction. All reactions were performed in triplicate.

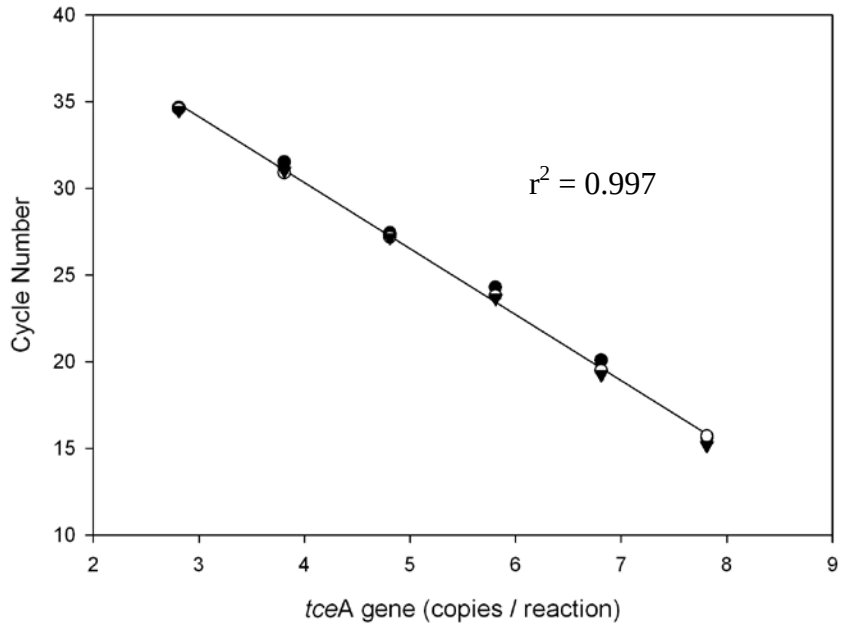

Figure 7.1.1.9. *Taq*man standard curve for vinyl reductase gene *tceA* (log copy number on x-axis). Target concentrations were linear between 4.7 x 10^2 and 4.7 x 10^7 copies/reaction. All reactions were done in triplicate.

Quantitative PCR assays were tested on two commercial bioaugmentation cultures: SDC-9 and KB-1. The KB-1 culture was composed primarily of *Dehalococcoides* species and had high copy numbers of the *bvc* and *vcr* genes but no detections of the *tce*A gene. The SDC-9 culture contained a comparable number of *Dehalococcoides* species, had similar copy numbers of *vcr* gene as KB-1 culture and no detectable copies of the *bvc* gene.

7.1.2. Quantitative PCR Assays Tested on Soil and Comparison of Two Commercial DNA Extraction Kits

Quantitative assays were tested on subsurface sediment from a site in Charleston, SC that is contaminated with chlorinated solvents. Soil cores were composed of sandy aquifer material interspersed with clayey bands. Sandy and clayey fractions were homogenized in a Waring blender. Clay samples were further separated from sand material by centrifugation at 10,000 rpm for 5 minutes. The upper clay fraction was removed and used for DNA extractions. DNA was extracted from homogenized clay or sand material using either a MoBio PowerSoil (Mo Bio Laboratories, Carlsbad, CA) or QiaAMP DNA Stool Extraction Kit (Qiagen, Inc. Valencia, CA). Quantitative PCR reactions were run in triplicate on triplicate dilutions of triplicate samples.

While the Qiagen method had a higher DNA yield than the MoBio method (10 and 2 ng/µl respectively), quantitative detection of Bacteria was highest using the MoBio kit (Table 7.1.2.1). No *Dehalococcoides* 16S rRNA or vinyl chloride reductase genes were detected in either fraction using the Qiagen method and total Bacteria were also 10x lower than those detected using the MoBio extracted DNA. The higher yield but lower quantitative detection generated by the Qiagen method suggests that it was less effective at removing PCR inhibiting compounds than the MoBio method. In addition, *Dehalococcoides* 16S rRNA and *vcr*A genes were detected more frequently and in far higher concentrations in clay than in sand samples using the MoBio method.

Table 7.1.2.1 Total Bacteria 16S rRNA (Bac), *Dehalococcoides* spp. 16S rRNA (Dhc), and VC reductase genes *bvcA* and *vcrA* detected by qPCR using two different DNA extraction methods. Copy numbers are per gram of aquifer sediment.

	Mo Bio Power Soil		Qiagen	
	Sand	Clay	Sand	Clay
Bac	1.25×10^8	1.56×10^8	2.29×10^7	4.80×10^7
Dhc	5.11×10^5	2.11×10^8	None detected	None detected
bvcA	None detected	None detected	None detected	None detected
vcrA	7.52×10^7	3.74×10^8	None detected	None detected

7.1.3. Effect of DNA Extraction and Sample Mass on Quantification of *Dehalococcoides* spp. Dechlorination Genes

Effect of Sample Mass and Extraction Method on Nucleic Acid Yields and Purity

The highest yields of total nucleic acids extracted from all three sample masses were obtained using the $Al_2(SO_4)_3$ method, which extracted 23 times more than the Mo Bio method and 29 times more than the Qiagen method (Fig. 7.1.3.1). Yields with the FastDNA method were statistically lower ($P<0.05$) than the $Al_2(SO_4)_3$ method for 0.25 g samples. For the KB-1 and *E. coli tce*A inocula, the $Al_2(SO_4)_3$ method also extracted the highest levels of nucleic acids of the four methods. Sample mass had no effect on yield for the Mo Bio method, but yields from the 10 g samples were significantly different from the other masses for the $Al_2(SO_4)_3$ method. The absorbance spectra of nucleic acids extracted using the $Al_2(SO_4)_3$ method, both with and without cell separation steps, showed peaks at the absorbance wavelength of nucleic acids (260 nm) (Fig. 7.1.3.2). Conversely, impurities (above and below the 260 nm wavelength) were apparent in all of the kits except the Mo Bio MAXI kit (1 and 10 g samples, data not shown.)

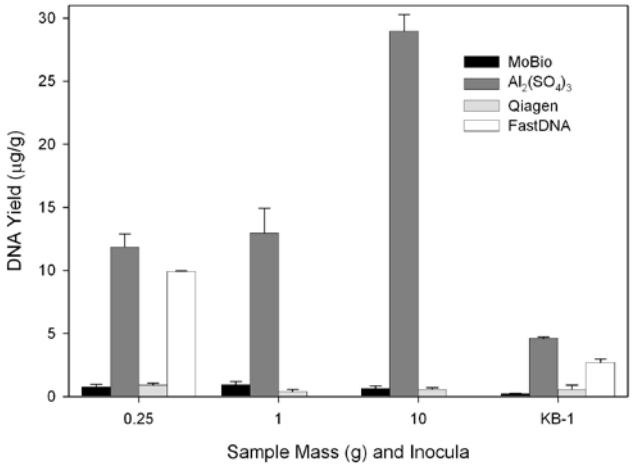

Figure 7.1.3.1. DNA yields from four extraction methods for three sample masses and from the KB-1 inoculum. DNA yields were determined from the inoculum added and are expressed as the amount of KB-1 DNA added per g of sediment. Error bars denote SD, n = 3 extracted samples.

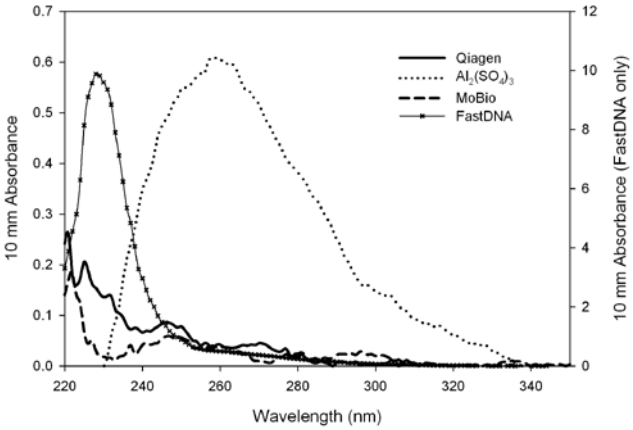

Figure 7.1.3.2. Sample DNA spectra from sediment inoculated with KB-1 and *E. coli tce*A cultures and extracted (0.25 g) using four methods: Qiagen, Mo Bio Powersoil, FastDNA, and the $Al_2(SO_4)_3$ method. Due to high contamination at the 230 nm wavelength, FastDNA spectra were plotted on the secondary Y-axis.

Effect of Sample Mass and Extraction Method on Gene Quantification by Q-PCR.

In the uninoculated sediment, no *Dehalococcoides* 16S rRNA or *tce*A genes were detected by the $Al_2(SO_4)_3$ and Mo Bio DNA extraction methods (n = 48 reactions). When the sediment was inoculated with KB-1 culture and with the *E. coli tceA* clone, the $Al_2(SO_4)_3$ method resulted in the highest densities of the two target genes (Dhc and *tce*A) for all sample masses. Results of the $Al_2(SO_4)_3$ method for all sample masses were not statistically different from each other, and were within the same order of magnitude as their respective inocula (Figs. 7.1.3.3 and 7.1.3.4). Also, the concentration of live *E. coli tce*A present in the inoculum by viable plate counts was 2.9 x 10^9 cells/g, which is similar to the concentrations detected in the inocula using the $Al_2(SO_4)_3$ method (3.3 x 10^9 cells/g).

Of the commercial kits, the FastDNA method detected the highest concentrations of the *Dehalococcoides* 16S rRNA gene in the 0.25 g samples and the Mo Bio 1 and 10 g samples detected the highest concentrations of the *tce*A gene but both were lower than the $Al_2(SO_4)_3$ method. Mo Bio had higher gene concentrations using 1 or 10 g (Mo Bio MAXI) than using 0.25 g (Mo Bio) samples for detection of both genes. The Qiagen 1 and 10 g samples produced no amplifiable *Dehalococcoides* genes (n = 9 reactions for each sample size) and very low copy numbers of the *tce*A gene.

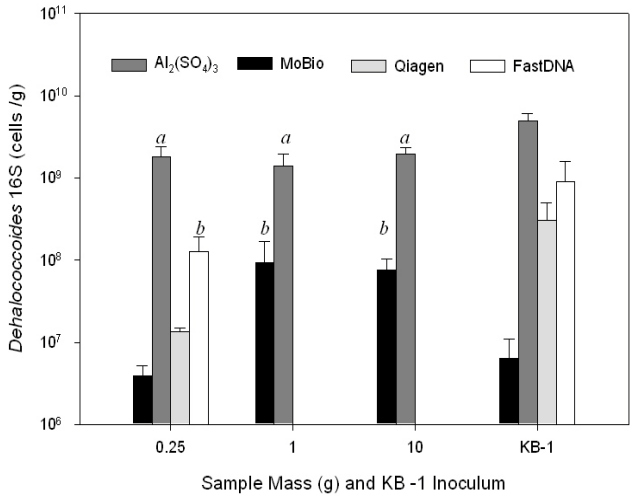

Figure 7.1.3.3. Concentrations of *Dehalococcoides* 16S rRNA genes in cells/g of sediment and in the KB-1 inoculum. For the inoculum, cell numbers were normalized to the amount inoculated per g of sediment. Bars with same letters denote no statistical differences. All other comparisons were significant at the 0.05 level. Error bars denote SD, n = 3 extracted samples with 3 Q-PCR reactions per sample.

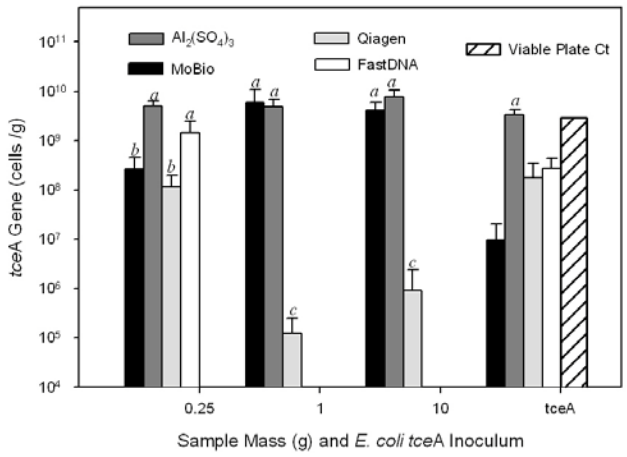

Figure 7.1.3.4. Concentrations of the *tceA* gene in cells/g of sediment and in the *E. coli tce*A inoculum. For the inoculum and viable plate counts, cell numbers were normalized to the amount inoculated per g of sediment. Bars with same letters denote no statistical differences. All other comparisons were significant at the 0.05 level. Error bars denote SD, n = 3 extracted samples with 3 Q-PCR reactions per sample.

Quantifying Recovery of Intracellular and Extracellular DNA using the $Al_2(SO_4)_3$ method

A live KB-1 culture was inoculated at a range of cell concentrations (0 to 4.0 x 10^9 cells/g) and a live *E. coli tceA* inoculum was added at a fixed concentration to allow for standardization of the

extraction efficiency between samples. Levels of recovered *Dehalococcoides* cells increased linearly with inoculum addition (R^2=0.98) and the average recovery was 20%.

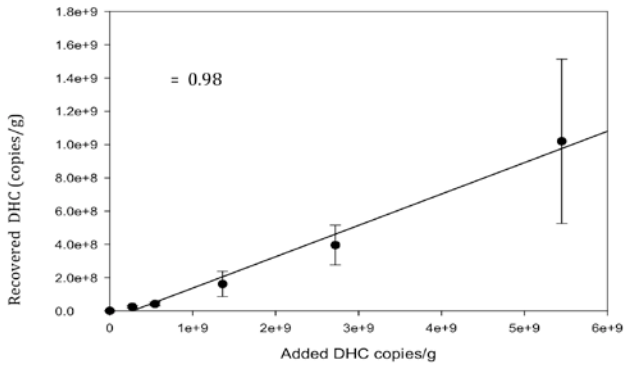

Figure 7.1.3.5. Recovery of added *Dehalococcoides* in soil

When extracellular DNA (extracted from a pure culture of *E. coli tce*A clone) rather than vegetative cells were inoculated into sediment, the $Al_2(SO_4)_3$ method removed almost all of the extracellular DNA while yielding high amounts of total DNA (39.8 ng/µl). No *tce*A genes were detected at additions of 3 x 10^7 copies or lower. For additions of 3 x 10^9 and higher, recovery was 0.1% or less.

7.1.4. Effect of Sample Mass and Extraction Method on Microbial Community Structure

The effects of sample mass and extraction method on the Shannon-Weiner Diversity Index were assessed using T-RFLP, and the results are shown in Figure 7.1.4.1. The highest diversity index was obtained for the aluminum sulfate method (10 g sample), and the lowest for the Qiagen method (0.25 g sample). Aside from the Qiagen and FastDNA methods, which gave the lowest Shannon-Weiner Diversities, the other methods and sample sizes yielded comparable indices. Another way of looking at this is to compare the individual T-RFs or phylotypes from each method. Such an analysis (data not shown) shows that all the dominant phylotypes were present in each method/sample size. The differences were in the phylotypes that contributed very small proportions to the overall community. This suggests that potentially all the methods/sample sizes (except for the Qiagen and FastDNA, which had the lowest diversities) will reveal the dominant phylotypes within the microbial community.

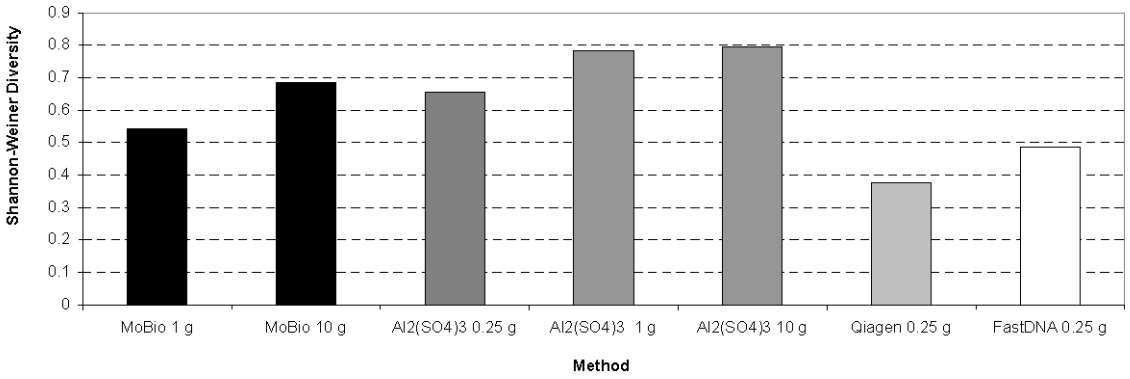

Figure 7.1.4.1. Shannon-Weiner Diversity Indices of various sample mass/extraction methods for Bacteria.

Non-metric multi-dimensional scaling with the Bray-Curtis similarity, which compares the microbial community similarity matrix based on presence/absence as well as abundance of specific T-RFs, shows that the FastDNA method was least similar to all other methods (Figure 7.1.4.2). The T-RF matrices for the Mo Bio, $Al_2(SO_4)_3$, and Qiagen 0.25 g methods formed tight clusters that separated from those of the KB-1 and uninoculated soil in three dimensional space. Qiagen 1 and 10 g samples were omitted because PCR with T-RFLP primers resulted in no amplification or generated PCR artifacts. While the FastDNA method clustered more closely to the KB-1 inoculum, this method did not detect more T-RFs present in the KB-1 inoculum than other methods.

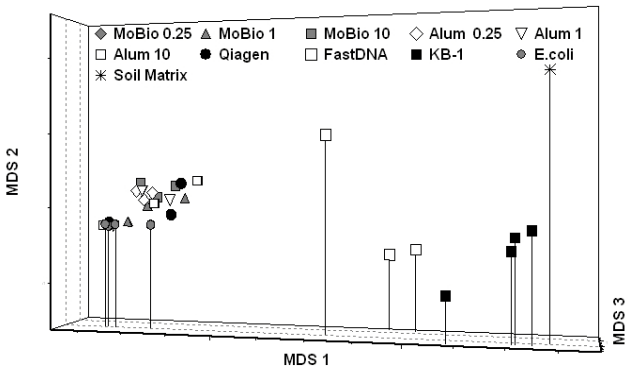

Figure 7.1.4.2. Multi dimensional scaling plot of the Bray-Curtis coefficient of bacterial T-RFLP matrices from sediment inoculated with KB-1 and *E. coli tce*A cultures and extracted using four methods: Qiagen, Mo Bio Powersoil, FastDNA, and the $Al_2(SO_4)_3$ method and three sample masses (0.25, 1.0 and 10.0 g). Qiagen 1 and 10 g samples were omitted due to PCR inhibition. KB-1 and *E. coli tce*A, and FastDNA are displayed with dropdown lines to show three dimensional scatter. The soil matrix (*) is the uninoculated soil.

The Shannon-Weiner Diversity and Bray-Curtis Similarity Indices for the Archaeal community were also determined using T-RFLP specific to Archaea (Figure 7.1.4.3). The results show that the Qiagen method had significantly higher diversity compared to the other methods. However, the Bray-Curtis similarity for the Qiagen method was the most different compared to "all methods", indicating that the Qiagen method results in a very different Archaeal community "fingerprint" (Figure 7.1.4.4). This is consistent with the analysis of the dominant and minor T-RFs (phylotypes) detected by the different methods. The Qiagen method detected many minor phylotypes not detected by the other methods, but did not detect the major phylotypes with the same abundances as the other methods. The results illustrate how the microbial community analysis can be skewed by the DNA extraction kit used. The results also show that the aluminum sulfate method yields comparable results to the other DNA extraction kits with respect to uncovering microbial community structure.

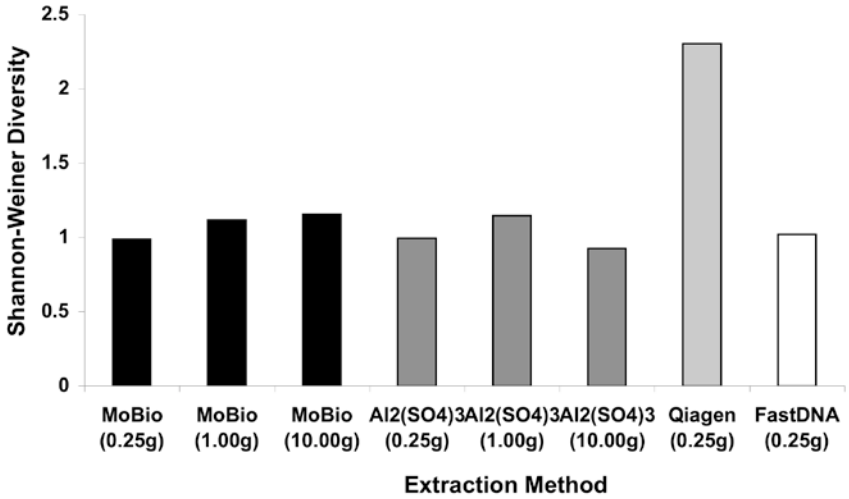

Figure 7.1.4.3. Shannon-Weiner Diversity Index for various sample sizes/extraction method combinations for Archaea

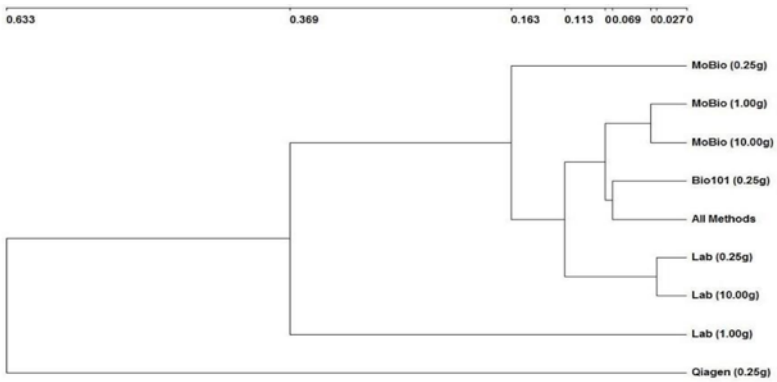

Figure 7.1.4.4. Bray-Curtis Similarity of various sample mass/extraction methods for Archaea.

All methods captured a similar number of T-RFs from the KB-1 culture with the highest being the Qiagen 0.25 samples (13 T-RFs; 61% of total T-RFs) and the lowest being the Mo Bio 0.25 g samples (7 T-RFs; 33%) (Table 7.1.4.1). Because only two replicates produced amplifiable DNA for the Mo Bio 0.25 and Qiagen 10 samples, these methods also showed a very high degree of dissimilarity within their respective replicates (93%) (Table 7.1.4.1). The Mo Bio 1 and 10 g samples, FastDNA 0.25 g, Qiagen 0.25 g and all $Al_2(SO_4)_3$ samples showed less dissimilarity within replicates (45% on average) although this level of dissimilarity is surprisingly high for homogenous replicates and instructive as to the natural scatter in community profiles. The KB-1 inocula and the FastDNA showed the greatest dissimilarity to the other methods (Table 7.1.4.1) and the highest R statistics (Table 7.1.4.2), which is consistent with MDS analysis (Fig. 7.1.4.2). With regards to the effect of sample mass by SIMPER, the Mo Bio 1 and 10 sample sizes and all three $Al_2(SO_4)_3$ sample sizes were similar to each other (>50%). The same pattern held true for ANOSIM except that the $Al_2(SO_4)_3$ 0.25 g samples were significantly different from the 10 g but with a low corresponding R statistic (0.30; not shown in table). One explanation for this may be the high replicate similarity of the 0.25 g samples (57%), which would result in a more tightly clustered similarity matrix for those samples.

Table 7.1.4.1. Similarity Percentage Analysis (SIMPER) of T-RFLP data from sediment inoculated with KB-1 and *E. coli tce*A cultures and extracted using four methods: Qiagen (Q), Mo Bio Powersoil (M), FastDNA (F), and $Al_2(SO_4)_3$ (A) and three sample masses (0.25, 1.0 and 10.0 g). The second column shows the percent of KB-1 T-RFs detected in the inoculum that were also captured by each extraction method. Dissimilarity Within shows the percent dissimilarity of the T-RFLP profiles within replicates of each method (Note: M 0.25 and Q 10 have only two replicates). Dissimilarity Between Methods describes dissimilarity between the T-RFLP profiles of paired methods.

		Percent Dissimilarity										
Methods	% KB-1 T-RFs	Within Methods	Between Methods									
			M .25	M 1	M 10	A .25	A 1	A 10	Q .25	Q 10	F .25
M .25	33	93									
M 1	50	42	65								
M 10	48	38	73	40							
A .25	57	31	72	40	36						
A 1	42	43	66	40	45	38					
A 10	48	49	65	41	46	44	39				
Q .25	61	51	64	44	50	46	41	44			
Q 10	40	93	87	79	78	81	83	79	80		
F .25	50	64	90	83	81	86	87	86	85	90	
KB-1		51	88	88	91	93	91	92	90	93	86

Table 7.1.4.2. Analysis of Similarity (ANOSIM) of bacterial T-RFLP data from sediment inoculated with KB-1 and *E. coli tce*A cultures. The R statistic for each pairwise comparison shows the dissimilarity between methods. The higher the R value the greater the replicate similarity within the method versus that between methods. Only pairwise comparisons that are statistically different in T-RFLP profiles at the 0.05 significance level are presented: Qiagen (Q), Mo Bio Powersoil (M), FastDNA (F), and the $Al_2(SO_4)_3$ (A) method and three sample masses (0.25, 1.0 and 10.0 g).

	R Statistic								
Method	M .25	M 1	M 10	A .25	A 1	A 10	Q .25	Q 10	F .25
Q .25	ns[a]	ns	0.52	ns	ns	ns			
F .25	ns	0.67	0.56	0.78	0.78	0.74	0.74	ns	
KB-1	0.71	1.00	1.00	1.00	1.00	1.00	1.00	0.82	0.80

[a] ns = not significant

7.1.5. Effect of Cell Separation During DNA Extraction

7.1.5.1 Effect on Yield and Gene Detection by Q-PCR

The $Al_2(SO_4)_3$ method was used in DNA extraction after cell separation by either a gentle method (vortexing) or a harsh (sonication) step. The sonication method produced the highest yields (100 μg/g), followed by the direct $Al_2(SO_4)_3$ method (93 μg/g) and then the vortexing (65 μg/g) method. The yields from the direct $Al_2(SO_4)_3$ method were not significantly different from either cell separation method but the cell separation methods were significantly different from each other (p = 0.05). The direct $Al_2(SO_4)_3$ extraction recovered statistically similar concentrations of total bacteria and *Dehalococcoides* 16S rRNA and *tce*A genes as the vortexing method, and of *Dehalococcoides* and *tce*A genes as the sonication method (Fig. 7.1.5.1). The sonication and vortexing methods were significantly different from each other for all target genes and for cell yield.

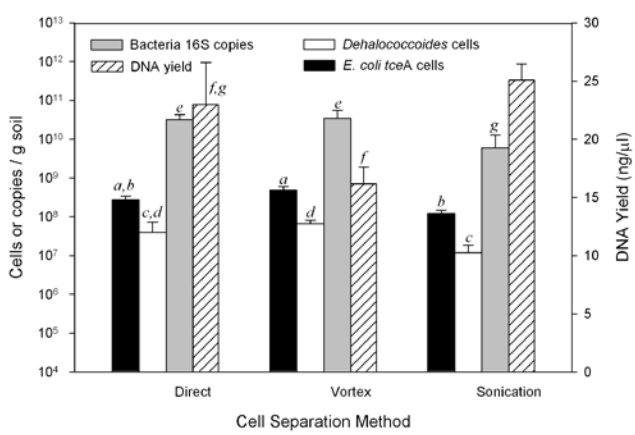

Figure 7.1.5.1. Quantitative PCR detection of *Dehalococcoides* 16S rRNA and *tce*A genes in DNA extracted from soil using the $Al_2(SO_4)_3$ method directly and with the addition of two cell

separation steps: vortexing and sonication. Bars with same letters denote no statistical differences. All other comparisons were significant at the 0.05 level. Error bars denote SD, n = 3 extracted samples with 3 Q-PCR reactions per sample.

7.1.5.2. Effect on Microbial Community Profiles

MDS analysis (Fig. 7.1.5.2) of bacterial T-RFLP similarity matrices on 1 g soil samples inoculated with KB-1 and *E. coli tce*A cultures showed little separation in two-dimensional space for the three extraction methods: direct $Al_2(SO_4)_3$ extraction and vortexing or sonication steps prior to $Al_2(SO_4)_3$ extraction. The two inocula clustered separately (shown in circles) as did the uninoculated soil. For Archaea (Fig. 7.1.5.3), the sonication method was most dissimilar, and thus showed a skewed Archaeal community fingerprint even though it gave the highest richness.

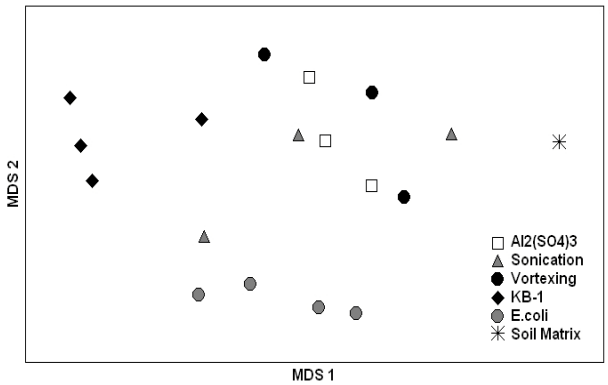

Figure 7.1.5.2. Multi dimensional scaling plot using the Bray-Curtis coefficient showing the effect of cell separation steps on bacterial T-RFLP similarity matrices. The direct $Al_2(SO_4)_3$ extraction was compared to $Al_2(SO_4)_3$ extraction with additional vortexing or sonication steps on soil inoculated with the initial KB-1 and *E. coli tce*A cultures and on uninoculated soil.

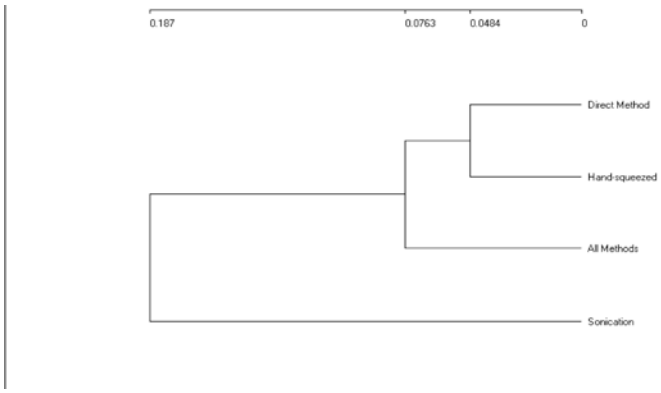

Figure 7.1.5.3. Bray-Curtis Similarity for Archaea, effect of cell separation steps.

R statistics and p values generated by ANOSIM for both bacterial and archaeal communities indicated that the community similarities between extraction methods as well as the uninoculated soil matrix were not statistically different from those within method replicates. For Bacteria, the uninoculated matrix was least similar to all inoculated samples than the inoculated samples were to each other. SIMPER analysis showed that the addition of cell separation steps had no effect on the bacterial or archaeal similarity matrices. Percent dissimilarity between the $Al_2(SO_4)_3$ method and $Al_2(SO_4)_3$ method plus the vortexing or sonication separation steps ranged from 66-84% for Archaea and 50-55% for Bacteria. In addition, the dissimilarity within sample replicates was high for bacteria (46-63%) and particularly high for Archaea (73-90%), which suggests that variation between replicates contributed a significant source of variation in resulting community profiles. The bacterial dissimilarity between the pure inocula and the inoculated samples was much higher (84-92%) although both direct and indirect methods captured a large fraction of T-RFs from both bacterial inocula: 17 KB-1 T-RFs for the vortexing method and 14 for the sonication method.

The aluminum sulfate pre-extraction treatment developed by Dong et al. (2006) differs from other approaches that attempt to remove PCR-inhibiting compounds in that removal occurs pre-lysis, thereby circumventing the concomitant removal of DNA from vegetative cells. This innovative approach addresses a key challenge to DNA extraction: sorption of DNA from live cells by humics and other compounds. Dong et al. (2006) found that aluminum sulfate addition removed extracellular DNA and our results support this finding. When extracellular DNA was added to soil at a range of gene copy numbers, the $Al_2(SO_4)_3$ method removed almost all non-viable DNA (>99.9%) even at high addition rates ($< 3 \times 10^9$ copies/g). The contribution of extracellular DNA to total DNA extracts is poorly understood but there is evidence that it is difficult to extract from soil. When Frostegard et al. (1999) inoculated phage λ DNA into samples, they could recover no more than 6 % of free DNA. If $Al_2(SO_4)_3$ removes extracellular DNA, this is an additional advantage of this method because the resultant DNA is almost entirely from viable cells.

A study by Persoh et al. (2008) corroborated the efficacy of the $Al_2(SO_4)_3$ pre-treatment for DNA recovery albeit only in the quantity and quality of DNA yield. Since the efficiency of the Dong et al. (2006) method can be largely attributed to the effects of $Al_2(SO_4)_3$ on DNA recovery, we designed a DNA extraction method that included this essential step and yet was basic, rapid and which produced consistently amplifiable DNA.

Unlike these previous approaches, we tested the method with downstream PCR applications—Q-PCR and T-RFLP. Nucleic acid yield is a commonly used parameter for evaluation of extraction efficiency. Persoh et al. (2008) found higher DNA yields and purity (by absorbance) using $Al_2(SO_4)_3$ addition which was highly similar to our findings. However, we also found that yields did not correlate to gene detection. For example, yields from the Mo Bio extractions were not statistically different for the three sample masses while quantitative detection of specific genes were. Conversely, the $Al_2(SO_4)_3$ method resulted in different DNA yields for different sample masses but similar quantitative gene detection. It should be noted that contaminants from the environmental matrix or from the extraction materials themselves may produce inaccurate absorbance readings. Also, variations in the extraction of non-target organisms likely exist for different methods. For example, the sonication + $Al_2(SO_4)_3$ method

yielded the most total DNA but recovered the lowest densities of total bacteria, *Dehalococcoides*, *E. coli tceA* gene, and KB-1 T-RFs compared to the $Al_2(SO_4)_3$ alone or the $Al_2(SO_4)_3$ + vortexing. Sonication at short exposures dislodges cells from surfaces but also lyses cells at longer exposures. Although a short exposure (30 s) was used in this study, *Dehalococcoides* and *E.coli* cells may be sensitive to sonication. Our findings suggest that nucleic acid yield is not as useful a measure of extraction quality as the ability to quantitatively detect the target population or other downstream molecular biological application for which it is intended.

The DNA extraction method had a much greater effect than sample mass on gene detection and microbial community structure. Sample masses between 0.25 to 10 g did not affect Q-PCR detection of the *Dehalococcoides* 16S or *tce*A genes when the $Al_2(SO_4)_3$ method was used. However, gene detection was inconsistent when the kits were used. DNA extracted from 1 and 10 g samples using the Qiagen method failed to amplify in *Dehalococcoides* 16S rRNA Q-PCR and in most T-RFLP PCR and *tce*A Q-PCR reactions. For Mo Bio Powersoil, *tce*A gene detection was similar for 1 and 10 g samples, which used the MAXI kit, but much lower for 0.25 g and inocula samples which used the standard kit. The gene densities using Mo Bio 0.25 g samples were also 1-2 log orders lower than densities in the corresponding 1 and 10 g samples, which suggests that the recovery efficiencies differ between these two mass-specific kits. SIMPER analysis for T-RFLP found that the Mo Bio 1 and 10 samples and all the $Al_2(SO_4)_3$ were similar. Regardless of sample mass, all extraction methods clustered together in T-RFLP patterns with the exception of the FastDNA method, which was statistically different from all other methods. Inspite of the dimensional proximity of the FastDNA samples to the KB-1 samples in the MDS plot, FastDNA did not capture a higher percentage of KB-1 T-RFs which suggests that this method preferentially isolated some other phylotype than the other methods. The $Al_2(SO_4)_3$ method performed best for all Q-PCR measurements and the Mo Bio MAXI kit produced comparable results for the *tce*A gene densities. T-RFLP results were less sensitive to extraction method than Q-PCR.

Numerous studies have compared different DNA extraction methods on DNA purity, yield and to a lesser degree molecular biological applications. This study differs in two important ways. Firstly, we applied Q-PCR and T-RFLP to soil samples that were designed to be homogeneous. Low biomass loamy sand was inoculated and homogenized with high densities of the KB-1 consortia and an *E. coli* standard to create a sample material that should presumably be highly similar in the dominant population profile. We found that most of the extraction methods that produced amplifiable PCR, with the exception of FastDNA, detected similar communities. The use of MDS combined with ANOSIM and SIMPER allowed for statistically rigorous analysis. Interestingly, the variation between inoculated, homogenized replicates was higher than expected which suggests that the natural scatter in community profiles between replicates in ecological systems would be even higher and that consistent use of unpooled sample replicates would offer a more accurate representation of the true spread in community analysis. This observation is supported by a recent study comparing the effects of pooling on soil microbial richness and diversity in which the authors found that pooling of either DNA or PCR products prior to hybridization significantly masked microbial diversity and reduced phylotype richness compared to unpooled samples (Manter et al., 2010).

Despite extensive research on improving nucleic extraction methods from soil, the quality and quantity of environmental DNA remains a major bias in molecular biological applications. Here we demonstrated the major impact that extraction method has on quantitative gene detection and to a lesser extent, community profiles. We measured the effect of DNA extraction method on quantitative gene detection of a class of dechlorinating bacteria that are widely used in bioaugmentation and bioremediation. Although consistent quantification of *Dehalococcoides* spp. and functional genes is a critical goal for assessing biodegradation projects, our results showed that the extraction method heavily biases quantitative information.

7.1.6. Summary of Results for Effects of Sample Size and Extraction Method

None of the commercially available kits were deemed suitable for quantitative analyses. The extraction method had a greater effect than sample size on gene abundance and community structure. Sample size did not affect qPCR results for the aluminum sulfate (lab-developed) method but gave inconsistent results when the kits were used. No correlation between DNA yield and quantitative detection of genes was observed for any method. The T-RFLP analysis also showed that some commercial kits resulted in strong biases with respect to microbial community structure. The aluminum sulfate method was deemed suitable for T-RFLP analysis. The aluminum sulfate method was able to distinguish between free DNA and DNA inside cells, potentially allowing only viable cells to be included in the molecular analysis. The direct method was compared to the indirect (cell separation) methods, and the results show that the direct method and the vortexing method yield comparable results with respect to Dhc and tceA detection as well as Bacterial and Archaeal community structure. However, the indirect method with sonication is not recommended as it yields slightly biased community structure as well as lower Dhc and tceA numbers.

7.2. Effect of Storage and Handling Conditions on Quantification of *Dehalococcoides* in Groundwater and Bacterial Community Structure

7.2.1. Effect of Storage on Quantitative Gene Detection

There were no significant differences between filtered or unfiltered groundwater when stored at 4 ºC or between filtered groundwater stored at either 4 ºC or 25 ºC for any of the 5 genes that were measured. Conversely, storage of unfiltered water at 25 ºC was significantly different (Table 7.2.1) from all other treatments for all five genes ($P = < 0.15$ for *tce*A and <0.05 for four other genes). Also, storage of unfiltered water at 25 ºC was significantly different from the control for all target genes.

The greatest effect of storage condition was on total bacterial densities, which ranged from 10^6 copies/ml in the control to 10^9 in the 25 ºC unfiltered water (Figure 7.2.1.1). All storage conditions produced significantly higher, log order increases in total bacteria with the 25 ºC unfiltered storage being by far the highest and with the other treatments being similar (10^7 copies/ml) to each other. Quantification of *bvc*A and *vcr*AB genes was less sensitive to storage conditions than other qPCR assays (Figure 7.2.1.2). There were no significant differences

between the control and samples stored at 4 ºC (filtered or unfiltered) or filtered at 25 ºC. Mean copy numbers of the *tce*A gene were significantly different between the control and the four storage conditions tested although gene densities remained within the same order of magnitude (1.9-6.9 x 10^5 copies/ml).

While the storage condition had a major impact on total bacteria numbers and we would expect to see a shift in the microbial community, the storage condition, with the exception of storage of unfiltered water at 25 ºC, and storage at 4 ºC for 14 days, had little effect on the detected number of *Dehalococcoides spp* and functional genes.

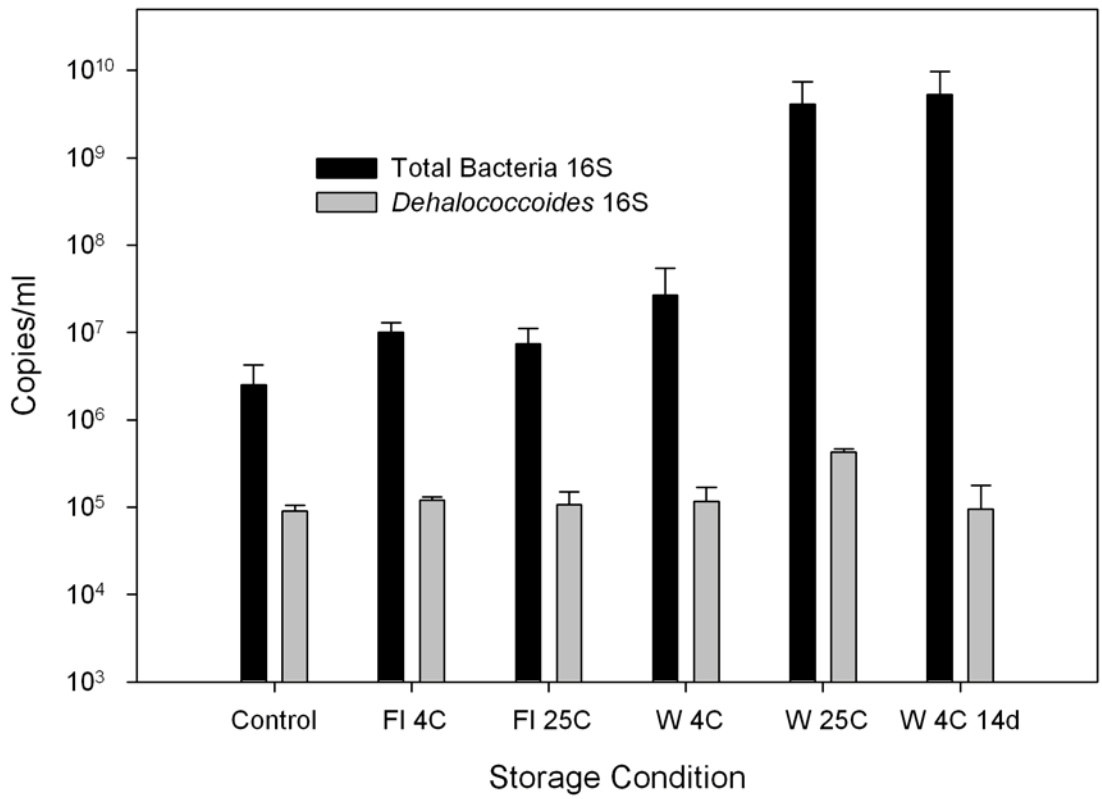

Figure 7.2.1.1. Effect of groundwater storage temperature and condition on qPCR detection of 16S genes targeting total bacteria and *Dehalococcoides spp*. Groundwater was stored either filtered (Fl) or unfiltered (W) at either 4 ºC or 25 ºC for 48 hours. Unfiltered groundwater was also stored at 4 ºC for 14 days (W 4C 14d). The control was filtered within 3 hours of collection and DNA was immediately extracted.

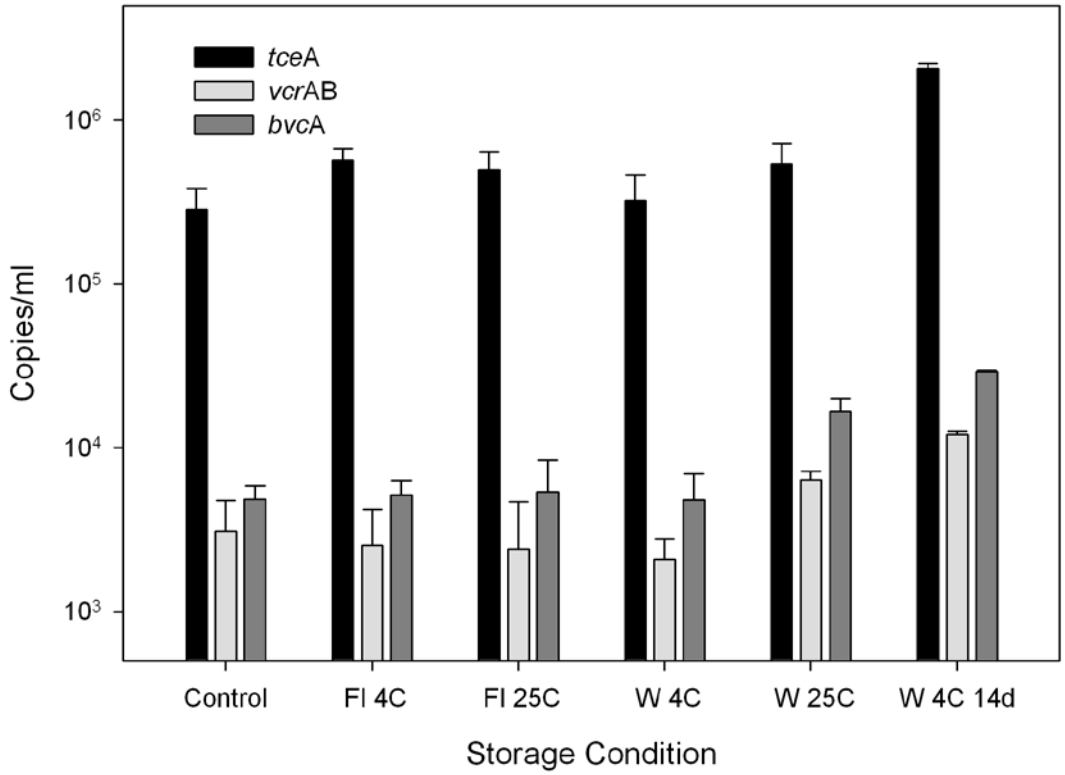

Figure 7.2.1.2. Effect of groundwater storage temperature and condition on qPCR detection of the dechlorinating functional genes *tce*A, *vcr*AB, and *bvc*A. Groundwater was stored either filtered (Fl) or unfiltered (W) at either 4 º C or 25 º C for 48 hours. Unfiltered groundwater was also stored at 4 º C for 14 days (W 4C 14d). The control was filtered within 3 hours of collection and DNA was immediately extracted.

Table 7.2.1. t-test comparisons of gene copy numbers detected in groundwater and stored under a range of conditions. Groundwater was stored either filtered or unfiltered and at either 4º C or 25 º C for 48 hours. The control was filtered within 3 hours of collection and DNA was immediately extracted. Probability values are for unpaired t-tests between control and treatments and within treatments. Dashes indicate comparisons where the differences between the means were not significantly different. P-values of < 0.05 indicate that the means were significantly different at the 95% confidence interval.

Unpaired t-test: P values					
Comparison	**Bacteria**	**Dhc**	***tce*A**	***bvc*A**	***vcr*AB**
Control + 4 º C Filter	< 0.05	< 0.05	< 0.05	__	__
Control + 25 º C Filter	< 0.10	__	< 0.05	__	__
Control + 4 º C Water	< 0.05	__	< 0.05	_	__
Control + 25 º C Water	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Control + 4 º C Water 14d	< 0.05	__	< 0.05	< 0.05	< 0.05
4 º C Filter + 4 º C Water	__	__	__	__	__
25 º C Filter + 25 º C Water	< 0.05	< 0.05	__	< 0.05	< 0.05
4 º C Filter + 25 º C Filter	__	__	__	__	__
4 º C Water + 25 º C Water	< 0.05	< 0.05	< 0.15	< 0.05	< 0.05
4 º C Water (14d) + 4 º C Filter	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
4 º C Water (14d) + 4 º C Water	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
4 º C Water (14d) + 25 º C Water	__	< 0.05	< 0.05	< 0.05	< 0.05

7.2.2. Effect of Storage on Bacterial Community Structure

Compared to the control (freezing of samples within 3 h of collection), storage of filtered or unfiltered groundwater for 48 hours resulted in a decrease in richness (number of T-RFs detected), and diversity (Table 7.2.2). Unfiltered groundwater stored at 25 °C for 48 hours resulted in a significant decrease in diversity. Storage for 14 days at 4 °C resulted in a drastic decrease in richness and diversity.

Table 7.2.1. Community indices of the groundwater samples from different storage conditions based on *Hha*I digestion profiles of 16S rRNA gene.

SAMPLE	Richness	Shannon Diversity (H)
Control	54	2.685
Filter (4C; 48h)	50	2.484
Filter (25C; 48h)	34	2.651
Water (4C; 48h)	37	2.404
Water (25C; 48h)	22	1.928
Water (4C; 336h)	24	1.418

A closer analysis of the T-RFLP patterns shows the effect of storage temperature on filtered groundwater. While the same dominant T-RFs were detected in the filtered samples as in the control, several T-RFs showed significant changes in relative abundance. The changes at 25 °C were more drastic, with some minor T-RFs (~1% abundance) increasing by 3 or 4-fold (Figure 7.2.2.1).

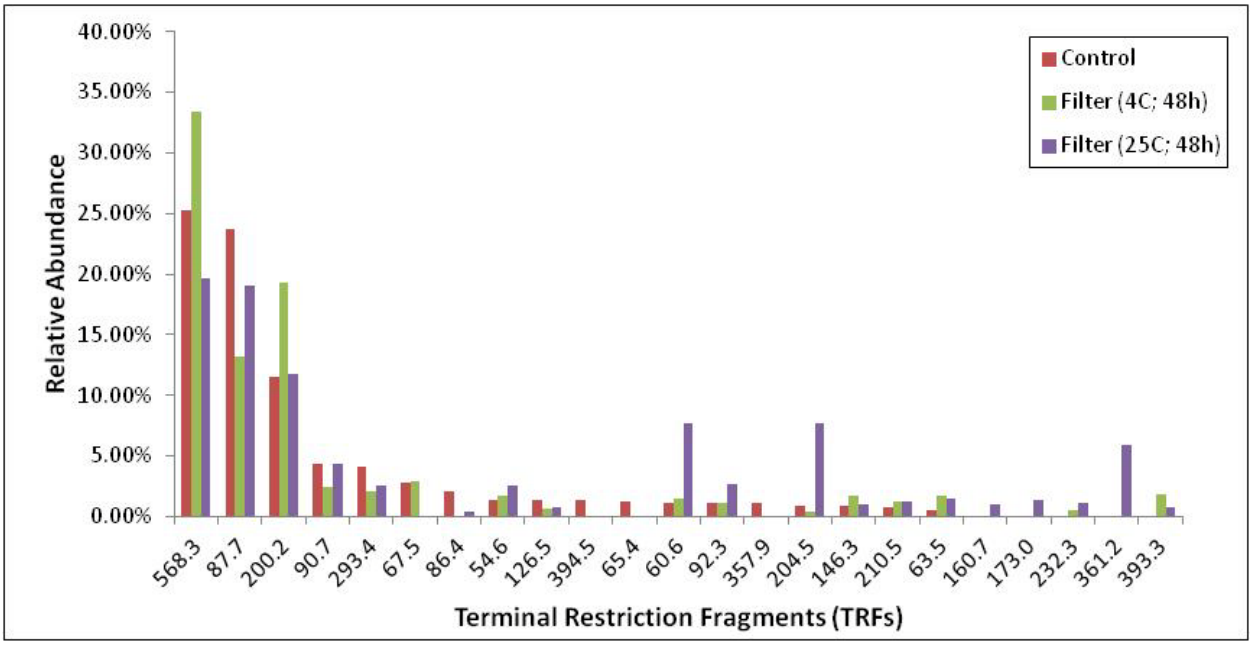

Figure 7.2.2.1. Dominant (>1%) Bacterial TRF profile based on *Hha*I digestion of 16S rRNA gene of filtered groundwater samples under varying storage conditions.

Similarly, the effects of storage temperature and time were revealed by analysis of the T-RF abundance pattern (Figure 7.2.2.2). While water at 4 °C had similar patterns to the control, increasing the temperature led to increases in minor T-RFs. Increasing the storage time led to a drastic increase in one T-RF, indicating growth of a specific ribotype.

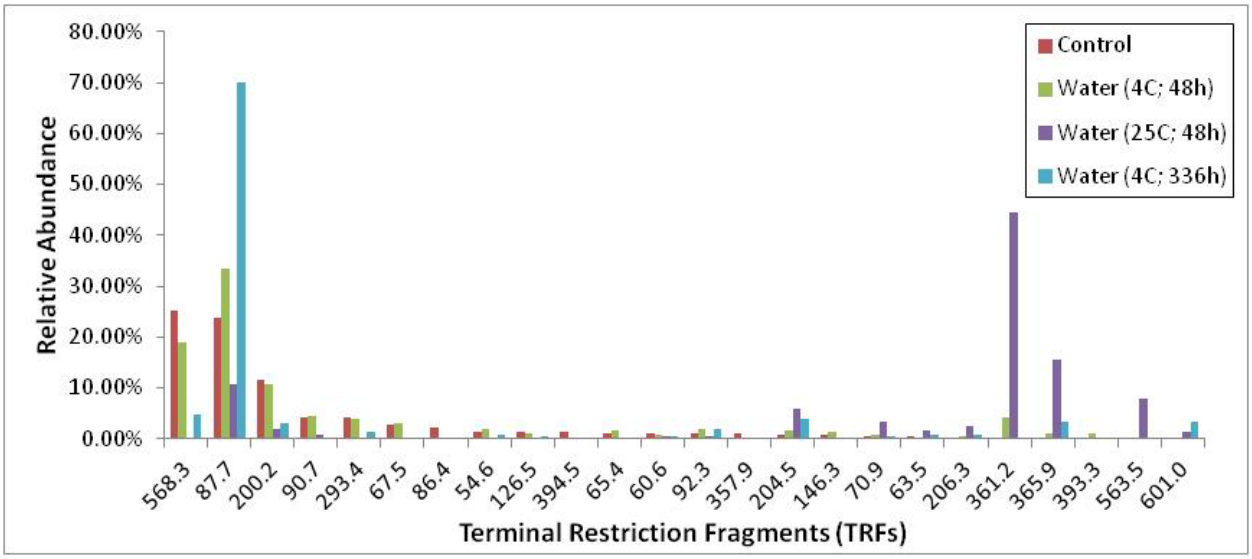

Figure 7.2.2.2. Dominant (>1%) Bacterial TRF profile based on *Hha*I digestion of 16S rRNA gene of stock groundwater samples under varying storage conditions.

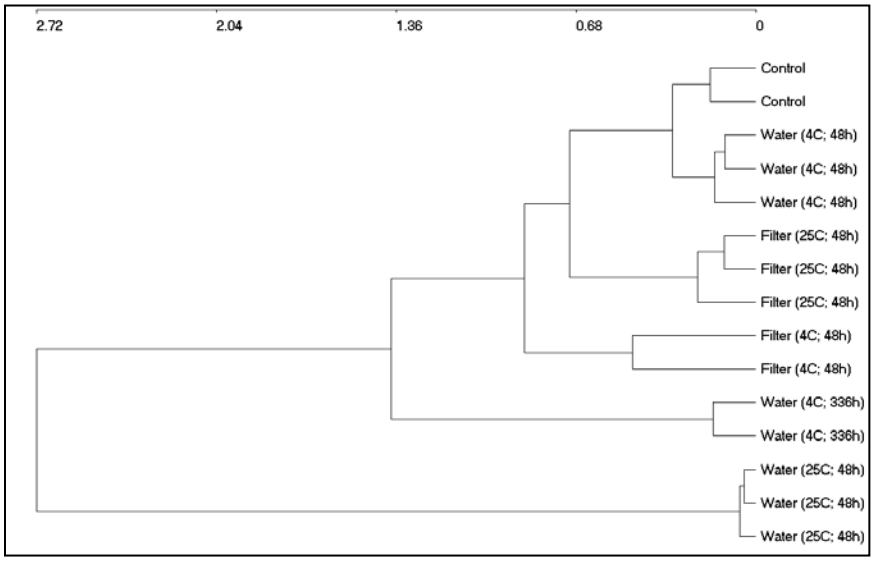

Figure 7.2.2.3. Bray-Curtis dendogram showing similarity of T-RFLP patterns of bacterial 16S rDNA extracted from groundwater samples under varying storage conditions.

Analysis of Bray-Curtis similarities (Figure 7.2.2.3) shows that storage of groundwater at a low temperature and for shorter periods before freezing or DNA extraction is the best option for minimizing changes to the bacterial community structure. Interestingly, filtration seems to preserve the original community structure even at 25 °C. Storage of unfiltered groundwater at 25 °C for 14 days resulted in a drastic change in bacterial community structure.

The inferred phylogeny of T-RFs was determined using the Phylogenetic Analysis Tool (PAT)(Kent et al., 2003) and is presented in Figure 7.2.2.4. The shifts in microbial communities associated with each storage condition are clearly seen, and support the similarity analysis. Filtered samples show less shifts compared to the control, while unfiltered water show more drastic shifts. For example, a decrease in putative *Dehalococcoides* (red column) is accompanied by a large increase in putative *Actinobacillus*, *Francisella*, and *Pasteurella* when stored at 25°C for 48 hours. Storage for 14 days at 4 °C resulted in an increase in the Lactococcus group, as well as an increase in the fraction of "unknown" or "unidentified" ribotypes. While such putative identification is not specific to species level, the inferred phylogeny analysis reveals how the community shifts, and indicates which putative groups change with different conditions.

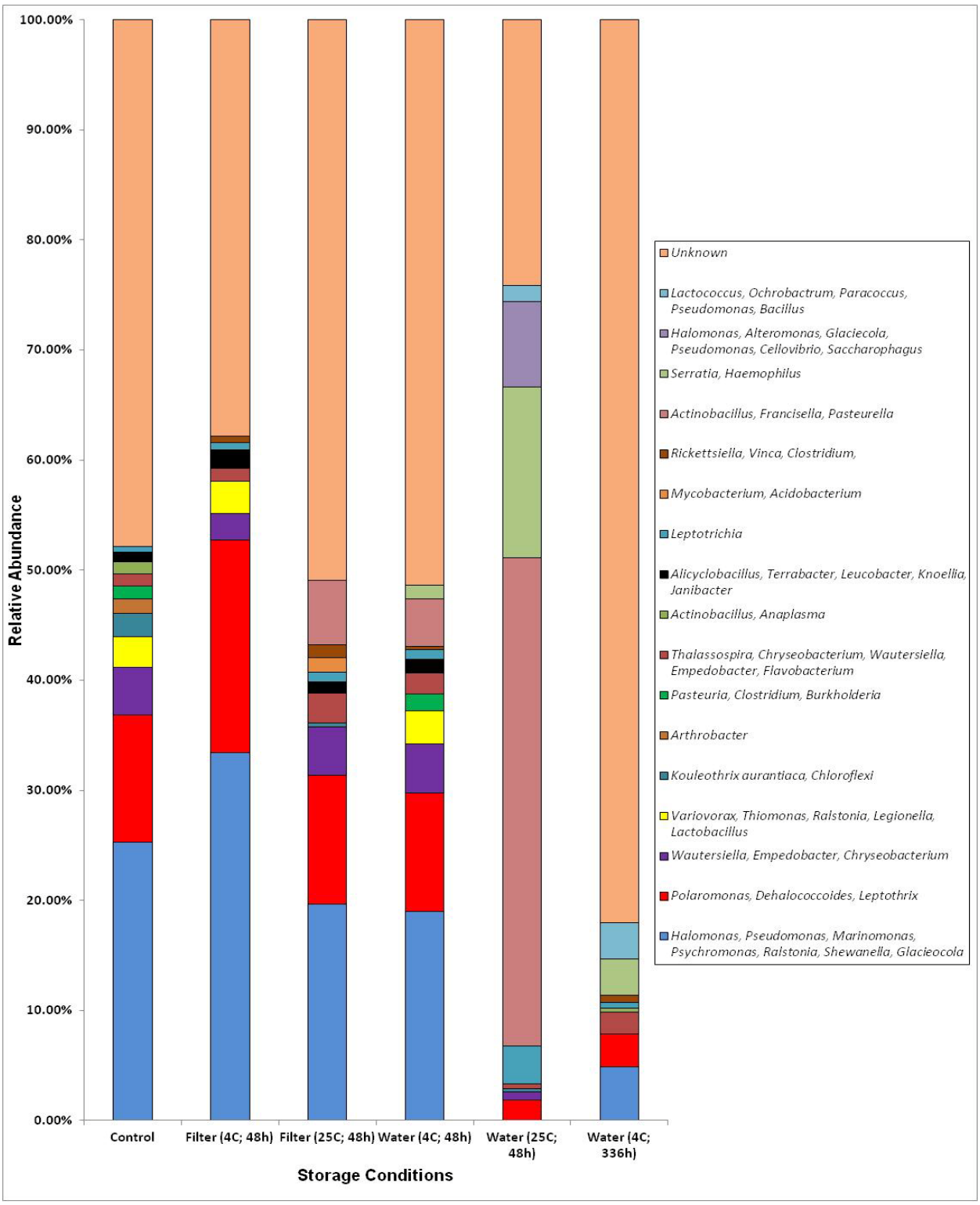

Figure 7.2.2.4. Relative abundance of inferred bacteria based on various T-RFs obtained from *in silico* restriction enzyme digests (using *Hha*I, *Msp*I, and *Rsa*I) from groundwater samples stored under varying conditions.

8. CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH

The results of molecular biological techniques are affected by how DNA is extracted from the bulk matrix, whether in sediment or groundwater. Quantitative results from Q-PCR studies are heavily biased by the choice of commercial DNA extraction method. The commercial methods yield Q-PCR copy numbers for specific organisms (e.g., *Dehalococcoides*) that are significantly different from each other (up to 1 order of magnitude) and from spiked samples (up to 2 orders of magnitude). Thus, comparing actual numbers of organisms derived from different studies using different DNA extraction techniques would be misleading. However, if a research lab uses the same procedure for extracting DNA from environmental samples, then it is still possible that the relative changes in cell concentrations can be monitored. Results of Q-PCR are also sometimes used to estimate dehalogenating activity or potential per cell. Again, depending on the DNA extraction method used, these numbers can be off by an order of magnitude or more.

Qualitative assessment of microbial community structure (the fractions of different phylotypes, as assessed by T-RFLP) in sediment and groundwater samples is also affected by DNA extraction method. While most commercial extraction kits resulted in similar communities, one kit yielded a dissimilar structure for Bacteria, and another kit yielded a dissimilar structure for Archaea. A table showing the advantages and disadvantages of the different extraction methods tested in this study is shown below.

A method for extracting DNA that yielded better results was developed. The new method, based on precipitation of humics using aluminum sulfate, resulted in higher yields, less contamination, and higher numbers of *Dehalococcoides* genes compared to commercial kits. The microbial communities derived from the new method were similar to the "combined" results of all methods tested, showing that there was no significant bias in T-RFLP results. The new method also was able to eliminate the recovery of non-cell associated ("free") DNA in sediment, and was shown to have an average recovery of 20% of spiked cells. Thus, the new method has potential for use in soil studies, where humics and other contaminants may affect and bias PCR amplification. In addition, the new method may be more accurate in determining levels of DNa from active cells, as inactive, extracellular DNA is eliminated (less than 0.1% recovery even at 10^9 copies added).

Storage conditions were found to affect the numbers of total bacteria, and to a lesser extent, the *Dehalococcoides* 16S and functional gene numbers. More extreme storage conditions (4°C for a long time, or 25°C for a short time) affected the Q-PCR results, and shifted the microbial communities drastically. Thus, samples should be frozen as soon as possible after collection. If samples are to be shipped on ice, processing should be performed no later than 48 hours after collection. Filtration of groundwater samples mitigates the microbial community shift, but it appears that storage at room temperature should be avoided to prevent changes in the microbial community structure. Future research studies should consider the impacts of nucleic acid extraction and storage and handling on their results. At the minimum, extraction techniques and storage and handling conditions should be described. In the absence of standardized internal controls for Q-PCR, research laboratories should report how they optimized these conditions.

Table 8. Advantages and Disadvantages of DNA extraction methods used in this study

DNA Extraction Method	**Advantages**	**Disadvantages**
Mo Bio Power Soil	• Kits available for several sample sizes • Most rapid DNA extraction method (<20 minutes per sample) • Involves minimal reagent preparation • At higher sample sizes, produces bacterial community profile similar with most of the DNA extraction methods used	• Very small DNA yield regardless of sample size used • Produces DNA extracts with high impurity
FastDNA Spin Kit for Soil	• Relatively high DNA yield • Relatively rapid DNA extraction method (<30 minutes per sample) • Involves minimal reagent preparation	• Sample size non-scalable (sample size limited to only 0.25g) • Produces DNA extracts with high impurity • Yielded dissimilar bacterial community profiles compared to other extraction methods used
QiaAMP DNA Blood Kit	• Kits can be customized for several sample sizes • Involves minimal reagent preparation	• DNA extracted with high impurity • Slow DNA extraction (<40 minutes per sample) • High PCR inhibition at larger sample sizes resulting to failed downstream PCR processes
Lab Method	• Scalable to desired sample size • Produces highest DNA yield compared to tested commercial kits • Produces high DNA purity • Produces bacterial community profile similar with most of the DNA extraction methods regardless of sample size used • Produces highest QPCR results for spiked samples • Can remove extracellular DNA	• Relatively more labor-intensive method • Involves several reagent preparations • Slowest extraction method (<50 minutes per sample)

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10. APPENDICES

List of Technical Publications

Hicks, K., M. J. So, R. C. Borden, and F. L. de los Reyes III. Effect of DNA extraction method and sample mass on quantification of *Dehalococcoides* spp. dechlorination genes and microbial community structure in soil. (submitted to *J. Microbiol. Methods*)

Hicks, K., M. J. So, R. C. Borden, and F. L. de los Reyes III. Effect of groundwater sample storage and handling on on quantification of *Dehalococcoides* spp. dechlorination genes and microbial community structure in soil. (to be submitted to *J. Appl. Microbiol*.)

Hicks, K., N. R. Smith, M. J. So, R. C. Borden, and F. L. de los Reyes III (2008) Optimizing DNA extraction and Sample Size of Groundwater Sediment for Molecular Biological Analysis, American Society for Microbiology 108th Annual Meeting, Boston, MA, June 1-5, 2008.