FINAL REPORT

Standardized Procedures for Use of Nucleic Acid-Based Tools

SERDP Project ER-1561

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LIST OF ACRONYMS

°C	degrees Celsius
%	percent
µg/µl	micrograms per microliter
µL	microliter
µM	micromolar
B. diminuta	*Brevundimonas diminuta*
bp	base pairs
B. subtilis	*Bacillus subtilis*
bvcA	VC-RDase gene
CCMP	Provasoli-Guillard National Center for Culture of Marine Phytoplankton
CFU	colony forming units
CN	cellulose nitrate
Dhc	*Dehalococcoides mccartyi*
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ds	double stranded
E. coli	*Escherichia coli*
EPA	Environmental Protection Agency
ft	feet
g	grams
g/L	grams per liter
IAC	internal amplification control

IC	internal control
L	liter
LB	lysogeny broth
luc	luciferase gene
MBT	molecular biological tools
mg/L	milligrams per liter
MIAC	microbial internal amplification control
mL	milliliters
mL/min	milliliters per minute
M. luteus	*Micrococcus luteus*
mm	millimeter
mM	millimolar
ng	nanograms
ng/L	nanograms per liter
nm	nanomole
NTC	no template control
PBS	phosphate buffered saline
pg	picogram
P. marinus	*Prochlorococcus marinus*
PES	polyethersulfone
QAPP	quality assurance project plan
QA/QC	quality assurance / quality control
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid

rRNA	ribosomal ribonucleic acid
SERDP	Strategic Environmental Research and Development Program
SON	statement of need
SOPs	standard operating procedures
STD	standard
USARC	United States Army Reserve Command
UV	ultraviolet
v/v	volume/volume
Wt	wild type

KEYWORDS

calibration

chlorinated solvents

Dehalococcoides mccartyi

Dhc

DNA

E. coli

E. coli strain TOP10 attTn7:luc

Escherichia coli

extraction

groundwater

inhibition

internal controls

internal standards

Luciferase

microbial partitioning

PCR

QA/QC

qPCR

quality control quality assurance

quantification

quantitative polymerase chain reaction

remediation

sampling methods

SYBR Green

TaqMan

transgene insertion

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1 ABSTRACT

Objectives

The use of molecular biological tools (MBTs) for the detection and quantification of microbial biomarkers (e.g., DNA/RNA) in environmental samples is rapidly increasing as remedial practitioners seek to improve the design, field performance and monitoring of bioremediation.

In 2005, the Strategic Environmental Research and Development Program (SERDP) developed a Statement of need (SON) in response to the status of MBT analysis, particularly for the presence and abundance of *Dehalococcoides* (*Dhc*) in environmental samples using quantitative real-time polymerase chain reaction (qPCR) methods, which were to:

1) Develop a better understanding of the effects on the efficacy of MBTs of all steps in the sampling process, including sample collection, transport, storage/preservation, and processing.

2) Develop improved sampling and processing techniques for groundwater and associated saturated soil samples that would support the use of biomarkers for environmental remediation.

3) Develop a better understanding of the relationship between the density of gene copies or other relevant biomarkers in a groundwater or associated saturated soil sample and the true density of microorganisms in the aquifer, and the impacts that sampling procedures may have on establishing such a relationship.

SERDP project ER-1561, *Standardized Procedures for Use of Nucleic Acid-Based Tools for Microbial Monitoring*, focused on identifying and minimizing the causes of variability during qPCR enumeration of genes of interest in groundwater, with the goal of developing a foundation for the development of standardized methods for collection, preservation, transport, storage and processing of environmental qPCR samples collected from contaminated sites.

Technical Approach

A technology review on the status of MBTs was performed at the beginning of the project to determine MBT use in other industries. The review focused project goals and activities, which included:

1) Comparing qPCR to non-PCR-based enumeration methods to validate and increase confidence in qPCR methods;

2) Comparing and assessing baseline variability within and between laboratories at the outset of the project using a multi-laboratory “round robin” approach;

3) Development and testing of a microbial internal amplification control (MIAC), for use in spike and recovery protocols, in the extraction and qPCR process to assess biomarker extraction efficiency and matrix interference;

4) Assessment and optimization of methods including sampling, biomass collection, nucleic extraction, and qPCR protocols;

5) Assessment of inter-laboratory variability after integration of the MIAC and optimized methods; and

6) Determining the distribution of *Dhc* cells between aquifer solids and groundwater to better understand and interpret groundwater enumeration results.

Results and Discussion

Significant results of the project included.

1) Obtaining agreement of qPCR methods with non-molecular methods such as plate counts and microscopy, these speak to the fundamental accuracy of qPCR.

2) The development of an effective MIAC, consisting of a modified *E. coli* with a chromosomal insertion of a firefly luciferase gene, for monitoring by qPCR. The MIAC can be used quantify biomass losses, qPCR inhibition and flag suspect samples/analyses.

3) Optimization of methods and protocols so that five independent labs were able to generate similar *Dhc* enumeration results for identical groundwater samples with maximum variability decreasing from as high as 40-fold to as little as 1.1-fold over the course of the study.

4) An improved understanding of the distribution of *Dhc* between aquifer solids and groundwater derived from column experiments. This study indicated that aquifer solids associated *Dhc*, comprised the majority of cells in the presence of growth substrates and cells were mainly planktonic in the absence of growth substrates.

Benefits

MBT such as qPCR have the potential to accurately enumerate microbial cells, including *Dhc*, in groundwater samples if proper procedures and appropriate controls are integrated into the process. The development of optimized methods, including the use of a project developed MIAC, allowed significant improvements in data quality and the ability to better detect and correct for matrix interference. Understanding the distribution of *Dhc* between sediment and groundwater will allow more accurate conclusions to be drawn regarding total *Dhc* biomass in aquifers using groundwater data. The ability to better quantify *Dhc* at a variety of sites with high

precision, in multiple labs, will increase remediation practitioners' confidence in remediation focused qPCR analysis and their ability to manage bioremediation projects effectively.

2 BACKGROUND AND OVERALL OBJECTIVES

The use of molecular biological tools (MBTs) for the detection and quantification of biomarkers (e.g., specific nucleic acid sequences, peptides, proteins, lipids) in environmental samples is increasing rapidly. Remedial practitioners are using these tools as they seek to improve the design, field performance and monitoring of biologically-based remediation technologies. While academic research facilities and several commercial entities routinely provide these analyses, there are currently no MBT-focused standardized methods (i.e., equivalent to Environmental Protection Agency [EPA] methods) for collection, preservation, transport, storage or processing of environmental samples collected from contaminated sites.

The ultimate goal of environmental MBT application is to measure temporal and spatial changes in a target organism's cell titer (i.e., abundance) and possibly activity. Such quantitative information is required to interpret and predict the biodegradation/biotransformation potential for contaminants of concern. Currently, the methods used to quantify key organisms of interest (e.g., *Dehalococcoides mccaryti* [*Dhc*] a critical microbe in the degradation of chlorinated ethenes) are not standardized. Concerns exist that changes in measured cell titers or activities may not reflect the actual site conditions because the analysis is confounded by variance or biases caused by sampling, microbial biomass amount, nucleic acid extraction, analytical procedures, and matrix effects (i.e., polymerase chain reaction [PCR] inhibition), thus making data interpretation ambiguous. These procedural deficiencies have implications at the most fundamental level, such as the establishment of any relationship between the true titers of the target microorganism(s) in the aquifer and their measured nucleic acid biomarker abundance in groundwater samples.

Figure 2-1 presents the general steps used to sample and quantify nucleic acid biomarkers using MBTs; these are:

1) Sampling of biomass (groundwater/aquifer solids);

2) Shipment of samples to the analytical laboratory;

3) Biomass collection (primarily for groundwater);

4) Extraction of nucleic acids (DNA/RNA); and

5) qPCR enumeration of gene targets.

There are factors at each of these steps that could add variability to the quantitative information and affect qPCR data interpretation. These factors have the potential to reduce the accuracy and precision of the analysis, and thus, reduce the likelihood that qPCR will provide an accurate reflection of actual conditions. In addition, the resulting variability can obscure relationships between qPCR results and microbial activities in the field. The goal of project ER-1561 was to assess and quantify variability and biomarker losses at each step in the analytical process so that

results obtained in different laboratories, reflect the true abundance of target gene(s), and can be interpreted with confidence.

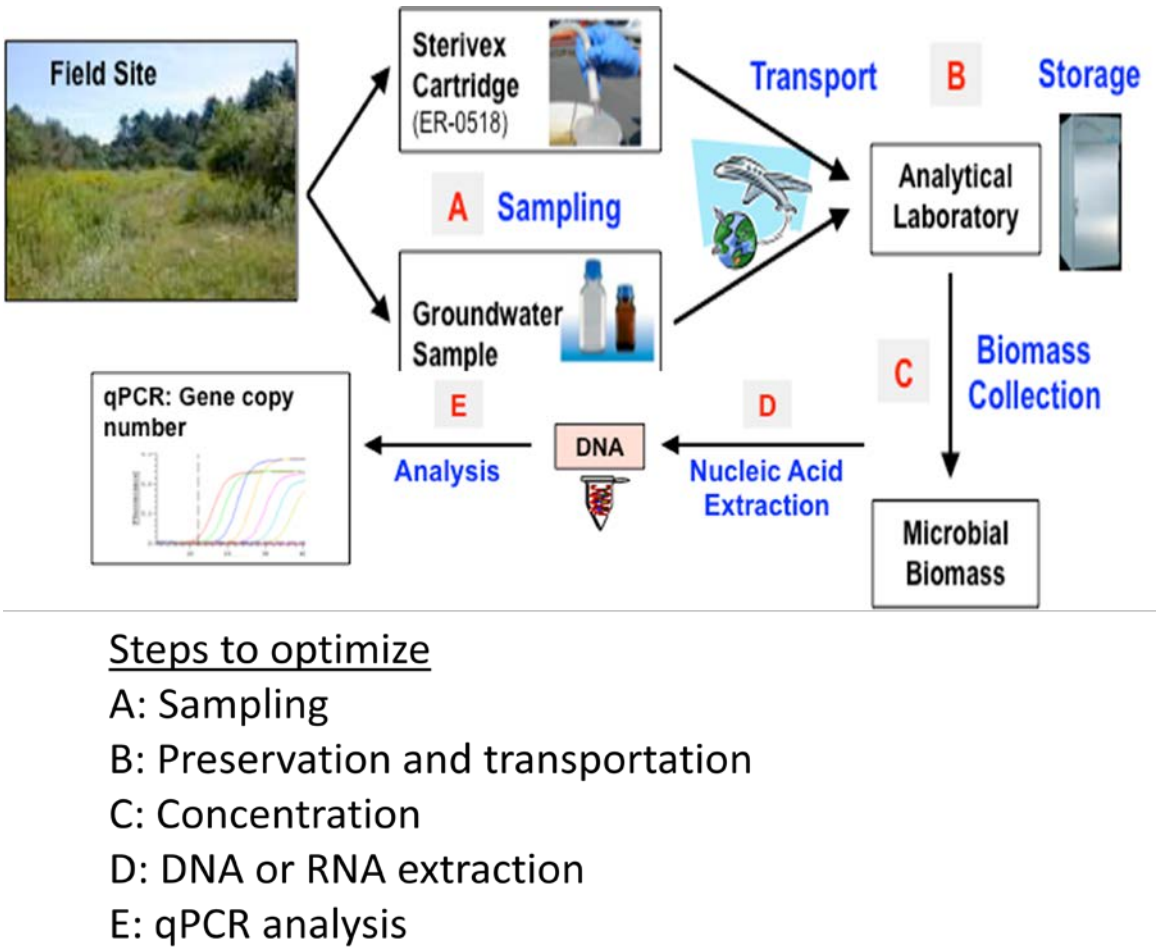

Figure 2-1: Overview of MBT activities/procedures with potential variability impacts on MBT analysis.

2.1 Specific Project Objectives

In fiscal year 2005, the Strategic Environmental Research and Development Program (SERDP) developed a statement of need (SON [ERSON-07-05]) in response to the current status of MBT analysis, particularly for the presence and abundance of *Dhc* in environmental samples using quantitative real-time polymerase chain reaction (qPCR) methods. The explicit needs identified in the SON were:

1) Develop a better understanding of the effects on the efficacy of MBTs of all steps in the sampling process, including sample collection, transport, storage/preservation, and processing.

2) Develop improved sampling and processing techniques for groundwater and associated saturated soil samples that would support the use of biomarkers for environmental remediation.

3) Develop a better understanding of the relationship between the density of gene copies or other relevant biomarkers in a groundwater or associated saturated soil sample and the true density of microorganisms in the aquifer, and the impacts that sampling procedures may have on establishing such a relationship.

The first two aims required improved quality assurance/quality control (QA/QC) methods for MBT procedures, and optimized analytical processes to correct for losses and variability at each step of the analysis. The final aim required an improved understanding of the relationship between *Dhc* quantified in a sample and the true number of *Dhc* in the aquifer. As groundwater sampling is typically used to assess contamination in aquifers, this also requires understanding the impact of groundwater sampling methods and site conditions on the abundance of *Dhc* determined by qPCR methods. To meet the SON goals, the overall technical objectives of this work were to:

1) Evaluate factors affecting quantification of nucleic acid biomarkers in groundwater samples (and thus the interpretation of qPCR data and site management decisions); and

2) Develop the foundation for a standard methodology (but not to establish the standard methodology itself).

These objectives were met through:

1) Identifying and quantifying the causes of variability during sample collection, transportation, storage and processing; and

2) Developing an internal standard approach so that biomarker losses during sample-processing and matrix effects causing PCR inhibition can be quantified.

By understanding and controlling or correcting for the inherent variability at each step of the analytical process, we can: (1) optimize sample collection, preservation, transport, storage and processing of environmental samples; and (2) detect real and relevant changes, temporally and spatially, in biomarker abundance, regardless of the laboratory that performs the analysis.

Efforts to standardize MBT procedures face similar challenges to those associated with development of standard methods for chemical analyses of environmental samples. Until chemical analyses were standardized, it was impossible to determine whether results were biased by sampling techniques, matrix interferences, laboratory procedures, analytical instruments or human error. The key difference between the analysis of chemicals and the analysis of microbial

biomarkers in environmental samples is the lack of suitable internal controls (IC) used in a spike and recovery approach during biomarker gene analysis.

Effective ICs are a prerequisite for elucidating factors that affect the accuracy and precision of the analysis, and for developing refined procedures and standardized methodologies. Using ICs allows controlled comparisons of how differences in procedures at each analytical step affect the efficiency of capturing and quantifying the target biomarker(s), and could lead to optimized methods with increased reproducibility (precision) and accuracy (the true abundance of biomarkers in the sample). The use of effective ICs to assess and correct for the differences in biomarker loss at each analytical step will ultimately allow comparison of results generated in different laboratories, detect real spatial and temporal trends in samples from the same wells, and reveal differences between wells from the same site and between sites. ICs are the basis upon which robust and verifiable analytical procedures are developed, so that, precision and accuracy are achieved within and between laboratories when analyzing the same samples. Accuracy and reproducibility are critical parameters that any analytical procedure must meet, and are required to establish confidence in the use of MBTs for environmental monitoring. The availability of ICs will support widespread MBT implementation and acceptance of MBT data by remediation project managers (RPMs) and regulators.

The technical approach of SERDP project ER-1561 focused primarily on the analysis of *Dhc* biomarker genes for several reasons. *Dhc* play key roles in the detoxification of chloroorganic contaminants, including chlorinated ethenes. *Dhc* targeted MBTs are already commercially available, and are currently used for assessment and monitoring at many chlorinated solvent sites, including Department of Defense (DoD) sites. Hence, the development of a rigorous protocol for MBT application to quantify *Dhc* cells is of immediate value to the DoD, and will serve as a template for the design of similar protocols for other target microbes relevant to DoD mission needs. The team focused on the analysis of groundwater samples for practical and economic reasons. Groundwater sampling is feasible at most sites through existing infrastructure (i.e., monitoring wells), and groundwater interrogates a larger portion of the aquifer compared to solids samples collected from discrete zones. Moreover, groundwater is the de facto sample matrix provided by practitioners for the application of *Dhc* biomarker-targeted MBTs.

Tasks defined at the outset of this project included:

1) Preparation of a white paper to review the status of nucleic acid-based MBT use in various fields that compiles and evaluates current information regarding sample collection, handling and processing techniques (Task 1);

2) Development of an IC to systematically evaluate the biomarker extraction efficiency of each step during sample collection, handling, processing and analysis (Tasks 2A/B);

3) Evaluation of the effects of each step of the entire sampling and analytical process on biomarker integrity and quantification (Task 3);

4) Evaluation of the relationship between *Dhc* biomarker gene abundances in groundwater versus soil samples (Task 4);

5) Assessment of factors affecting the variability of nucleic acid biomarker gene content in groundwater samples (Task 5); and

6) Documentation and integration of all information including, sample collection, processing, reporting, and data interpretation, from which to derive standard EPA or EPA-equivalent methodologies for the analysis of nucleic acid biomarkers in groundwater samples (Task 6).

Further definition of the specific research goals and approaches was possible after completion of the white paper, which included a review of then current procedures and practices used by participating commercial and non-commercial laboratories. The results of this review and the specific research objectives are summarized in Section 3.

3 LITERATURE AND TECHNOLOGY REVIEW

The use of MBTs to detect and quantify key microorganisms often employs more sophisticated methodologies in other industries and disciplines than those used in the environmental remediation field. The disciplines with well-developed methodologies for MBTs include medical testing, food quality surveying, source tracking, as well as criminal forensics. Numerous peer-reviewed journal articles discuss factors affecting reproducibility, sensitivity, and accuracy of MBTs when applied in the aforementioned fields. At the outset of the project Task 1, a literature review and summary of commercial methods (i.e., a technology review) was performed. The purpose of this review was to assess the current state of practice and the applicability of various technologies and approaches used in other industries for use in monitoring groundwater remediation. The report *An Overview of Current Approaches and Methodologies to Improve Accuracy, Data Quality and Standardization of Environmental Microbial Quantitative PCR Methods* (Lebrón et al., 2008) summarized the team's findings, confirmed the team's strategy, and prioritized the project objectives.

3.1 Literature Review Summary

One of the goals of the literature review was to confirm the projects team's strategy and approach, and identify additional promising approaches and technologies that could be incorporated to address the project objectives. Of particular interest was the evaluation of:

1) Methods that are currently available and/or emerging;

2) Quality assurance/quality control (QA/QC) procedures associated with these methods, specifically internal and reference standards;

3) Factors that affect sensitivity of the analysis, and the variability within/between methods; The impact of field heterogeneity on MBT results and data interpretation; and

4) Groundwater/soil sampling techniques.

Information was obtained by surveying the peer-reviewed scientific and technical literature with a focus on the methods used in other disciplines utilizing qPCR including the medical, agricultural/food, forensics, and environmental fields. In addition, ancillary topics such as groundwater sampling procedures and biomass collection from groundwater were reviewed. Methods and practices of the major commercial entities providing qPCR testing of bioremediation samples, specifically SiREM (www.siremlab.com) and Microbial Insights (www.microbe.com) were also reviewed.

The review identified that unique challenges are associated with groundwater samples, including the potential for high variability in terms of biomass, geochemistry and the presence of remediation amendments, challenges associated with representativeness (i.e., are samples

consistent and the same as aquifer groundwater?) biomarker losses during sample processing and extraction, and matrix interference leading to PCR inhibition. Recommendations for assessing and addressing these challenges included the development of internal microbial controls (i.e., microbial internal amplification controls [MIAC]) to: a) assess current approaches for sampling, shipping, storage, biomass collection, nucleic acid extraction, and data analysis/interpretation, and b) identify areas where methodological improvements may be required.

The literature review indicated that the use of quality control measures relevant to qPCR testing are well developed in disciplines such as pathogen detection, medical testing and criminal forensics, but that the methods have not been fully applied to environmental remediation testing.

Specifically the review indicated that the bioremediation industry would benefit from the use of internal controls and specifically microbial internal amplification controls. Internal controls are standards that are added in known quantities to the assay or sample materials and are co-monitored throughout the analytical process to quantify losses and recognize interferences. Microbial internal amplification controls (MIAC) are whole cell internal controls that are co-quantified with the test target (e.g., *Dhc*) and function to assess losses throughout sample processing and analysis.

3.2 Refined Research Objectives

The purpose of the literature/technology review was to identify bioremediation-relevant technical issues and to review promising methodologies for MBT application developed in other disciplines. In contrast to MBT applications in medicine and food industries, bioremediation uses environmental samples (i.e., soil, sediment and groundwater) that present unique sampling and analytical challenges due to high heterogeneity and other characteristics.

The goal was to survey and adopt existing technologies for improving MBT application in a bioremediation context. The aspects of the technology review that were examined further under the project were those deemed most important based on the project team's review, experience and unpublished results. Provided below are the key research activities and the relevant sections related to the original project task:

Baseline Variability, Section 4: (Applicable to Project Task 3): (i) Comparison of qPCR to non-PCR-based enumeration methods in order to validate and increase confidence in qPCR methods. (ii) Comparison and assessment of variation for methods and laboratories at the outset of the project using a multi-laboratory testing "round robin" approach;

Development and Testing of Internal Controls, Sections 5 and 6: (Applicable to Project Tasks 2A/2B): Development and testing of ICs for use in spike and recovery protocols in the extraction and qPCR process to assess biomarker extraction efficiency and sample-specific effects including matrix interference;

Assessment and Optimization of Laboratory Methods, Section 7 (Applicable to Project Task 3): Assessment and optimization of methods including biomass collection, nucleic extraction, and qPCR protocols;

Assessment of Groundwater Sampling Preservation and Storage Methods, Section 8 (Applicable to Project Task 5): Assessment of preservation methods including on site versus in lab filtration, high and low flow sampling approaches;

Multi-Lab Variability Assessment after Method Optimization, Section 9 (Applicable to Project Task 3): Assessment of variability after integration of microbial internal amplification controls (MIAC) and optimized methods; and

Solids Groundwater Partitioning, Section 10 (Applicable to Project Task 4): Assessment of partitioning of *Dhc* cells between aquifer solids and groundwater to better understand and interpret groundwater enumeration results.

The following sections provide background information and an overview of experiments carried out to accomplish these tasks. Detailed materials and methods are provided as supplemental information and are provided in the Appendices and Attachments.

4 BASELINE VARIABILITY ANALYSIS

After the literature review, the project team deemed it essential that the accuracy and precision of the initial methods and participating labs be determined. This analysis would provide a starting point from which future improvements could be quantified.

While variability can occur in any analytical method, qPCR for the enumeration of microbes in environmental samples is likely more susceptible for several reasons including:

1) The nucleic acid analyte (e.g., DNA) must be extracted from microbial cell populations, which may have varying properties depending on the type of cells and their physiological status. Nucleic acids (in particular RNA) are subject to enzymatic hydrolysis (i.e., nucleases).

2) DNA can exist in several forms (e.g. linear /supercoiled/ nicked) that can impact its performance in PCR assays.

3) The qPCR assay is dependent on an enzymatic process that is susceptible to matrix inhibition by substances (e.g., humic compounds) sometimes found in soil and groundwater samples.

4) The high dynamic range of the qPCR analysis (6 to 7 orders of magnitude) is wider than many standard analytical methods (e.g., VOC quantification) predisposing the analysis to quantitatively higher variability.

Due to the above factors, variability with qPCR methods is expected to be higher than for chemical analytical methods. In some respects qPCR data is more comparable to microbial plate counts, where substantive variability is assessed on a logarithmic as opposed to a linear scale.

The following specific questions with respect to variability of qPCR assays were made at the outset of this project including:

1) How close could non-PCR methods be compared to qPCR results under ideal conditions? This speaks to the fundamental/potential accuracy of qPCR.

2) How do results vary with the initial approaches (i.e., before method optimization) with respect to sample to sample (within lab variability) and between lab variability?

3) If significant variation in the results were observed, could critical steps contributing to this variability be identified and optimized (e.g., extraction, analysis, calibration etc.)?

4) What would be considered acceptable between labs and sample to sample variability?

These questions were addressed in the experiments described in the following sections.

4.1 Comparison of qPCR Enumeration with Direct and Plate Count Methods

Verification of qPCR results using non-MBT based methods would demonstrate that a qPCR method is accurate and not subject to consistent or fundamental biases. This approach of comparing a new technique to an existing one is the standard approach for demonstrating that the new technique meets measurement needs. The approach in this project used non-MBT based methods, which rely on fundamentally different underlying approaches (e.g., growing cells/counting them directly) versus extracting and quantifying deoxyribonucleic acid (DNA) molecules (as qPCR does). Obtaining similar results between qPCR methods and PCR-independent enumeration methods, including microscopy and plate counts, would ultimately increase our confidence in qPCR analysis.

4.1.1 Approach

For enumeration of cells independent of molecular tools, direct counts using microscopy (*Dhc* and *Escherichia coli* [*E. coli*]) and colony counts (*E. coli*) were employed. To verify *Dhc* levels, the *Dhc* strain BAV1 culture was used. Unlike an environmental sample, the BAV1 culture is solely compromised of *Dhc* organisms, and thus, organism counts and qPCR measurements should theoretically be equal. Direct cell counting (20 fields each) using a microscope and acridine orange cell staining were conducted on the same *Dhc* strain BAV1 culture, from which DNA was extracted for qPCR (for detailed methods see Appendix A). Direct counts (10 fields) using an alternate cell staining method (SYBR Gold) were also performed at the University of Delaware (See Appendix A). Enumeration of cells by qPCR (6 replicate 5 mL samples) using three distinct primer sets targeting the *Dhc* 16S RNA gene, the BAV1 VC-RDase (*bvcA*) gene and universal ("all bacteria" 16S rRNA) were compared to the direct microscopic counts.

For *E. coli* enumeration, five independently inoculated *E. coli* (strain TOP10 attTn7:*luc*) cultures were grown at 37 degrees Celsius (°C) with shaking for 15 hours. Two (2) milliliters (mL) of each culture were used for DNA extraction prior to qPCR assays. Both a single copy of the *luc* gene inserted into the *E. coli* genome (See Section 5.3) and the single-copy *E. coli dxs* gene were used as targets for qPCR assays (for detailed methods see Appendix A). Due to the fact that both of these qPCR targets occur as single copy genes on the *E. coli* chromosome, the gene copy number was expected to approximate the total cell numbers in actively growing cultures.

4.1.2 Results

Results for quantification of *Dhc* and *E. coli* cultures by qPCR and non-molecular methods are summarized in Figure 4-1. *Dhc* quantification by microscopy typically ranged from 0.5-5 x 10^7 cells per mL, using any of the methods available (Figure 4-1A/B). Acridine orange and SYBR Gold based counts represent the mean cell numbers, and similar abundances were recorded for the culture in several independent experiments. *E. coli* plate counts, which quantify viable organisms, were also very similar to both microscopic and qPCR results targeting the *dxs* or the *luc* genes (Figure 4-1C).

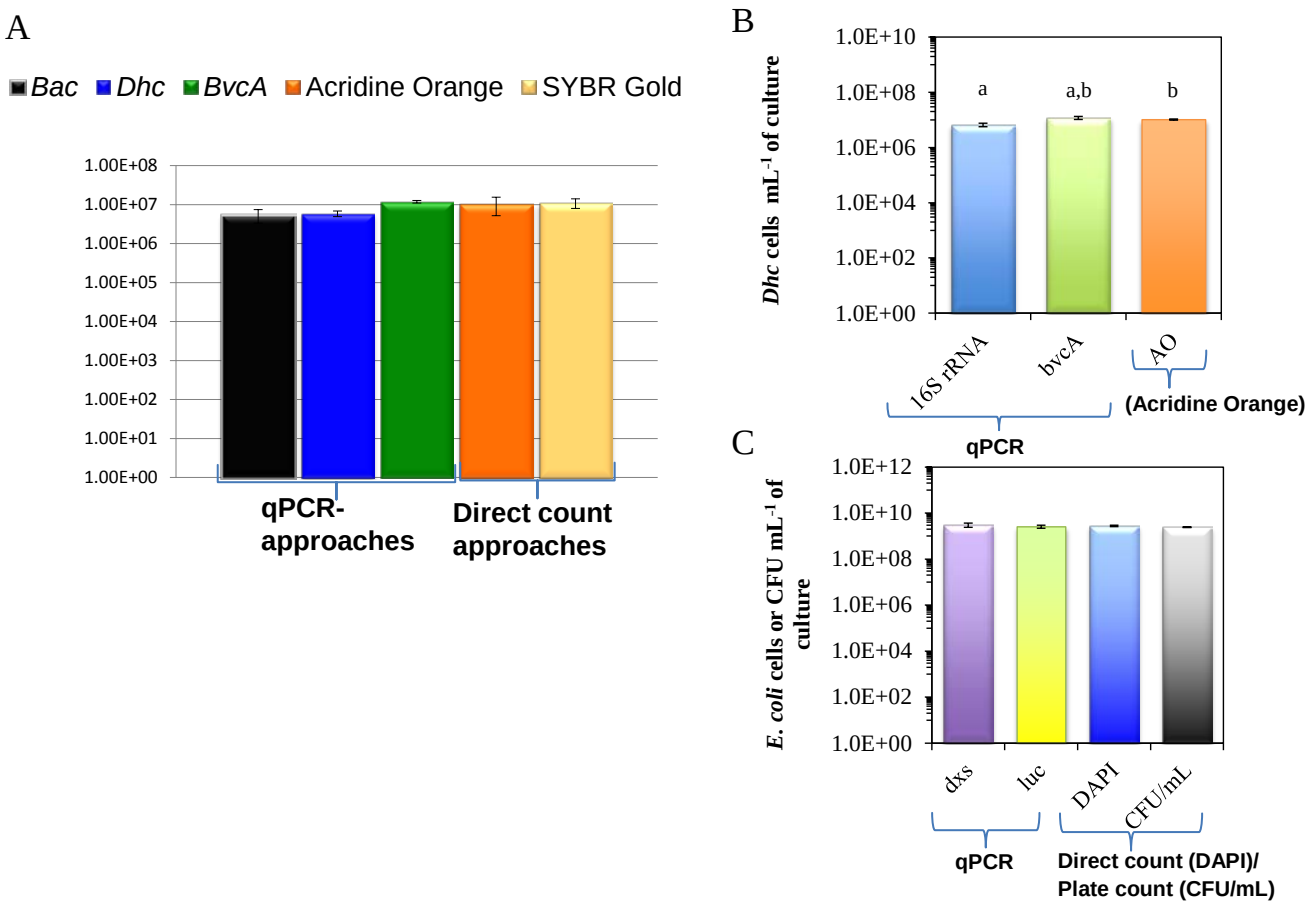

Figure 4-1: Summary of enumeration results of *Dhc* and *E. coli* by qPCR and other microbiological methods. Each panel represents an individual experiment performed with different samples. Panel (A): enumeration of a *Dhc* cells in a *Dhc* strain BAV1 culture by qPCR methods with 3 primer sets targeting general *bacteria* 16S rRNA genes (black), the *Dhc* 16S rRNA gene (blue) and the *Dhc bvcA* functional gene-specific target (green). Results for the three primer sets were virtually identical and were very similar to direct microscopic counts (orange and gold bars). Panel (B): qPCR results for *Dhc* using the 16S rRNA and *bvcA* targeted primers were virtually identical to direct counts using acridine orange dye Panel (C): enumeration of *E. coli* cultures using qPCR methods targeting the *dxs* and *luc* genes by qPCR methods (purple

and yellow bars) indicated similar enumeration results to microscopy using DAPI staining and plate counts (colony forming units [CFU]/mL). The combined results of these experiments indicated qPCR methods and classical enumeration methods provided similar results for both *Dhc* and *E. coli* cultures.

The experiments conducted comparing qPCR methods to microscopy and plate counts, indicated that for pure cultures maintained in the laboratory (i.e., not environmental remediation samples) it was possible to obtain similar enumeration results for *Dhc* and for *E. coli* using direct microscopic counts, plate counts (for *E. coli*) or qPCR. Encouragingly, these data suggested that no significant obvious bias associated with the qPCR approaches for these microorganisms that would make ongoing enumeration using qPCR challenging. Two follow up questions related to the accuracy of qPCR methods included:

1) Do environmental remediation samples (as opposed to pure laboratory cultures) pose additional challenges to accurate enumeration of *Dhc*?

2) Can different laboratories consistently and accurately quantify *Dhc* in groundwater samples?

These questions were addressed in the following experiments and summarized in the following sections.

4.2 Multi-Laboratory Analysis to Assess Baseline qPCR Method Variability (DNA)

A series of multi-laboratory analyses (i.e., "round robins") were carried out, which involved the distribution of test materials (DNA, cells, groundwater, etc.) to the following five labs:

1) Edwards Lab (University of Toronto [UofT]);

2) Löffler Lab (Georgia Institute of Technology [GT], and University of Tennessee [UTK]);

3) Microbial Insights (Rockford, Tennessee [MI]);

4) SiREM (Guelph, Ontario); and

5) Yeager Lab (Savannah River National Laboratory [SRNL], then Los Alamos National Laboratory [LANL]).

In reporting the round robin results, the labs are identified only by numbers or letters to maintain focus on overall variability/consistency as opposed to the results obtained in any particular laboratory. Note number or letter designations were not necessarily consistent throughout the study (i.e., 'Lab 1' 'Lab 3' etc. are not necessarily the same lab between different round robins).

The goal of the first inter-laboratory comparison (Round Robin 1) was to assess the existing variability of qPCR data generated in the different analytical laboratories using the same DNA sample. DNA was used first because it eliminates differences in sampling, extraction and biomass handling effects. The purpose of this study was to determine if significant differences were associated with just the analytical (i.e., qPCR) steps, as compared to variation associated with the entire sampling and analysis chain including biomass collection, cell lysis, and DNA extraction. Variation with identical DNA could be expected due to:

1) qPCR calibration approach;

2) PCR chemistry (SYBR Green Versus TaqMan detection chemistry); and

3) Other variables including-pipetting accuracy, laboratory personnel technique, instrument and PCR reagent differences.

4.2.1 Approach

Briefly, identical desiccated DNA samples at three unknown concentrations M1, M2, and M3 (i.e., it was a blind study) were shipped to the five participating labs. Upon arrival, the samples were suspended in an identical volume of buffer and quantified using each lab's standard qPCR method. Each lab quantified the three samples and the mean results from three qPCR replicates were reported. For detailed methods refer to Appendix B. The data was analyzed using a 2-way Analysis of variance (ANOVA) considering lab and concentration. In addition to the main effects, an interaction term for lab and *Dhc* abundance was also included (Appendix C).

4.2.2 Results

The qPCR results obtained for Round Robin 1, using plasmid DNA containing the *Dhc* 16S rRNA gene, are summarized in Figure 4-2.

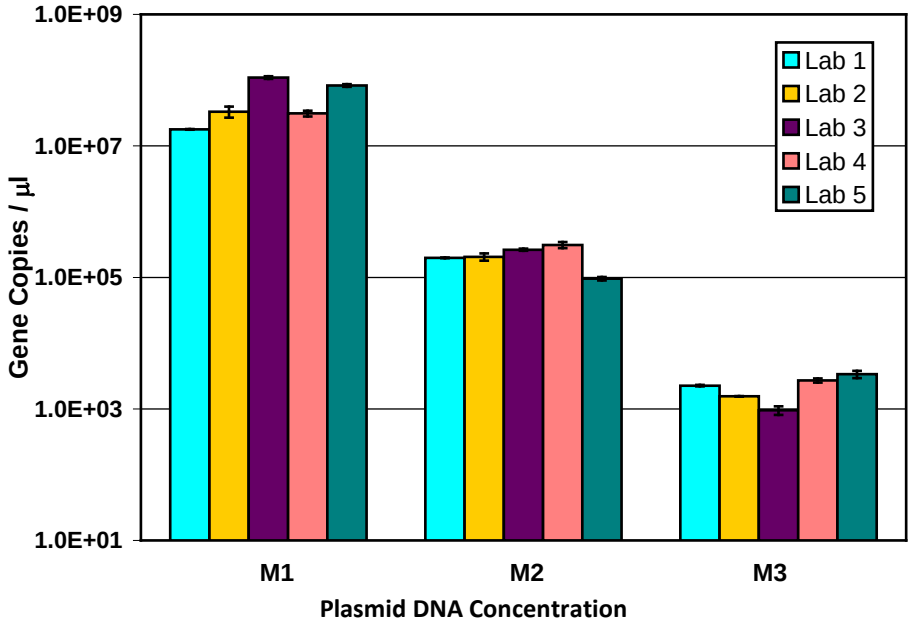

Figure 4-2: Round Robin 1, qPCR quantification of plasmid DNA containing the *Dhc* 16S rRNA gene at three concentrations distributed to five participating laboratories. The results indicated that sample to sample variability (within labs) was relatively low (0.34 fold) whereas between labs variability was higher with up to 6-fold differences in means between the highest and lowest reporting labs. Based on total DNA quantification using NanoDrop in the source lab the expected concentrations of the standards were M1: 1.7 x 10^7 gene copies μL^{-1}, M2: 1.7 x 10^5 gene copies μL^{-1} and M3: 1.7 x 10^3 gene copies μL^{-1}.

The results of Round Robin 1 indicated (See Appendix C):

1) No lab consistently produced high or low results across the range of concentrations considered;

2) Within laboratory variability was relatively low with a maximum variation between replicates within the same laboratory of 39 % (0.39-fold);

3) Inter-laboratory (between lab) variability was more significant with a maximum of 600% (6-fold) variation between the highest and the highest and lowest mean values reported; and

4) Overall, the data indicated that differences between labs were statistically significant.

The results of Round Robin 1 suggested that the laboratories produced quite consistent data (high precision) but that there were significant differences between the highest and lowest results between labs. The reasons for the observed differences could have included differences in methodology/equipment (qPCR chemistry, qPCR reagents, qPCR instruments plastic-ware or calibration approaches) or personnel and methodological differences such pipetting, and differences in the re-dissolving of DNA stocks and the stability and incubation times of DNA stocks. Many of these issues were examined in later stages of the project. A further examination of laboratory differences was carried out in a second round robin (Round Robin 2), where whole *Dhc* cells were distributed to each participating lab (Section 4.3).

4.3 Multi-laboratory Quantification of Whole *Dhc* Cells

Building on the data obtained in Round Robin 1 (DNA) a second round robin using whole *Dhc* cells spiked into artificial groundwater was performed. The purpose of this test was to determine baseline variability within and between labs when the entire process including biomass collection, DNA extraction and qPCR analysis were performed. Due to the more complicated logistics of this experiment a Quality Assurance Project Plan (QAPP) entitled *Round Robin Simulated Groundwater Dhc Analysis* was developed under the guidance of the Environmental Protection Agency (EPA). The purpose of this document was to provide a framework for a multi-laboratory comparison of samples spiked with *Dhc* cells (Attachment 2). The QAPP was submitted to Mr. Scott Jacobs of the US EPA for review, and was approved on 3 September 2008.

4.3.1 Approach

Briefly, *Dhc* (KB-1 culture) spiked simulated groundwater samples and blanks were prepared as described in the QAPP (for detailed methods see Attachment 2). Each lab was provided with 12 bottles, each containing 500 mL of a simulated groundwater sample. These 12 samples included 5 replicate samples at high *Dhc* abundance, 5 replicate samples at low *Dhc* abundance, and 2 samples with no *Dhc* (blanks). Samples were shipped from the source lab (University of Toronto) on 29 September 2008 to the four other participating labs. The receiving laboratories were not informed of the expected *Dhc* abundance in each of the samples (i.e., it was a blind study). Upon completion of the experiments by the different laboratories, the qPCR data were submitted to the EPA for compilation and preliminary statistical analysis. One laboratory concentrated the samples by two methods Sterivex cartridge and centrifugation to compare the impact of different biomass collection approaches, the other four labs used filtration only.

4.3.2 Results and Discussion

Data were summarized according to the procedures outlined in Attachment 2. The data are presented below in Figure 4-3 that exhibits the replicates for the blanks (negative control-no added *Dhc*) and the high and low *Dhc* spikes for each of the five participating labs. Laboratory 2,

which collected biomass using both centrifugation and the filtration method, returned similar results for the two methods although centrifugation netted somewhat lower overall enumeration.

Figure 4-4 presents mean *Dhc* enumeration of the high and low spikes in order to more easily compare the overall quantitative performance of each of the labs. In this figure, identical geometric symbols indicate statistically similar quantification. Statistical similarity in this context is defined as being within a 3-fold difference of the mean at a 95% confidence level. This definition of reasonable (3-fold) variability for between lab data was agreed upon in discussions within the project team. A 3-fold difference was considered low enough to fall below what remediation practitioners are likely to consider consequential, and results within 3-fold are effectively considered the same for practical purposes.

Overall the variability observed in Round Robin 2 (whole *Dhc* cells) was higher than that observed in Round Robin 1 (DNA only) and the following observations were made:

1) The whole *Dhc* cell round robin demonstrated that replicate simulated groundwater samples were a better measure of data variability compared to "pseudoreplicates" (multiple aliquots from the same DNA extract removed immediately before qPCR measurement). This is most likely because biomass concentration and nucleic acid extraction introduces variability. Therefore it is important to collect and analyze multiple samples to understand the variability in MBT measurements.

2) Based on Power curves analysis of 5 replicates would be sufficient to distinguish results between 2 labs in most cases.

3) False positives (positive results in blanks) were not reported by any of the labs, false negatives were reported by one lab (Lab 5) in 2 out of 10 samples;

4) Within lab variability was much higher than observed in the DNA round robin with sample-to-sample variability of up to 1,000-fold observed in two out of five labs, the high variability observed in these labs was most likely due to DNA extraction issues, due to methodological errors, or other technical reasons that are not fully understood;

5) When the means of each lab's data were reported, maximum variability was over 40-fold between labs for low *Dhc* spikes and 12-fold for high *Dhc* spikes, significantly higher than maximum 6-fold between lab variability observed in Round Robin 1 (DNA); and

6) Based on the increased variability observed between the whole cell round robin (40-fold) and the DNA round robin (6-fold), it is evident that cell concentration and DNA extraction have substantial impacts on the overall variability of the analysis.

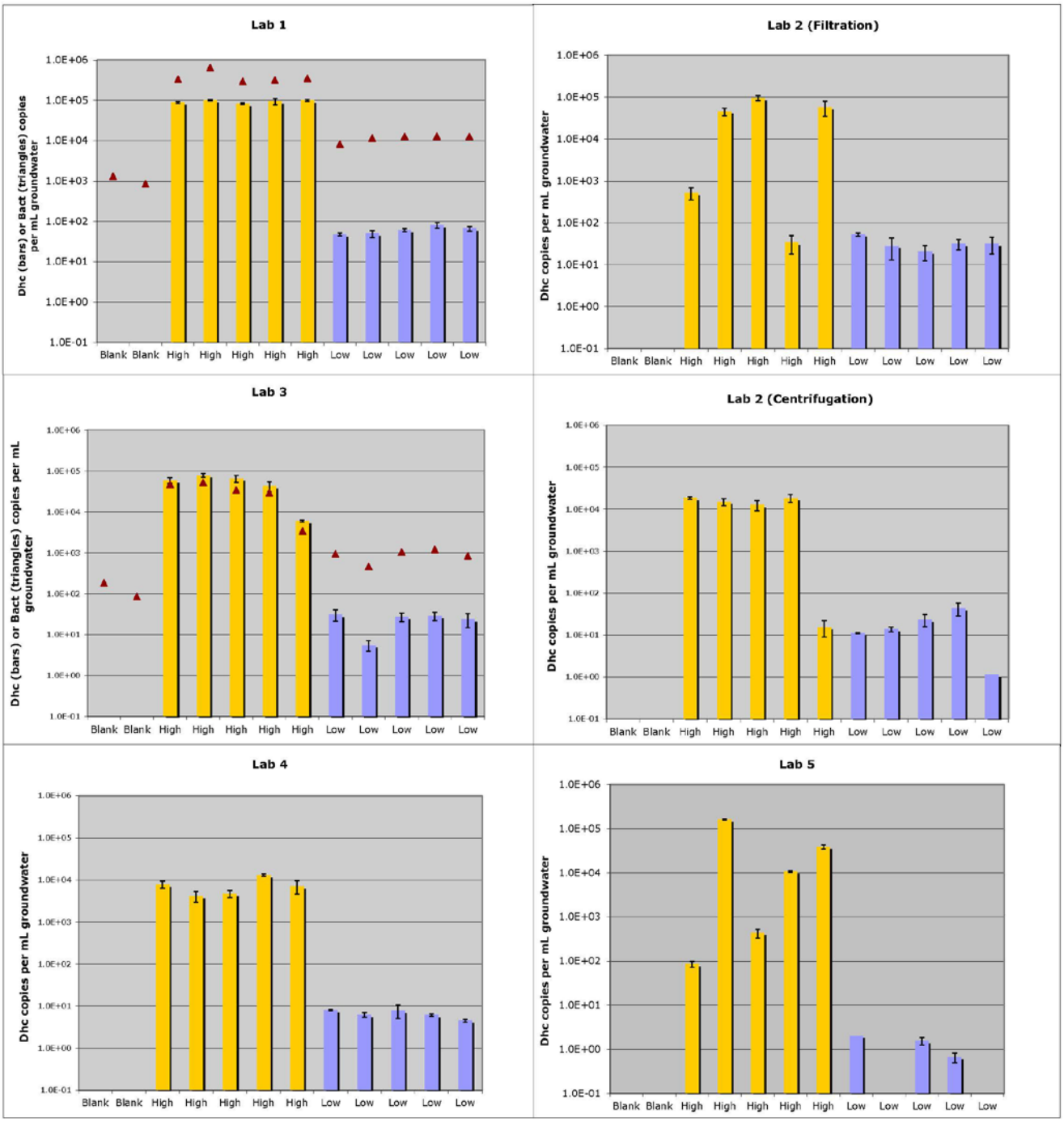

Figure 4-3: Summary of results for Round Robin 2 (whole *Dhc* cells). Five labs participated; one lab (Lab 2) reported data for both filtration/centrifugation biomass collection methods. Each

lab used their own sample preparation and qPCR analytical methodologies to determine *Dhc* 16S rRNA gene copies per mL in 12 samples that included 5 replicates at low *Dhc* abundance, 5 replicates at high *Dhc* abundance, and 2 blanks with no added *Dhc* (i.e., *Dhc* would not be expected unless it was as a contaminant). Error bars indicate variation associated with qPCR [pseudo] replicates. Two labs also enumerated the total bacterial 16S rRNA gene copies (red triangles).

Figure 4-4 provides the mean values of the five replicates presented in Figure 4-3. Labs reporting results where the ratio of mean *Dhc* enumeration, between labs, fell within a factor of three (based on t-tests performed at a 5% level of significance) are indicated by identical geometric shapes. Four out of five labs (circles) returned results within 3-fold for the high *Dhc* spike and three out of five labs (triangles) returned results within 3-fold for the low *Dhc* spike.

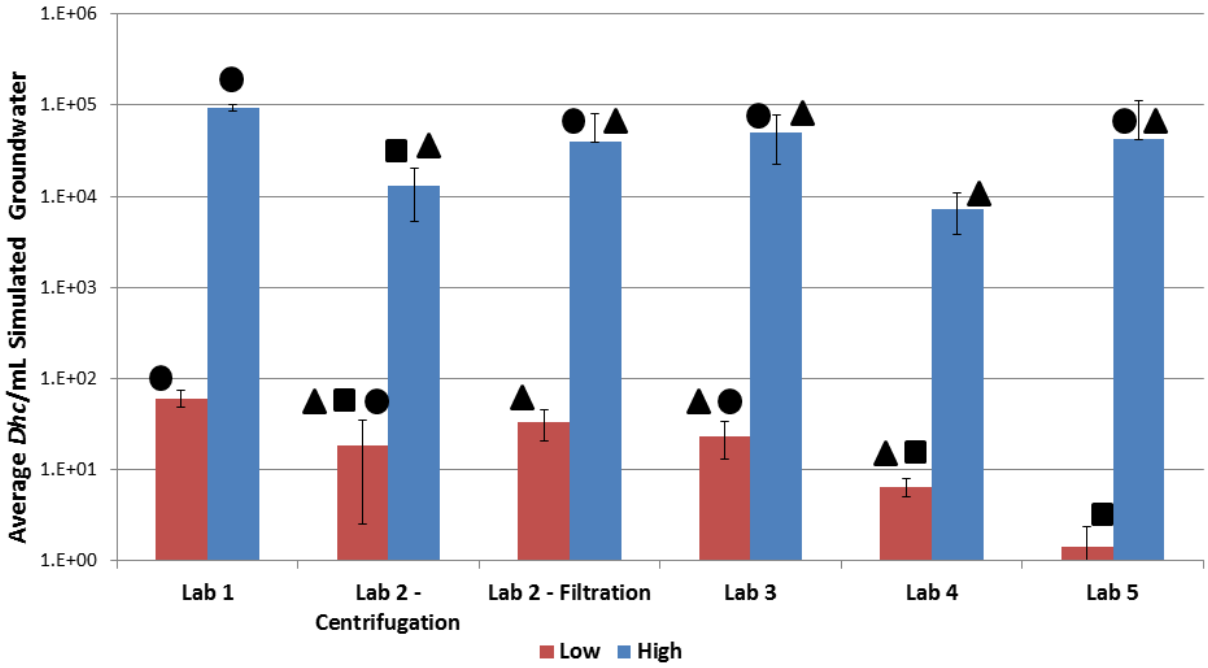

Figure 4-4: Mean *Dhc* enumeration for high and low *Dhc* samples in simulated groundwater for five labs (Round Robin 2). Mean values for the low *Dhc* spike varied by more than 40-fold between Lab 1 and Lab 5 and approximately 12-fold between Lab 1 and Lab 4 for the high *Dhc* spike. Identical symbols indicate labs with no statistically significant differences (exceeding a factor of 3 [i.e., a maximum 3-fold difference]) in mean *Dhc* quantification.

These data indicate overall variability was proportionally lower for the high abundance *Dhc* samples. The data also indicate that centrifugation and filtration for cell concentration returned essentially identical results for Lab 2.

4.4 Conclusions of Baseline Variability Analysis

At the outset of the project, the accuracy of qPCR methods in terms of how they compare with other accepted methodologies and how consistent results were within one laboratory and between different laboratories were not well characterized. The baseline variability analysis indicated that:

1) qPCR can closely mirror other accepted microbial enumeration results such as microscopy and plate counts in pure cultures.

2) qPCR methods can accurately quantify genes of interest, including *Dhc* biomarker genes.

3) In some cases differences in mean *Dhc* enumeration both between and within labs was higher than optimal.

The overall assessment at the conclusion of baseline variability analysis was that while qPCR methods have the potential to accurately quantify *Dhc*, variability at the beginning of the project was higher than optimal (i.e., greater than 3-fold). Efforts to better understand and control variability would improve confidence and the interpretability of qPCR analysis as applied to environmental samples. These efforts are summarized in Sections 5-9 of this report and include method optimization and development of internal controls.

5 DEVELOPMENT OF INTERNAL CONTROLS

A key need identified in the project proposal (Tasks 2A/B) was a requirement for incorporating an internal control (IC) to improve the accuracy and interpretation of results and confidence of the end user in the data provided by MBTs applied to environmental samples.

ICs are added directly in known quantities to the assay, or sample materials, and are co-monitored with the analyte of interest throughout the extraction and testing procedure to quantify losses. Therefore, ICs can provide crucial information including the:

1) Assessment of analyte losses (e.g., sorption, degradation) in the extraction and analytical process;

2) Assessing matrix inhibition (e.g., PCR inhibitors); and

3) Ongoing assessment of test effectiveness including assessment of laboratory personnel and techniques.

In the technology/literature review, a number of studies reported the use of ICs in qPCR methods. Internal controls have been used to compensate for incomplete recovery of biomarkers, sample deterioration (i.e., biomarker degradation), and the presence of PCR inhibitors (Muska et al., 2007), all of which can compromise data quality in qPCR analysis.

Internal controls in qPCR often consist of non-cell associated DNA such as plasmids *(Cubero et al.*, 2001; Koike et al., 2007), salmon sperm DNA (Haugland et al., 2005), synthetic oligonucleotides (personal communication, Dora Ogles, Microbial Insights), Lambda DNA (Mumy and Findlay, 2004) or M13 Phage DNA (Sum et al., 2004). DNA internal controls are typically added to the extracted (i.e., purified) sample DNA or directly to the qPCR assay to quantify losses or inhibition during amplification. Clearly, this approach does not provide information about losses occurring prior to, or during, DNA extraction including incomplete recovery of target cells from the environmental sample material, incomplete cell lysis, and inefficient DNA extraction, etc.

It was identified by the project team that the shortcomings of DNA internal controls could be addressed through the use of whole cell internal standards that could be added directly to the environmental sample or during the DNA extraction process. Reports of the use of whole cell internal controls in the peer-reviewed literature were less common than DNA internal controls. Nevertheless, their use was reported in the literature for qPCR analysis. In one study, the yeast *Geotrichum candidum* was added to the DNA extraction bead tube in a procedure to quantify pathogenic *Candida* species (Brinkman et al., 2003). *E. coli* with a plasmid containing a target DNA sequence was used as whole cell internal control in a method for the quantification of *Salmonella* (Klerks et al., 2006). *E. coli* was also used as a whole cell internal control in an assay for *Helicobacter pylori* in drinking water (Sen et al., 2007). The testing and development

of various strategies for a microbial internal amplification controls (MIACs) is provided in the following sections.

5.1 Introduction to Microbial Internal Amplification Controls (MIACs)

A microbial internal amplification control (MIAC) is a specialized type of internal control used in qPCR assays. An MIAC consists of whole microbial cells with an appropriate target gene sequence, which can assess the efficiency of the entire testing process from cell recovery, nucleic extraction and qPCR analysis. MIACs are analogous to chemical surrogates (target chemical analyte labeled with deuterated hydrogen) used in standard analytical chemistry methods.

An MIAC could be introduced into a sample immediately following groundwater sampling, or at the analytical laboratory prior to, or following, biomass collection. Thus, the MIAC could be carried with the groundwater sample through the entire analytical procedure including shipping, storage, biomass collection, nucleic acid extraction, and qPCR.

Specifically, a properly designed and used MIAC could be beneficial for:

- Determining the efficiency (i.e., percent recovery) of individual processing steps and the overall procedure;
- Detecting matrix interferences caused by humic acids, metals and other compounds present in groundwater;
- Flagging, and potentially correcting for human error; and
- Ongoing (non-PCR-based) external verification of PCR methods, because the MIAC culture, unlike the target *Dhc* cells, are readily assayed in a plate count assay or other non-PCR-based assays such as microscopy.

The use of MIACs was found not to have been incorporated in commercial methods for the quantification of environmental remediation samples as we surveyed the literature.

When a MIAC is needed for environmental sample analysis, the project team identified that the MIAC would ideally have the following characteristics:

- MIAC nucleic acid sequence(s) would not be present in groundwater;
- The filtration and sedimentation, as well as the cell wall characteristics of the MIAC organism would be similar to the target cells (e.g., *Dhc*) to ensure similar cell recovery and cell lysis efficiency;
- Unique nucleic acid sequences in the MIAC would be quantifiable using qPCR methods;

- Nucleic acid sequences within the MIAC would not interfere with quantification of target microorganism;
- A PCR-independent method for enumeration of the MIAC organism would be available (e.g., plate counts or microscopy methods); and
- The organism must be non-pathogenic and not a spore former.

Efforts to develop a MIAC with the above characteristics are summarized in the following subsections.

5.2 Development of MIACs

A number of different MIAC approaches were tested under the project with a modified *E. coli* ultimately proving most effective. The following options were tested for their utility as a MIAC in *Dhc*-focused qPCR tests:

1) Naturally occurring (non-*Dhc*) microorganisms (Section 5.2.1);
2) An *E. coli* carrying a plasmid with a mutated *Dhc* 16S rRNA gene (Section 5.2.2); and
3) A genetically modified *E. coli*, with its chromosome carrying a non-microbial gene suitable for enumerating the host cells (Section 5.3.3).

Naturally-occurring microorganisms have the advantage of not requiring genetic modification and have a wide variety of size and cell wall characteristics. Due to our ability to genetically manipulate *E. coli*, MIACs based on this microbe could include custom inserted genetic sequences that make detection of the MIAC highly specific. Details of experiments carried out to test the above options are provided in the following subsections.

5.2.1 Naturally-Occurring Microorganisms as MIACs

Natural MIACs are defined under this project as non-genetically modified microorganisms, which possess characteristics that make them suitable for use as internal controls in an assay of interest. For *Dhc* assays, several candidates for naturally-occurring MIACs were considered based on the following properties:

- A cell size similar to *Dhc*;
- The ability to enumerate using plate counts;
- Availability in cell culture collections; and

- Expectations the microbe would not be found in groundwater.

Based on the above criteria, three microorganisms were identified as potential natural MIACs for *Dhc* assays. These were included *Brevundimonas diminuta* (*B. diminuta*), *Micrococcus luteus* (*M. luteus*) and *Prochlorococcus marinus* (*P. marinus*). These three microorganisms are spherical or coccoid organisms not unlike *Dhc*, which is small and disk shaped.

B. diminuta is small and approximately 200 nm in diameter (Lee et al., 2002). For this reason, *B. diminuta* (ATCC-1946) is used in standard protocols to validate the performance of 0.2 µm (micrometer) i.e., 200 nanometer (nm) filters (ASTM, 2007). *M. luteus* (ATCC-4442) is a somewhat larger (1,000 nm) spherical bacterium (Madigan et al., 2006). *P. marinus* is a small marine photosynthetic cyanobacterium (Ahlgren, 2006) and as this microorganism requires light to grow, it was expected to be absent from subsurface environments, including aquifers.

M. luteus, B. diminuta and *P. marinus* met many of the criteria for MIAC in that they have similar cell sizes as *Dhc*, were culturable, and therefore could be enumerated by PCR-independent methods such as plate counts, were available in culture collections, and were non-pathogenic. Further testing was required to determine if these organisms would be absent from groundwater.

Approach

B. diminuta and *M. luteus* were purchased from the American Type Culture Collection (ATTC), which also provided media and protocols for propagation of these microorganisms]; *P. marinus* was purchased from Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP) including growth media and genomic DNA from related microorganisms.

16S rRNA gene based PCR methods targeting *B. diminuta* and *M. luteus* were developed by the project team including primer sequences and optimized PCR methods (Appendix D). PCR methods for *P. marinus* were performed as described by Ahlgren et al. (2006). These methods were used to determine if these microorganisms could be detected in groundwater samples from a variety of contaminated sites.

Figure 5-1: *P. marinus* is a photosynthetic marine organism and was one of three natural microorganisms tested under the project as a possible natural MIAC for qPCR assays.

Results

In groundwater screening tests, both *B. diminuta* and *M. luteus* 16S rRNA genes were widely detected in groundwater samples. The analysis indicated that a majority (18/25) of the groundwater samples tested positive for *B. diminuta* and around half (12/25) tested positive for *M. luteus* DNA (Appendix D). Due to the common presence of *B. diminuta* and *M. luteus* in groundwater DNA, these two microorganisms were not examined further for use as a potential MIAC as they violated one of the key requirements not being present in groundwater.

In an initial screening with 25 randomly selected groundwater samples, *P. marinus* were not detected (Appendix D). However, further screening of groundwater samples obtained from coastal areas in California, Washington, North Carolina, Florida, Texas, and Maryland revealed the presence of *P. marinus* in 65 of the 77 groundwater samples (Data not shown Personal Communication Dora Ogles, Microbial Insights). These samples tested positive for amplification with the *P. marinus* 16S rRNA gene-targeted primer set (Ahlgren, 2006). Thus, the majority (greater than 85%) of groundwater from coastal states contained *P. marinus* or a related organism, violating a key criterion for an acceptable MIAC. The frequent detection of *P. marinus* in aquifers of coastal states led to the conclusion that *P. marinus* would also not be a suitable MIAC and the natural MIAC approach was abandoned.

5.2.2 Modified *Dhc* 16S rRNA Gene as an Internal Amplification Control

The difficulty finding naturally-occurring MIACs, due to the presence in groundwater of all tested microorganisms, led the project team to consider a different approach. An alternative MIAC consisting of a genetically modified *E. coli* strain with a mutated *Dhc* 16S rRNA gene was tested. The likelihood of the artificially mutated sequence being present in groundwater samples would be very low; however, possible cross reactivity with wild-type (non-mutated) *Dhc* 16S rRNA gene had to be evaluated.

This approach involved introducing a plasmid (a small circular extrachromosomal piece of DNA commonly found in bacteria) carrying a mutated version of the *Dhc* 16S rRNA gene into *E. coli.* The cells could then be added to samples and be tracked independently from the wild type *Dhc* 16S rRNA gene. One advantage of this approach is that the internal control amplicon shares the same primer binding sites with the analyte (wild type *Dhc* 16S rRNA gene) thus avoiding PCR biases associated with multiple primers sets.

Ideally, the use of TaqMan probes carrying different colored fluorophores would allow multiplex applications to enumerate both target sequences simultaneously in the same PCR tube using the approach demonstrated in Figure 5-2.

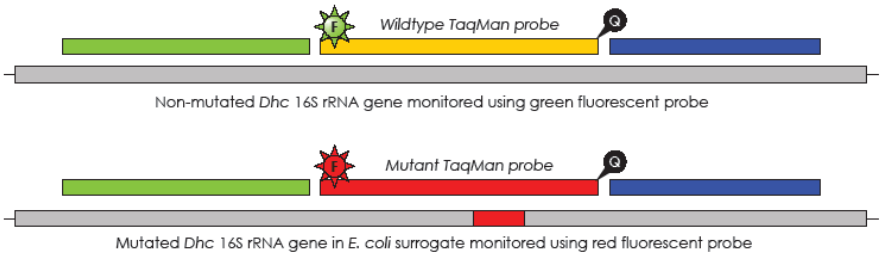

Figure 5-2: Use of multiplex PCR for monitoring mutated and non-mutated (wild-type) *Dhc* 16S rRNA genes simultaneously. Primers (green/blue) are the same for both target sequences. The probe labeled with the green fluorophore binds only the wild type *Dhc* sequence, whereas the probe labeled with red fluorophore binds only the mutated *Dhc* sequence. This approach could allow co-monitoring of the internal control (red fluorescence) and the *Dhc* target (green fluorescence) in the same qPCR reaction tube.

Approach

A mutated internal control version of the *Dhc* 16S rRNA gene was generated (see Appendix E for detailed methods). This was accomplished by mutating the *Dhc* strain BAV1 16S rRNA gene and introducing the mutated gene into an *E. coli* host using a single copy plasmid vector. The mutated 16S rRNA gene carried in this vector was expected to be maintained in *E. coli* at one copy per cell. In qPCR analysis, the *Dhc* 16S rRNA wild-type gene and the *Dhc* 16S rRNA mutated gene were both amplified with the same primers but detected with different TaqMan probes. A genetically modified *Dhc* 16S rRNA gene was constructed by introducing a four-base pair mutation in the wild-type sequence targeted by the TaqMan probe. Experiments with qPCR verified that the TaqMan probe targeting the wild-type *Dhc* 16S rRNA gene did not bind to the mutated 16S rRNA gene. Conversely, the probe targeting the mutated 16S rRNA gene did not bind to the wild-type *Dhc* 16S rRNA gene. A multiplex TaqMan approach was tested using probes targeting the wild-type and mutated *Dhc* 16S rRNA genes carrying different fluorophores (Figure 5-2).

Results

In singleplex assays, both the wild-type and mutated *Dhc* 16S rRNA gene targets were accurately quantified. The multiplex approach also quantified both the wild-type and the mutated *Dhc* 16S rRNA genes; however, the dynamic range of target gene detection in the presence of the competitive mutated sequence was limited. Specifically, quantitative data for both mutated and wild-type target genes were only obtained when both template DNA sequences were present at abundances within 1% of each other. If one of the populations fell below 0.1% of the total target DNA, the one of lesser quantity was not detected.

The use of a single primer set and two different probes for quantitative assessment of the *Dhc* 16S rRNA gene target (i.e., the wild-type *Dhc* 16S rRNA gene target) and the IC in a multiplex qPCR assay yielded accurate quantification only when the wild type target and the IC template were present in similar abundances (less than a 10-fold difference). When either template exceeded the amount of the other by a factor of 10 or greater, preferential amplification of the more abundant target occurred and accurate quantification of the low abundance target was not possible. Hence, this approach only worked when the IC was added in abundance ranges within 10-fold below or above the actual *Dhc* target gene amount (Appendix E). Obviously, this requires *a priori* knowledge of the abundance of the *Dhc* target gene, and hence, is applicable to very few sites. Since *a priori* knowledge of *Dhc* abundance is not always available, another approach that targets the *Dhc* 16S rRNA gene and an artificial gene introduced into *E. coli* serving as an MIAC was explored as a more practical solution.

5.3 Development of a Genetically Modified Microbial Internal Amplification Control Containing the Luciferase Gene

As an alternate approach for developing a MIAC, the project team considered the use of a genetically modified *E. coli*. In this case the *E. coli* would be modified to contain a plasmid based, or a chromosomal insertion (Hatt et al., 2013), that would allow for its unambiguous identification in an environmental sample. These unique gene sequences would ideally be present in a single copy per genome, and the *E. coli* "mutant" would be easily cultured in the laboratory.

The approach used was to develop an *E. coli* construct that contained the *Photinus pyralis* (firefly) luciferase gene (*luc*). The advantages to this approach are:

- The luciferase (*luc*) gene encodes an enzyme involved in light production in bioluminescent organisms and is highly unlikely to be found in groundwater as was the case for natural MIAC tested;
- *Dhc* target genes and the luc gene are amplified with different primer sets therefore avoiding preferential amplification of the more abundant target.

A potential drawback of the above approach is that the amplification efficiency of target (*Dhc*) and the MIAC *luc* templates could differ. Therefore, primer design had to ensure specific amplification of the *luc* gene with comparable efficiency compared to the *Dhc* target gene amplification. The *luc* gene is available commercially for both DNA and ribonucleic acid (RNA) control purposes and is a common reporter gene used for assessing gene regulation in biomedical research (O'Shaughnessy et al., 2002; Lee et al., 2012; Hatt & Löffler, 2012). A plasmid pGEM-*luc* carrying the *luc* gene that can be used for generating standard curves is commercially available from Promega Corporation.

Two approaches to introduce the *luc* gene into *E. coli* for use as an MIAC were tested:

1) The use of an *E. coli* transformed with a plasmid construct (Section 5.3.1); and

2) The introduction of the *luc* gene directly into the E. coli chromosome (Section 5.3.2).

5.3.1 Testing of an *E. coli* construct carrying the *luc* gene on plasmid (extrachromosomal element) as an internal control

Approach

The *E. coli* strain EPI300 containing a single copy pCC1 plasmid carrying the *luc* gene target (pCC1-Luc) was isolated for use as an MIAC. To explore if the *luc* gene in the transformed *E. coli* strain could be accurately quantified, the engineered strain was cultured in LB at 37ºC with shaking and 1 mL samples were taken during early-log phase (OD600 ~ 0.1), mid-log phase (OD600 ~ 1.0) and late-log phase (OD600 ~ 2.0). To mimic the treatment of environmental groundwater samples, culture samples were filtered using 0.2 µM Durapore GVWP filters and DNA from biomass collected on the filters was isolated using the MOBIO PowerSoil® DNA Extraction Kit. Gene copies for a single-copy *E. coli* gene encoding the D-1-deoxyxylulose 5-phosphate synthase protein (*dxs*) and the *luc* gene were determined by qPCR using SYBR Green and TaqMan chemistries, respectively, for each gene. Accurate quantification of both gene targets would result in a one-to-one ratio of *dxs* to *luc* gene copies.

Results

Three independent experiments indicated that the ratio of *dxs* to *luc* ranged from 2 to 8-fold greater than the expected 1:1 ratio, suggesting that one or both of the qPCR assays produced inaccurate results. Differences in the detection chemistry (i.e., SYBR Green vs. TaqMan) could have accounted for the differences observed. Alternatively, plasmid loss during the DNA extraction could have resulted in lower gene copies of the plasmid based *luc* gene compared to the *dxs* gene, which is located on the *E. coli* chromosome. Previous studies have reported that plasmid gene copy numbers show plasticity in fast-growing bacteria (i.e., the number of plasmids per cell are variable). These data indicated that the *E. coli-luc* MIAC, carrying the *luc* gene on a

plasmid, did not meet the criteria for an MIAC as there was not a 1:1 relationship between the *luc* target gene and the *E. coli* cell.

5.3.2 Generation of *E. coli* MIAC with *luc* gene integrated into the chromosome

The insertion of the *luc* gene into the chromosome (i.e., not a plasmid) generates a stable construct with single *luc* gene copy per cell. Genomic DNA is the type of DNA typically extracted from groundwater and assayed in qPCR analysis; therefore the MIAC quantification would use genomic DNA as with the target microbe (e.g., *Dhc*). More importantly, plasmid copy number per cell can vary and plasmids can be lost from the cell, as was observed in experiments outlined in Section 5.3.1. A chromosomal insertion is a much more stable target for quantification of microbes by qPCR.

Approach

An *E. coli* strain that carries the firefly *luc* on the chromosome was engineered using a transgene insertion vector pGRG36 (McKenzie and Craig, 2006) that utilizes the site-specific recombination machinery of the transposon Tn7 (for detailed methods see Appendix F). This vector facilitates a non-disruptive insertion of any gene into the site-specific Tn7 insertion site on the *E. coli* chromosome (Figure 5-3).

The chromosomal integration of the *luc* gene in the engineered strain was verified by screening for the presence of the *luc* gene using qPCR. Comparison of plate counts, microscopy and qPCR indicated a 1:1:1 relationship indicating that just one copy of the *luc* gene was inserted into the *E. coli* chromosome. The modified microorganism is referred hereafter as *E. coli-luc* MIAC, or simply the MIAC.

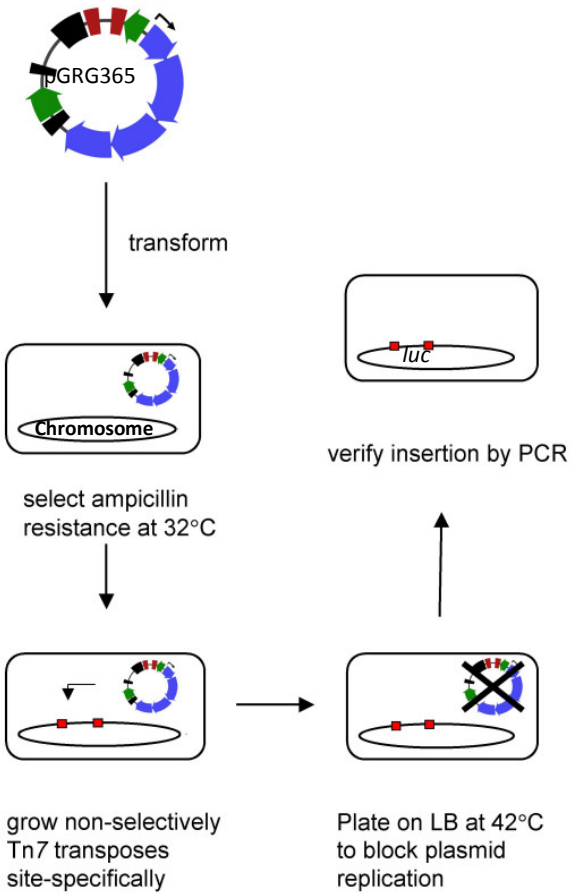

Figure 5-3: Process for inserting a gene (*luc*) into the *E. coli* chromosome using the transgene insertion vector. At the conclusion of the process, the *E. coli* contained one copy of *luc* in the bacterial chromosome, which was used to enumerate the organism by qPCR. Modified from McKenzie and Craig (2006).

Specificity testing of MIAC quantification and interference with *Dhc*-targeted qPCR

Tests to determine if the addition of the *E. coli-luc* MIAC to groundwater interfered with the quantification of *Dhc* biomarkers using a SYBR Green qPCR assay were performed by spiking varying amounts of the MIAC genomic DNA into qPCR reactions (in triplicate) with *Dhc* genomic DNA at 1.6×10^4 16S rRNA gene copies (Table 5-1). The data indicated no apparent interaction of the *E. coli-luc* genomic DNA on *Dhc* biomarker gene quantification based on the observation that the *Dhc* gene copy enumeration was virtually identical irrespective of the spiked amount of *E. coli-luc* DNA. This suggested that spiking whole cells of the MIAC into groundwater or MIAC DNA prior to DNA extraction would not affect the quantification of *Dhc* biomarkers and overcame a major barrier observed for the mutated *Dhc* sequences tested previously (Section 5.2). *E. coli-luc* spikes representing 1×10^4 copies per qPCR reaction was determined to represent an acceptable spike quantity sufficiently high to assess losses (2-3 orders of magnitude above qPCR detection limits).

Table 5-1: Enumeration of *Dhc* 16S rRNA genes in the presence of MIAC genomic DNA at varying abundances.

Spiked MIAC genome copies /reaction	Recovery of 1.6E+04 *Dhc* gene copies/reaction	Standard deviation of *Dhc* enumeration
1E+06	1.7E+04	5.9E+03
1E+05	1.4E+04	1.4E+03
1E+04	1.8E+04	2.0E+03
1E+03	1.6E+04	2.7E+03
0	1.6E+04	2.3E+03

Based on the verification that the *luc* gene was present in one copy per cell and, did not impact the quantification of *Dhc*, further testing and verification were performed to determine if the MIAC could meet the additional MIAC criteria and be handled consistently in multiple labs (Section 6.0).

5.4 Conclusions

Several approaches were tested to develop an effective MIAC for *Dhc* targeted qPCR assays; these included:

1) Testing of several naturally-occurring microorganisms determined their presence in groundwater indicating that they were unsuitable as MIACs;

2) The development of a plasmid-borne, mutated *Dhc* 16S rRNA gene was unsuitable due to its interactions with wild-type *Dhc* targets during PCR amplification;

3) The development of a *E. coli* with a plasmid borne *luc* gene which was found not to be present at a 1:1 ratio with *E. coli* cells and;

4) The development of a chromosomally-modified *E. coli* containing a luciferase gene.

Only the *E. coli-luc* MIAC with the chromosomal insertion of the *luc* gene proved to be useful for qPCR enumeration of spiked *E. coli* DNA without interference with *Dhc* biomarker gene enumeration. Based on the above, this MIAC was moved forward to additional testing summarized in Section 6.

6 TESTING AND VERIFICATION OF MICROBIAL INTERNAL AMPLIFICATION CONTROL

The data presented in this section were derived from the same multi-lab round robins, in which *Dhc* data were also collected (Sections 4 and 9). The MIAC specific aspects of this work are detailed separately in this section to highlight the testing and development of protocols for using the MIAC.

A chromosomally modified *E. coli*, with an inserted *luc* gene, compatible with qPCR for *Dhc* (i.e., the MIAC [Section 5.0]) was carried forward for additional testing to determine:

1) At what point in the extraction process should the MIAC be applied? (Section 6.1);

2) Can the MIAC be grown consistently and applied and quantified consistently in different labs? (Section 6.2);

3) Can the MIAC be quantified by multiple methods so that qPCR enumeration can be verified? (Section 6.3);

4) Can the MIAC be stored, making it practical for ongoing use in the analytical laboratory setting? (Section 6.4); and

5) Can the MIAC be used to detect PCR inhibition by compounds that might be found in some groundwater samples? (Section 6.5).

6.1 Impact of Point of Application of MIAC

The use of the MIAC requires that it be added to a site sample at some point in the analysis. Options include adding the MIAC directly to:

1) Groundwater samples (i.e., before filtration);

2) Sterivex cartridges used in onsite filtration protocols and containing concentrated biomass; or

3) The DNA extraction kit bead tube (after filtration).

The point of addition of the MIAC would allow losses associated with different steps to be assessed. For example, if the MIAC were added to the groundwater samples, it could be used to correct for losses associated with filtration. In contrast, if the MIAC were added to the DNA extraction bead tube (a post-filtration step), losses associated with filtration would not be quantified. The experiment described below assessed the impact of addition of the MIAC in different points in the analysis and the impact on the recovery efficiency.

6.1.1 Approach

A MIAC culture was quantified and was enumerated at 8.45 x 10^8 cells/mL using plate counts. Therefore assuming 100% DNA extraction efficiency a similar enumeration by qPCR would be expected. The experiment described below varied the addition point of the MIAC in the extraction process analysis to determine if the resultant qPCR enumeration was impacted by the point of addition (see Appendix G).

Briefly, simulated groundwater was spiked with a *Dhc* culture (KB-1) and was amended with the MIAC as follows:

1) MIAC was spiked directly into simulated groundwater followed by biomass collection with Sterivex cartridges; or

2) MIAC was spiked directly (i.e., preloaded) into Sterivex filters followed by biomass collection from simulated groundwater using the preloaded cartridge; or

3) MIAC was added directly to the DNA extraction kit bead tube.

DNA extractions were performed using the Ultra Clean®Soil DNA isolation kit (Mo Bio Laboratories Inc.) and the MIAC was enumerated using qPCR targeting the *luc* gene. For detailed methods see Appendix D.

6.1.2 Results

The proportion of the MIAC recovered was dependent on the point, at which the MIAC was added during sample processing. The following observations were made which are summarized in (Figure 6-1). Addition of the MIAC directly to simulated groundwater (treatment 1) resulted in a mean recovery of 28.4% ± 2.6 (high *Dhc*) and 28.4% ±7.6 (low *Dhc*). Addition of the MIAC to the Sterivex cartridge prior to groundwater filtration (treatment 2) resulted in mean recoveries of 6.1%+/-2.1% (high *Dhc*) and 10.0+/-8.5% (low *Dhc*). Finally, when the MIAC was added directly to the DNA extraction bead tube (treatment 3), the mean recovery was 76.1%+/-20 (high *Dhc*) and 52.7%+/-12 (low *Dhc*). The recovery of the MIAC was similar in the presence or absence of *Dhc* and the other microorganisms in the KB-1 culture suggesting that the presence of other biomass did not impact MIAC recovery.

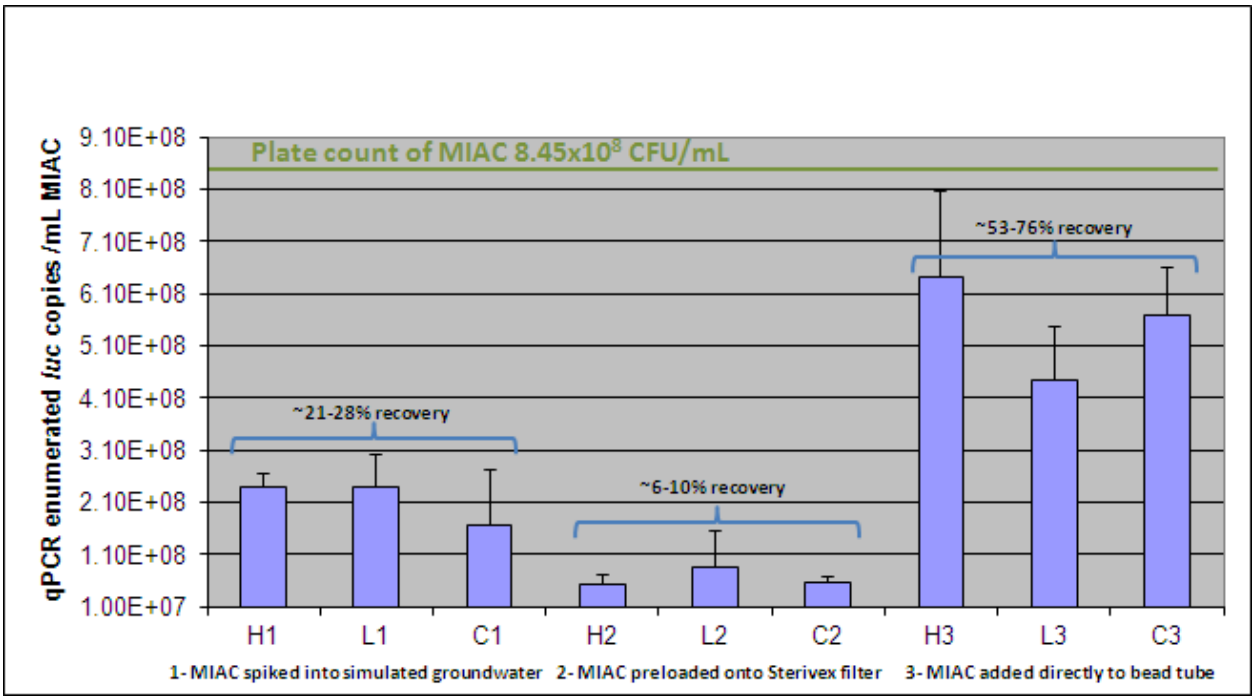

Figure 6-1: MIAC recovery experiments for application at different points in the analysis. Blue bars indicate mean *luc* enumeration values for qPCR on a MIAC culture determined to be 8.45 x 10^8 CFU/mL based on plate counts. MIAC was added at different points in the biomass concentration extraction process: 1; MIAC spiked into simulated groundwater prior to filtration, 2; MIAC preloaded onto the Sterivex cartridge, 3; MIAC added directly to the DNA extraction bead tube together with the biomass collected from groundwater. Note: (n=5 for H [high *Dhc*] and L [low *Dhc*]; n=2 for C [no *Dhc* controls]).

6.1.3 Conclusions

Application of the MIAC at different points in the analysis indicated that losses varied depending on the point in the analysis at which the MIAC was added. While total recovery percentage is an important consideration, consistency of recovery (i.e., the width of the error bars) is also important, as this impacts our ability to categorize samples as "normal recovery" or flag as "low recovery". Based on high consistency, any of the MIAC addition options could be viewed as viable. Further and ongoing use of the MIAC will provide additional information as to which application options represent the best for real world and assessment of matrix inhibition and target microorganism losses.

6.2 Multi-lab Testing of the MIAC

The utility of an internal control ultimately depends on the ability to deploy it effectively and consistently in analytical laboratories. To determine if the MIAC could be consistently grown and deployed, two round robins were performed to demonstrate the performance and value of this internal control in multiple laboratories.

6.2.1 Approach

Simulated groundwater, made as described in Attachment 3, was spiked with *Dhc* and distributed to five labs designated 1 through 5. Five identical "low *Dhc*" and "high *Dhc*" samples and two *Dhc*-negative samples were shipped to each of the five labs in a similar fashion as for Round Robin 2 (Attachment 2). Prior to extraction, all labs spiked whole MIAC cells at 1.5 x 10^3 - 4.5 x 10^3 cells/mL into the simulated groundwater samples (targeting 10^4 gene copies per qPCR reaction). Note, one laboratory (Lab 4) used frozen aliquots of the MIAC (see Section 6.4). Each lab used their established workflows to collect cells, extract DNA and quantify *luc* by qPCR (Figure 6-1).

Results

All labs reported relatively consistent sample to sample recoveries (i.e., low variability) for the spiked MIAC, which ultimately would allow its use in detecting matrix interference or other losses in the analytical process. Between laboratory recovery ranged from ~4 to ~30% (Figure 6-2), suggesting that variable losses of target genes occurred in all laboratories. Within labs, approximately the same proportion of the MIAC was recovered from high *Dhc* and low *Dhc* samples, suggesting that the MIAC is applicable to samples harboring a wide range of *Dhc* abundance. The inter-lab differences in *luc* recovery and *Dhc* abundance estimates may reflect differences in lab-specific workflows, including biomass harvesting and DNA extraction procedures. For example, Lab 4 used frozen *E. coli* cells which could have increased recovery by weakening the cell wall, however, in subsequent experiments other labs not using frozen cells had relatively high recoveries compared to Lab 4 (Figure 6-3) suggesting that the use of frozen cells for the MIAC may not be a key factor impacting recovery.

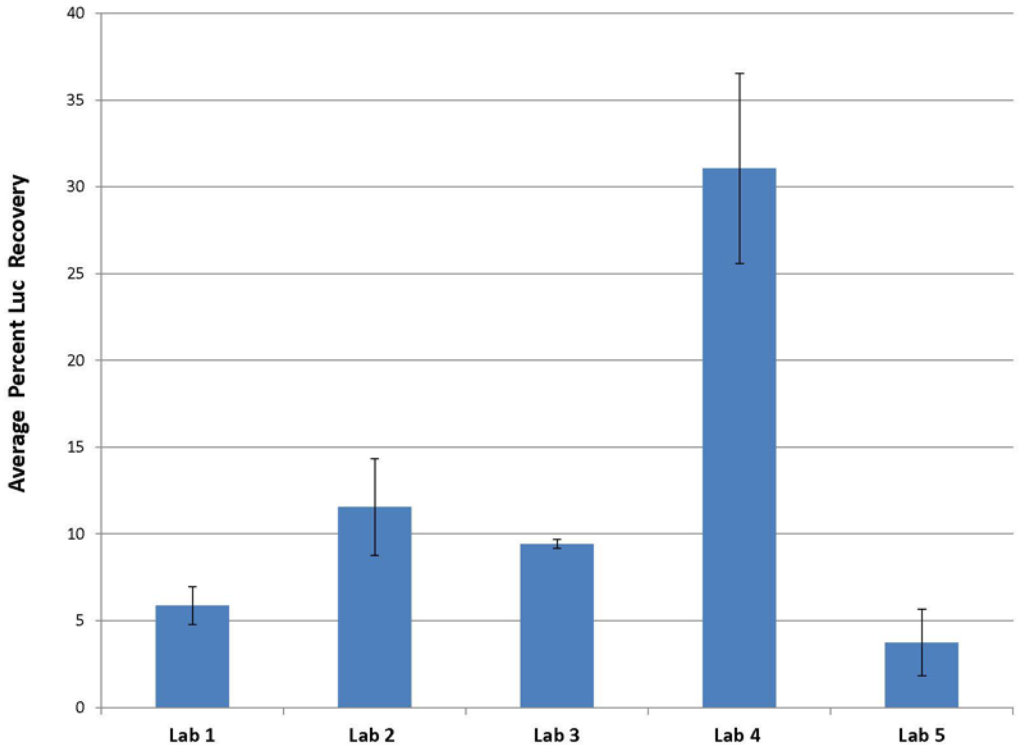

Figure 6-2: Mean percent recovery of the MIAC in five independent laboratories in simulated groundwater (Round Robin 3). A known amount of *E. coli* cells harboring the *luc* gene (i.e., the MIAC) were spiked into 10 artificial groundwater samples, 5 with "high" *Dhc* (10^6 *Dhc* /mL) and 5 with "low" *Dhc* (10^3 *Dhc* /mL) and enumerated by qPCR targeting the *luc* gene. Recovery ranged from 4-30% indicating that MIAC recovery varied between labs.

Error bars represent 95% confidence intervals for the mean *luc* recovery calculated for 10 samples in each lab. If there were no abnormal loss/error or matrix inhibition than recovery for a spiked sample would be expected to fall within the error bars 19 times out of 20. See Appendix H for detailed MIAC recovery data.

6.2.2 Recovery of MIAC from Site Groundwater Samples

Multi-lab testing of performance of the MIAC in simulated groundwater was followed by performance testing of the MIAC in groundwater samples obtained from the Bachman Road site in Oscoda, Michigan.

Approach for Testing the MIAC in Site Groundwater Samples

As part of Round Robin 4 (Attachment 4) replicate 500 mL groundwater samples were collected from two wells (ML-3 and AML-3) at the Bachman Road site. In addition, biomass was sampled on site using Sterivex cartridges. The MIAC was added to the 500 mL groundwater sample immediately prior to filtration in the lab. For the Sterivex cartridges, the MIAC was directly

added to the DNA extraction bead tube together with the filter membrane containing the biomass.

The recovery of the MIAC in the different labs from groundwater and Sterivex cartridges was determined using qPCR for the introduced *luc* gene in the MIAC, as noted above *Dhc* results for this round robin (RR4) are discussed in Section 9.

Results of Testing of the MIAC in Site Groundwater

Recovery of *luc* in groundwater from Bachman Road site groundwater (well ML-3) is provided in Figure 6-3. Additional data for a second well (A-ML-3), and for replicate samples, are provided in Appendix H. In general, the recovery of the *luc* in this experiment was higher than observed in the initial experiment in simulated groundwater (Figure 6-2) in 4 out of 5 labs. Lab 4, which used the same frozen cell aliquots for both experiments, had essentially identical recovery in both tests.

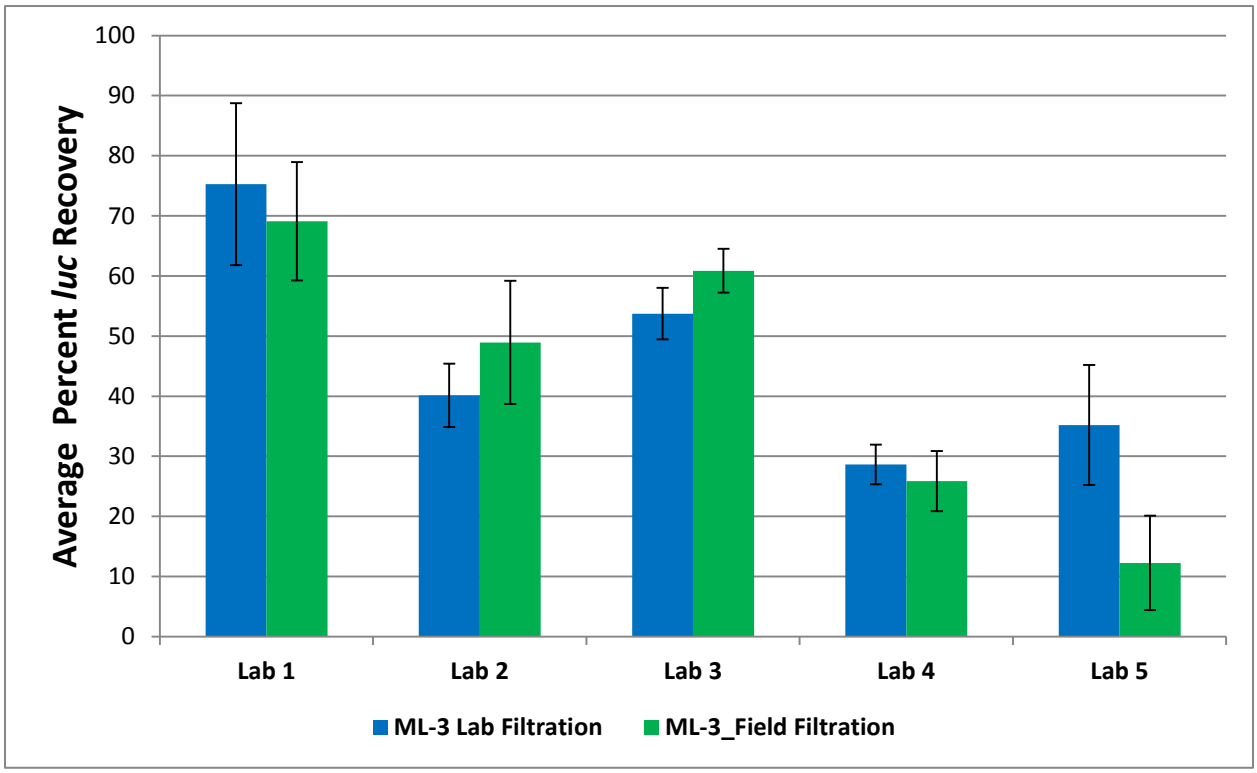

Figure 6-3: Spike and recovery of the MIAC in Bachman Road site groundwater (Round Robin 4). Samples were either bulk groundwater (blue bars) or were collected by on site filtration with Sterivex cartridges (green bars). Five participating labs added the MIAC to the bulk groundwater or to bead tubes (for Sterivex cartridge extractions) *luc* gene copy number was enumerated by qPCR after DNA extraction. Error bars represent 95% confidence intervals for the mean *luc* recovery calculated for 5 samples processed in each lab. Overall, *luc* recovery was higher in this test (for lab filtration [~29-75%]) compared with ~4-31% in Round Robin 3 (Figure 6-2).

The majority of labs reported consistent recovery when the MIAC was added to groundwater and directly in the bead tube, suggesting losses of *E. coli* cells and DNA due to filtration were negligible. The results obtained in Round Robin 4 (Figure 6-3) indicate recoveries of the MIAC in the range of 30-75% in site groundwater. Furthermore, variability between replicate samples (as indicated by the error bars in Figure 6-2 and 6-3) was generally low enough to assign a lower limit to normal variation. Therefore, samples falling below the low end of the error bar have a high probability of suffering from possible losses or inhibition. Overall, the results of these tests indicated the MIAC can be spiked into groundwater samples and recovered with reasonable efficiency, and sufficiently low variability, so that the MIAC could be used to gauge target biomarker gene losses.

6.3 Quantification of the MIAC by Multiple Methods

One of the characteristics of an ideal MIAC is the ability to enumerate cells by methods other than qPCR, thus allowing the MIAC to verify qPCR enumeration. This approach is not feasible with many environmental microorganisms (such as *Dhc*) as the cells do not grow on agar plates and are not easily enumerated with microscopic methods (they are small /grow in mixed cultures). In the tests detailed below, the MIAC was enumerated by three different methods to determine the feasibility of these approaches and the consistency of results. The methods used to enumerate the MIAC were:

1) Plate counts that enumerate viable cells which are both living and able to grow;

2) Total genomic DNA quantification which counts cells by quantifying the total DNA extracted using fluorometry then calculating he number of cells by determining the number of cellular genomes which equals the number of cells; and

3) qPCR targeting the inserted *luc* gene.

The comparison of the qPCR methods to non-qPCR methods provides an opportunity to verify the accuracy of the qPCR method and could be used for ongoing method validation. For example, if the MIAC plate counts and *luc* qPCR numbers ever diverged significantly, this could flag a problem with the qPCR method. This is not possible with *Dhc*, which cannot be enumerated by plate counts. Multi-method quantification also provides additional assurance that qPCR methods are free of biases to the extent they agree with non-qPCR methods (see Section 4.1).

6.3.1 Approach

Cultures of the MIAC were grown and enumerated at two dilutions using plate counts in triplicate on LB agar as described in Appendix I. Genomic DNA was also extracted from the culture using the QIAamp kit (Qiagen Inc.), which is optimized to extract DNA from *E. coli*

cultures (the MIAC is *E. coli*). Extracted DNA was quantified using PicoGreen fluorometry and qPCR targeting the chromosomally inserted *luc* gene (for detailed methods including sample calculations for determining the *E. coli* titer see Appendix I). Assuming the cells were viable, that DNA extraction was 100% efficient, and the qPCR methods were accurate, it would be expected that the plate counts and the two DNA-based enumerations would be consistent. Generating the same result with three fundamentally different methods would increase confidence that each of the methods is accurate.

Results

Table 6-1 summarizes the results obtained for multiple methods of quantifying the *E. coli-luc* MIAC. The three distinct methods (plate counts, fluorometry and qPCR) all provided counts (green cells in Table 6-1) for the MIAC culture that were similar ranging between 3.3-3.9 x 10^7 cells per mL.

Table 6-1: Enumeration of the MIAC by multiple methods including qPCR targeting the *luc* gene, genomic DNA quantification and plate counts.

qPCR		Genomic DNA Quantification		Plate Counts	
Frozen aliquot	*luc* gene copies/mL	Total DNA ng/mL quantification by fluorometry	Calculated *E. coli* genome copies/mL	Plate count replicate	Colony forming units (CFU)/ml
1	3.61E+07	215	4.18E+07	1	3.26E+07
2	3.61E+07	169	3.29E+07	2	2.72E+07
3	4.06E+07	205	4.00E+07	3	3.42E+07
4	4.33E+07	139	2.70E+07	4	3.65E+07
5	3.93E+07	144	2.80E+07	5	3.30E+07
6	3.98E+07	148	2.88E+07	6	4.00E+07
7	3.79E+07	--	--	--	--
Average=	3.90E+07	--	3.31E+07	--	3.39E+07
% recovery	115%	--	98%	--	100%
Comment	qPCR with primers targeting the chromosomally inserted *luc* gene	Total DNA ng/mL extracted from 0.4 mL of frozen MIAC cells	Enumeration of cells by calculating the genome copies/mL-see Appendix I for sample calculation	--	Measures viable cells

Plate counts were arbitrarily defined to represent the true number of cells in the culture (i.e., 100% recovery). The different enumeration methods exhibited minimal variation from plate counts,-2% [98% recovery] for fluorometry and +15% [115% recovery] for qPCR. The high degree of similarity obtained using molecular methods and plate counts confirmed that the MIAC met the requirement of being quantifiable by non-PCR methods. This property provides a convenient means of verifying qPCR protocols for the MIAC against another accepted

enumeration method (i.e., plate counts). The agreement of qPCR with plate counts provides further verification that qPCR has the ability to accurately enumerate cultures (*E. coli* and *Dhc*) compared to classical (i.e., non-molecular methods) as was observed in the experiments outlined in Section 4.

6.4 Storage and Stability of the MIAC as Frozen Cell Stocks

In order for a MIAC to be practical for day-to-day use, it has to be stable and storable as the process of re-growing and quantifying is time and labor intensive and not compatible with routine analysis. Therefore the ability to produce and store a MIAC is an important consideration. The following properties affect the usefulness of preserved MIACs:

1) Does storage affect the subsequent viability and performance of the MIAC (e.g., does storage affect cell integrity and other properties)? ;

2) Can consistent aliquots be produced for use in routine analysis ?; and

3) Are these aliquots stable over the long term (i.e., for several months)?

The following sections provide information on the effectiveness of using frozen cell cultures as an MIAC.

6.4.1 Approach to Preserving Frozen Aliquots of MIAC

The MIAC culture suspension was diluted 100-fold (two 10-fold serial dilutions in phosphate buffered saline (PBS)) and ninety 400 micro liter (µL) volumes of the 1:100 diluted suspension were aliquoted into 0.5 mL screw cap micro-tubes (Diamed Lab Supplies, Mississauga, ON) after which 30 µL (7% volume/volume [v/v]) of dimethylsulfoxide (DMSO), a compound commonly used for cryopreservation of microbial cells, was added and mixed thoroughly by repeated inversion. The cell-DMSO mixture was quick (flash) frozen in a dry ice-isopropanol bath and stored at -80°C. Non-frozen and frozen *E. coli* were quantified by colony counts on LB agar plates to explore if the freezing process affected cell viability.

6.4.2 Viability of Frozen Cell Stocks for use as MIAC

If *E. coli* cells were undamaged by the DMSO/ freezing process, the expected viable cell count would be expected to be approximately the same for non-frozen and frozen cells. Alternatively, significant declines for frozen cell counts would indicate cell damage possibly affecting lysis properties. The lysis properties of cells used as an MIAC are important, as one of the functions of the MIAC is to test the efficiency of cell lysis. Inconsistent or altered lysis of a MIAC after freezing could ultimately make interpretation of qPCR results difficult.

Table 6-2 provides plate count data comparing unfrozen *E. coli*- MIAC to frozen *E. coli*-MIAC and indicates less than an approximate 20% decline in cell counts (cfu/mL) for the frozen cells. The reduced plate counts seem to indicate only a modest decline in cell viability after freezing. Furthermore, there was no apparent increase in the variability of the plate counts after freezing. Both of these findings suggest that freezing cell aliquots in this way does not unduly affect cell integrity.

Table 6-2: Comparison of plate count results for fresh (unfrozen) and frozen MIAC cells

Plate Counts		
Plate Count Replicate	Fresh Cells (cfu/mL)	Frozen Cells (cfu/mL)
1	3.26E+07	2.72E+07
2	2.72E+07	2.87E+07
3	3.42E+07	2.55E+07
4	3.65E+07	3.48E+07
5	3.30E+07	2.73E+07
6	4.00E+07	2.51E+07
--	**3.39E+07**	**2.81E+07**
	100%	**83%**
--	--	Freezing/DMSO treatment of cells marginally affects cell viability

The cells in the frozen aliquots were determined to be stable with consistent recovery in qPCR assays and plate counts over several months. This approach to preserve the MIAC makes day-to -day use in analytical laboratories feasible as the labor intensive preparation of the MIAC does not have to be performed on a day to day basis.

6.5 Testing the Ability of the MIAC to Detect Matrix Interference

Matrix inhibition of PCR has the potential to produce false negative results or underestimates of the actual biomarker gene abundance in qPCR-based tests. Compounds reported to inhibit PCR include lipids, proteins, metals (e.g., calcium), polysaccharides, proteins and phenolic compounds (Cankar et al., 2006); urea (urine); bile salts and complex polysaccharides (fecal matter); and humic substances (soil) (Nolan et al., 2007). Inhibitory substances can also be introduced with chemicals used during the DNA extraction process or even from chemicals leaching from laboratory plasticware (Fox et al., 2007).

PCR inhibition has often been diagnosed during the nucleic acid quantification process by dilution of the extracted template DNA sample. Dilution of the DNA sample containing inhibitors may lead to positive amplification whereas the undiluted samples yield negative

results. In the literature, the dilution approach was the most common method for assessing inhibition (e.g., Koike *et al.*, 2007). While generally effective for assessing inhibition, the dilution method has disadvantages it (i) increases the detection limit of the assay (ii) is reagent intensive, as several dilutions may be required and (iii) may not be effective for all samples. A MIAC-based approach could offer more flexibility than dilution (it does not require the analyst to predict in advance the number of dilutions required to overcome inhibition), could be integrated into standard protocols and would not require a second round of testing of samples (i.e., testing numerous dilutions beyond the after the initial tests), which also increases analysis turnaround times.

One of the major classes of PCR inhibitors relevant to environmental samples are humic acids often referred to as "humic compounds" or just "humics". Humic acids are an amorphous collection of complex, dark colored organic compounds formed through the decomposition of organic matter in soil (Matheson et al., 2010).

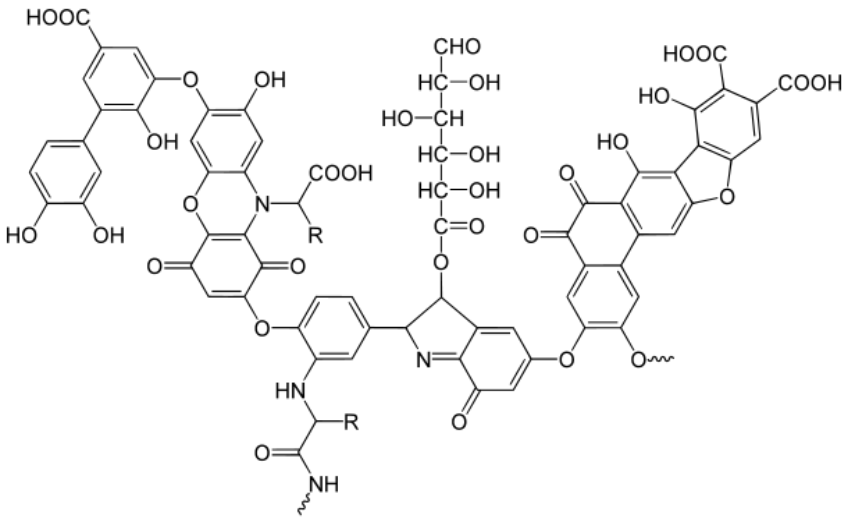

Figure 6-4: Structure of a humic acid. Humic acids are a class of large molecules with variable structures associated with degradation of organic compounds in soil. Humic acids can cause PCR inhibition (http://en.wikipedia.org/wiki/Humic_acid).

Humic compounds tend to be difficult to remove from DNA preparations. Humic compounds are thought to inhibit PCR through two primary mechanisms.

1) Template inhibition, in which humic compounds bind to DNA template preventing it from being amplified by PCR; and

2) Enzyme inhibition, in which humic compounds prevent the activity of the Taq polymerase used in qPCR (Matheson et al., 2010).

Approximately 0.7 - 3.3 micrograms per microliter (µg/µl) of humic acids can be extracted from soil DNA preparations (Matheson et al., 2010) with much lower amounts, as little as 0.5 nanograms (ng) (Green and Field, 2012) to 10 ng (Tsai and Olsen, 1992) sufficient to cause PCR inhibition when spiked directly into a PCR tube. Therefore, inhibition by humic compounds is a major consideration when extracting DNA from soils but less significant when template DNA is extracted from groundwater.

While literature values for humic acid concentrations in groundwater are not widely reported, and undoubtedly vary widely, selected samples can have high concentrations. For example, Feng et al. (2007) reported 20 milligrams per liter (mg L^{-1}) in groundwater. A study by Mäkelä and Manninen (2007) concluded that around 40% of total organic compounds in groundwater were comprised of humic compounds. The concentration of humic acids in groundwater can vary widely depending on the properties of the terrestrial surface. In some cases concentrations as high as 100 mg L^{-1} were reported where groundwater infiltrated from wetlands. Humic acid concentrations were orders of magnitude lower at sites with different terrestrial surface properties, for example, conifer forests on sand (Ghabbour and Davies, 2004).

Humic compounds are common constituents of groundwater and soil and are present in groundwater in highly varying concentrations. Therefore it is important to consider the impact of humic compounds on PCR amplification as part of standard analytical procedures. The MIAC was identified as possible tool to detect the impact of PCR inhibitors, including humic compounds, in groundwater. The MIAC could be used in conjunction with sample dilution, which could be used to further assess inhibition in the samples flagged as inhibited by the MIAC. An MIAC could flag samples with a higher probability of being false negative (based on low MIAC percent recovery) or which have reduced (i.e., low biased) enumeration due to the presence of specific inhibitory compounds.

6.5.1 Approach

Commercially available humic acids were added directly into groundwater obtained from the Bachman Road site in Oscoda, Michigan (See Attachment 5 for more information). The goal was to evaluate the effectiveness of the MIAC at detecting PCR inhibition by humic compounds to determine if the MIAC was appropriate for measuring PCR inhibition and to better understand the concentrations of humic acid concentrations in groundwater that may cause inhibition of qPCR assays.

Groundwater from the Bachman Road site was amended at 10 mg L^{-1} and 100 mg L^{-1} of humic acids using stock solutions of humic acid sodium salt (Sigma Aldrich, St. Louis, MO) (6 grams per liter [g L^{-1}] and 60 g L^{-1}) that were shipped to the sampling site. And 10 mL were added to two carboys each containing 6 L of groundwater representing 10 mg L^{-1} and 100 mg L^{-1} final concentrations. It should be noted that the composition of commercially available humic acids differs from the humic substances found in many natural systems and the impact of these compounds on PCR inhibition could be different. The groundwater used for this test was

collected from monitoring well (MW-2A), which historically had moderate (10^4 *Dhc*/mL) *Dhc* abundances. The humic-spiked groundwater was then filtered in five 1 liter (L) sub-samples using Sterivex cartridges. The MIAC was added to the bead tube of the DNA extraction kit (PowerWater® Sterivex™) and analyzed in the laboratory in parallel with non-humic spiked groundwater samples from the same well.

6.5.2 Results & Discussion

Comparison of the results for the *Dhc* enumeration and the *luc* recovery percentage for the MIAC is presented in Figures 6-5 and 6-6, respectively. The performance of the qPCR tests in humic acid-spiked groundwater samples are compared to non-spiked samples.

To investigate the impact of humic compounds on the quantification of *Dhc*, all *Dhc* enumeration data obtained with samples from MW-2A were plotted in Figure 6-5. Five out of five samples with 100 mg L^{-1} of humics and three out of the five samples with 10 mg L^{-1} of humics had non-detectable *Dhc*. This indicated a clear PCR inhibition impact of humics at 100 mg/L and a variable impact at 10 mg L^{-1}, possibly indicating 10 mg L^{-1} is around the concentration where PCR becomes inhibited by humic compounds .

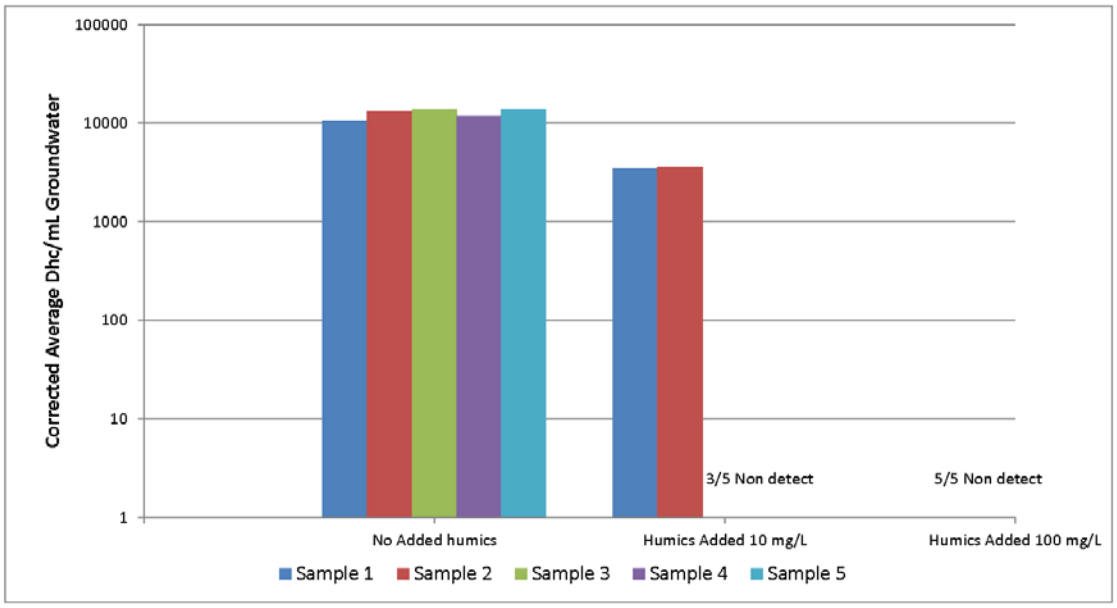

Figure 6-5: Effect of humic acids on *Dhc* enumeration in groundwater. Comparison of *Dhc* enumeration in Bachman Road site groundwater (no added humics) and the same groundwater spiked with 10 mg L^{-1} and 100 mg L^{-1} of humics. *Dhc* 16S rRNA gene were detected in all (5 of 5) samples without added humics, but in only 2 out of 5 samples spiked with 10 mg L^{-1} humics and *Dhc* was not detected in any samples spiked with a 100 mg L^{-1} humic acid.

The impact of humic compounds on the recovery of the MIAC was also tested in groundwater by spiking of whole MIAC cells into the bead tube of the DNA extraction kit. These results are summarized in Figure 6-5.

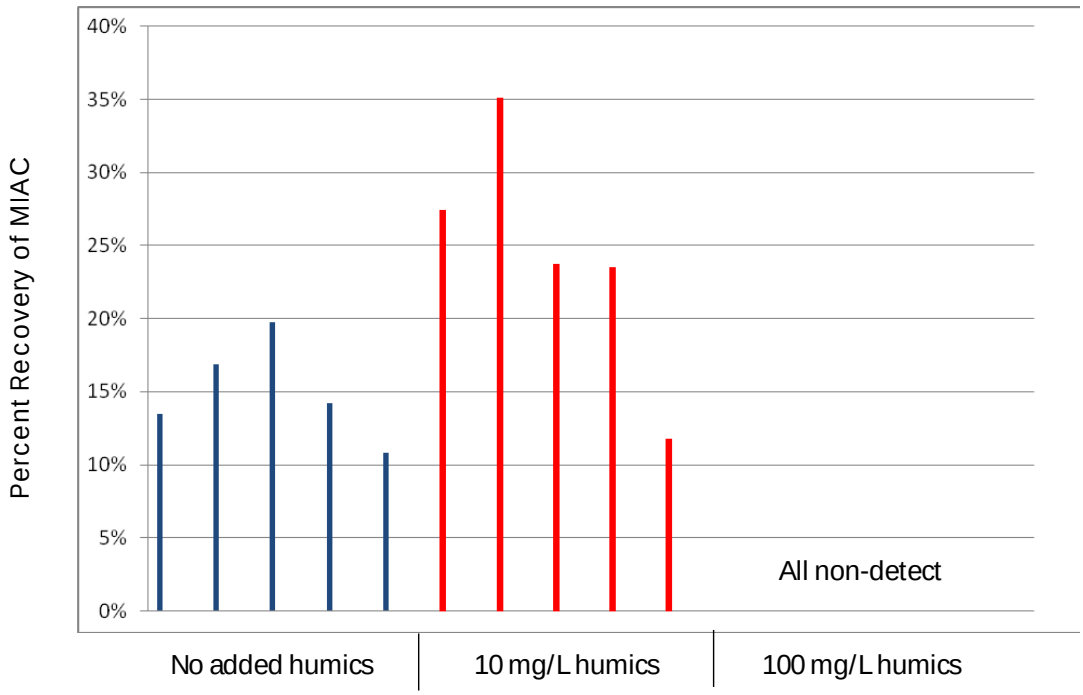

Figure 6-6: Effect of humic acids on recovery of the MIAC. MIAC percent recovery in Bachman Road site groundwater samples with no added humics (left-blue), 10 mg L^{-1} humics (middle-red) and 100 mg L^{-1} humics (right-no bars). The results indicated that 10 mg L^{-1} humics did not reduce the recovery of the MIAC, whereas 100 mg L^{-1} completely inhibited recovery of the MIAC (ND for 5/5 replicates).

Figure 6-5 indicates that the *luc* percent recovery was in the 10-20% range with no added humics and was approximately 10-35% in the 10 mg/L spike. This level of recovery is consistent with observations in previous experiments (see Figures 6-2/6-3). In contrast, a spike of 100 mg/L humic acids completely inhibited *luc* amplification resulting in non-detects. These data are consistent with observations that *Dhc* was also not detected in the 100 mg L^{-1} humics spike (Figure 6-5) and suggests the MIAC could be a useful control for detecting and quantifying matrix inhibition.

The impact of humics concentrations in the range between 10 and 100 mg L^{-1} requires further study. Based on observations of many groundwater samples, inhibitory compounds probably reach fully inhibitory concentrations in only a small percentage of groundwater samples. For example approximately 3-5 % of groundwater DNA samples submitted for commercial *Dhc* testing were not amplifiable with universal bacteria PCR primers (SiREM, unpublished data),

suggesting at most to 1 sample in 20 was fully inhibited or DNA was not extracted. Matrix inhibition in soil likely affects a larger proportion of samples, owing to soil's higher humic acid content. While complete inhibition of PCR analysis occurs in only a minority of samples, the proportion of groundwater samples imparting partial inhibition (and the potential for target microbe underestimates) is less clear and requires further study. Ongoing use of a MIAC in a commercial laboratory processing a large volume and variety of samples would be informative regarding this question.

6.6 Conclusions Regarding the MIAC

An ideal MIAC should have the following properties, it should be: quantifiable by multiple means (so as to verify qPCR methods); capable of being grown, applied and quantified consistently; able to be stored effectively (makes its use practical for ongoing use in a laboratory setting); able to detect PCR inhibition.

Furthermore, recovery (e.g., filtration and lysis properties) between MIACs and the target microbes would ideally be the same. Otherwise data adjustments made with a MIAC might be too high or too low, which could lead to inaccurate assessment of *Dhc* abundance in a sample. Future research Comparing target gene recovery for *Dhc* and *E. coli* cultures under a variety of conditions could be used to determine if differential recovery is an issue for these microbes in filtration and extraction methods.

The experiments outlined in this section indicated that the developed MIAC had the following attributes:

1) The MIAC can be applied at different steps in the extraction process, including directly to groundwater, or to the DNA extraction bead tube with sufficiently high and consistent recovery.

2) The MIAC was quantifiable by multiple methods and returned similar results with plate counts, genomic DNA quantification and qPCR analysis. This increased confidence in the qPCR method.

3) The MIAC can be frozen and stored effectively at -80°C making it practical for ongoing use in a laboratory setting.

4) The MIAC detected PCR inhibition by humic compounds at concentrations that might be found in groundwater samples.

The MIAC was deemed to be a useful tool based on observations of specificity and its absence from groundwater outlined in Section 5 and its successful use in multiple labs in Section 6. Use of the MIAC in optimization of cell concentration, DNA extraction and qPCR lab methods is outlined in Section 7. The use of the MIAC in interpretation of *Dhc* results is summarized in Sections 8 and 9.

7 ASSESSMENT AND OPTIMIZATION OF LABORATORY METHODS

Optimization of the basic laboratory methods used in the qPCR testing focused on the areas of the analysis considered by the project team to represent the highest potential for variability. These included:

- Biomass collection (i.e., groundwater filtration) (Section 7.1)
- DNA extraction (in particular cell lysis) (Section 7.2)
- PCR methods, i.e., qPCR chemistry and primer selection (Section 7.3)
- Calibration effects related to quantification of and type of standards used (Section 7.4)

For biomass collection, DNA extraction and qPCR methods and chemistry, significant challenges arise from the variability associated with environmental samples including wide ranges in pH, solids, biomass, and interfering substances such as humic acids, heavy metals and electron donors used in bioremediation. Furthermore, the microorganisms being quantified can have varying properties in terms of cell lysis and filtration collection efficiency. For these reasons, optimization of sample-specific and organism-specific protocols, are likely to be important. This is not only applicable for *Dhc*, but also other microorganisms requiring accurate quantification protocols.

The impact of calibration methods differs from sample related challenges as calibration generally occurs outside the realm of environmental samples. While calibration materials (plasmids, genomic DNA from pure cultures, cells etc.) are not typically derived from environmental samples, they can affect qPCR results and have been the subject of increasing focus outside the remediation community. One issue in particular is reported overestimation of qPCR methods caused by the use of supercoiled plasmids as calibration materials (Laghi et al., 2004; Chen et al., 2007; Hou et al., 2010 and Lin et al., 2011).

7.1 Impact of Filtration Methods on Quantitative Results of qPCR Analyses

Collection of biomass by groundwater filtration is often the first step in a qPCR analysis. This essential procedure concentrates biomass onto a filter membrane to allow processing in small format DNA extraction kits. In the past, time consuming, and impractical, groundwater centrifugation methods were also used. Filtration may be performed using positive pressure methods (e.g., peristaltic pump through Sterivex cartridge) performed on site during well sampling. Alternatively, filtration may be performed with groundwater samples shipped to the testing laboratory by either positive pressure or vacuum filtration methods.

7.1.1 Comparison of Vacuum Filtration/DNA Extraction Using Different Filter Membranes

Given the variety of possible filter membranes available for biomass collection, the impact of the choice of membrane on the efficiency of recovery of biomass from groundwater and subsequent extraction of DNA is not well understood. A preliminary experiment to determine if filter membrane type in vacuum filtration impacted quantitative analysis of *Dhc* in groundwater was performed. The goal of this experiment was not a comprehensive testing of filter types but rather to determine if differences due to filtration membrane could be observed by comparing two commonly used membrane filters. Filter membranes have the potential to impact the quantification of *Dhc* through several mechanisms including cell loss through the membrane, irreversible attachment of cells to the membrane, or inhibition of the DNA extraction process as the filter is typically crushed in the bead tube during extraction.

Two commonly used filter types were compared for their effectiveness in *Dhc* enumeration. The membrane types were compared using similar format disposable vacuum filters with a 250 ml reservoir and were:

1) Cellulose nitrate (CN), 0.22 µm pore size; and

2) Polyethersulfone (PES), 0.22 µm pore size.

The PES membrane is the same material used in the Sterivex™ cartridges (Sterivex™–GP 0.22 µm), which are typically used with positive pressure as opposed to a vacuum. The results reported here apply only to vacuum applications, and not necessarily to biomass collection using positive pressure and Sterivex cartridges.

Approach

Nine (9) *Dhc*-positive groundwater samples were selected from bioaugmented sites and identical groundwater volumes were vacuum-filtered side-by-side in the laboratory, one sub-sample with a CN membrane filter, the other with the PES membrane filter.

The CN membrane filter apparatus was secured to a 2 L vacuum flask, which was attached to a vacuum pump. The PES membrane filter apparatus was attached to a 1 L medium bottle and the same vacuum pump apparatus was used. Equal volumes of groundwater were filtered for each filter type (250 mL). Thorough mixing ensured that the groundwater samples applied to each filter type were consistent. The PES filters were separated from the filter housing using a scalpel. DNA extraction and qPCR analysis for the *Dhc* 16S rRNA gene were performed according to Attachment 2 [Attachment B.1]. All qPCR analyses were performed concurrently. Total extracted DNA was quantified in triplicate using a NanoDrop spectrophotometer according to the manufacturer's instructions (NanoDrop Inc., Wilmington, DE).

Results and Discussion

All groundwater samples tested positive for *Dhc*. Quantitative results suggested differences in performance between the CN and PES membranes. The PES membrane *Dhc* enumerations ranged from 4% to 65% of the CN membrane enumerations. The PES membrane mean *Dhc* quantification was 26% of the mean CN membrane *Dhc* quantification (Table 7-1). Total DNA recovered from the PES membrane was also lower and ranged from 16% to 99% of the CN membrane with a mean recovery of 40% when compared to the CN membrane. All differences observed between the PES and CN membrane were statistically significant at a 5% level of significance using a paired t-test on log transformed data.

Table 7-1: Comparison of the total *Dhc* enumerated and DNA recovery in groundwater samples collected using cellulose nitrate or polyethersulfone filters.

Groundwater Sample	*Dhc* Enumeration (16S rRNA Gene Copies/L)		*Dhc* Enumeration PES:CN Ratio	Total DNA Extracted (ng/L)		Total DNA PES:CN Ratio
	CN	PES		CN	PES	
A	9.6E+06	3.6E+05	4%	4443	2079	47%
B	1.8E+08	1.8E+07	10%	16209	3225	20%
C	1.5E+07	2.2E+06	14%	19740	3792	19%
D	3.9E+09	2.0E+09	51%	16374	12135	74%
E	1.9E+08	1.3E+07	6%	17352	2847	16%
F	3.8E+08	2.5E+08	65%	34644	34206	99%
G	9.4E+05	5.0E+05	53%	5664	2568	45%
H	2.3E+06	3.9E+05	17%	12186	2304	19%
I	8.3E+05	1.5E+05	18%	7682	1717	22%
		Average	26%		Average	40%
Standard Deviation	1.3E+09	6.8E+08				

The data suggest that differences in recovery of total biomass and *Dhc* biomass under the vacuum conditions and DNA extraction conditions tested could be attributed to the filter membrane used. This preliminary test suggests that filter membrane choice may be relevant to the performance of vacuum filtration protocols and is a suitable subject for further study. These studies could examine ideal membranes and/or groundwater filtration protocols for use with specific membranes. In addition, the use of a MIAC could flag losses or be used to correct for losses associated with filtration using different filtration membranes.

7.1.2 Impact of Vacuum Strength in Groundwater Filtration

Biomass concentration via filtration is one of the primary steps in qPCR analysis and is commonly performed by vacuum methods (typically laboratory based) and positive pressure approaches when biomass is obtained on site using Sterivex cartridges. The effects of filtration conditions were explored based on observations of low yields and large experimental

inconsistencies when different DNA extraction methodologies to recover *Dhc* DNA from polyethersulfone (PES) membrane filters were used.

In this experiment several aspects of filtration were examined, including:

1) Efficiency of filtration compared to centrifugation;

2) The impact of different vacuum strengths; and

3) Comparison of vacuum filtration versus positive pressure approaches.

These investigations demonstrated that vacuum strength applied during biomass collection onto membrane filters affected biomarker recovery.

Materials and Methods

KB-1®, a *Dhc*-containing bioaugmentation culture was used for these experiments. KB-1® frozen stocks (40-1.5 mL plastic tubes each containing equal volumes of the stock culture) were stored at -80°C. Cells from an individual tube were suspended in 100 mL sterile phosphate buffer (50 millimolar [mM], pH 7.0), and the resulting cell suspension was filtered through flat Millipore Express (PES, 47 millimeter (mm) diameter, 0.2 µm pore size) membranes under the following three conditions:

1) Strong vacuum; all manifolds closed (6 manifold system) except the one attached to the operational filter unit line, total filtration time was less than 1 minute;

2) Weak vacuum; all manifolds open, total filtration time was approximately 6 minutes; or

3) Peristaltic pump; the filter was placed in an in-line filter housing unit and the cell suspension (100 mL) was pumped through the unit at 10 milliliters per minute (mL/min) followed by an additional 100 mL of sterile phosphate buffer to flush all cells onto the membrane, total filtration time approximately 20 minutes.

The filters were cut in half following filtration. One filter half was immediately stored at -80°C for future use and the other half was cut into small pieces and placed into a MO BIO PowerSoil® DNA extraction kit bead beating tube. DNA extraction was performed immediately as per the manufacturer's instructions except that bead beating was performed in a FastPrep 24 bead beater (MP Biomedicals, Santa Ana, CA) for two 30 second intervals.

Biomass from stock tubes was also collected by centrifugation. The content of a stock tube was transferred to an empty PowerSoil® DNA bead beating tube, which was centrifuged at 10,000 x gravity (g) for 5 minutes. The supernatant was carefully removed and the contents of the bead beating tube (beads and extraction buffer) were returned to the tube containing the cell pellet.

DNA extraction was performed as described in the paragraph above. As a control, half of a sterile Millipore Express membrane without cells was cut into small pieces and added to a bead beating tube (prior to bead beating) containing the KB-1 biomass collected by centrifugation. Recovery of *Dhc* was assessed by 16S rRNA gene-targeted TaqMan® qPCR (Attachment 2 [Attachment B.4]).

Results and Discussion

Centrifugation appeared to have the highest total recovery, which suggests this method was an efficient cell harvesting method. Addition of filter pieces to bead beating tubes containing *Dhc* cells collected by centrifugation resulted in an approximate 1.5 fold decrease in cell *Dhc* DNA recovery. This indicates that the presence of polyethersulfone membrane slices during the initial steps of the PowerSoil® DNA extraction process had a minimal effect on *Dhc* DNA recovery.

In contrast, filtration strength (i.e., the vacuum strength with which water was pulled through the membrane filter) appeared to have had a strong influence on *Dhc* biomarker enumeration by qPCR (Figure 7-1). On average, recoverable *Dhc* DNA was 2 to 4 fold lower when a weak vacuum or peristaltic pump was used for filtration as compared to centrifugation. With the peristaltic pump, the *Dhc* recovery was variable. This was likely due to handling of the filter during the disassembly of the in line filter housing, and some cell loss may have occurred during this step. In contrast, the use of a strong vacuum to collect cells resulted in complete loss of *Dhc* (detection limit 50 to 100 *Dhc* 16S rRNA copies per qPCR assay). The reasons why *Dhc* could not be detected when strong vacuum conditions were applied were unclear. One explanation is that under strong vacuum conditions, increased pressure caused cell lysis and loss of DNA into the filtrate. Alternatively, increased vacuum strength could have irreversibly embedded *Dhc* cells into the filter membrane matrix, or completely pulled them through membrane holes into the filtrate. The peristaltic pump approach resulted in relatively higher variability in this trial compared to variability observed in multi-lab round robins performed using positive pressure approaches in field filtration (Section 9). This could be due to the different filters and or extraction methods used in the field trial (Sterivex filter/PowerWater® Sterivex™ DNA Isolation Kit [Mo Bio, Carlsbad CA]) versus the in line flat membrane (Millipore Express membrane) filter and DNA extraction (PowerSoil Kit, Mo Bio) used in these experiments.

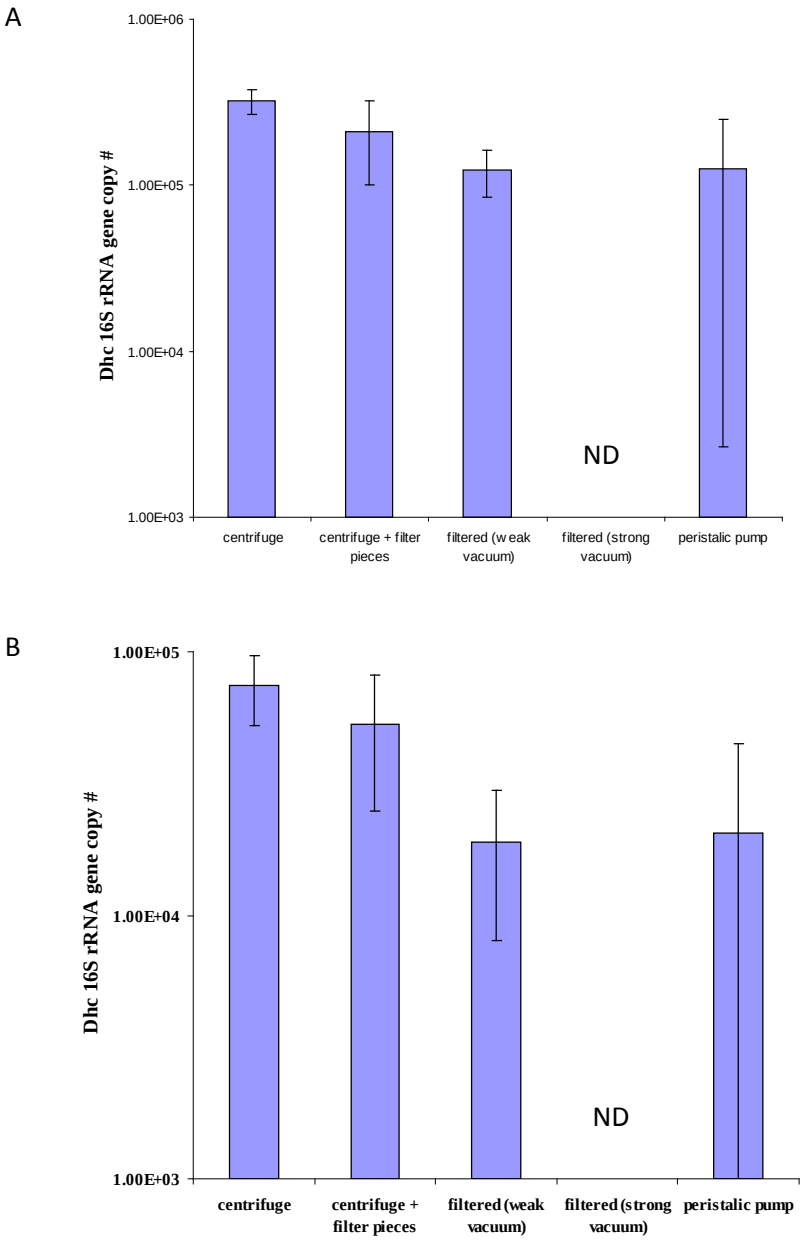

Figure 7-1: Effect of biomass collection method on *Dhc* quantification. Figure 7-1A presents data from filters and cells that were immediately processed and Figure 7-1B presents data from filters and cells that were stored frozen at -80°C prior to DNA extraction. Centrifugation produced the highest *Dhc* counts followed by weak filtration and then positive pressure filtration. In the strong filtration protocol, *Dhc* were not detected, indicating possible losses through the filter membrane. Overall *Dhc* enumeration was higher for filtered and immediately extracted samples (A) compared to filters frozen at -80°C prior to DNA extraction (B).

7.1.3 Conclusions and Implications for Future Research /Implementation

The variability obtained by performing filtration under different conditions highlights the potential influence of biomass concentration methods on quantitative *Dhc* results including the importance of:

1) Filter membrane type; and

2) Filtration protocols, particularly vacuum pressure.

Standardizing filtration procedures for *Dhc* quantification from groundwater samples has the potential to improve reproducibility of results. In particular, standardization and monitoring of the vacuum or positive pressure used when filtering samples in the lab or on site filtration protocols. Consistent use of the same filtration methods between samples (and sampling events) is also important to reduce variability in biomass concentration.

7.2 DNA Extraction-Impact of Bead Beating Intensity

Breaking open or lysing microbial cells is a critical step in the DNA extraction process. Frequently, cells are mechanically broken by vigorous shaking with inert beads (i.e., bead beating). Bead beating must be of sufficient duration and intensity in order to lyse cells, thereby releasing the DNA, without being unduly harsh. One mechanism that could degrade DNA is heat generation by bead beating, which could favor DNA hydrolysis. Therefore, bead beating may require optimization depending on the cell type in order to maximize recovery of intact DNA from the microbial targets of interest.

In this experiment, a previously optimized bead beater method (data not shown) was compared to the manufacturer recommended Vortex method for the MO BIO PowerSoil® DNA Isolation Kit (a widely used kit for extraction of DNA from groundwater). The tests were performed using groundwater samples to determine if increases in *Dhc* enumeration could be realized through the use of different bead beating methods.

7.2.1 Approach

The goal of this experiment was to determine if standard manufacturer recommended methods for DNA extraction kit widely used for groundwater (MO BIO PowerSoil® DNA Isolation Kit) were optimal for extraction of *Dhc* or alternatively if more intense bead beating could lead to increases in *Dhc* extraction efficiency. Nineteen groundwater samples from five different contaminated sites were vacuum filtered in parallel and the filters were placed into the bead tube of the MO BIO PowerSoil® DNA Isolation Kit. Bead beating was performed by either the:

1) Vortex method: 10 minutes on a Vortex Genie 2 (Fisher) operated at full speed (setting 8) fitted with a tube holder (Vortex Adapter, MO BIO Inc.).

2) Bead beater method: 2 minutes at 50% speed in the Mini-Bead Beater 8 (BioSpec Products Inc., Bartlesville, OK); "bead beater method."

A shorter duration was used for the bead beater method as it is more vigorous and previous optimization experiments indicated that bead beating for longer than 2 minutes did not lead to increases in *Dhc* enumeration. Total DNA extracted was determined with a NanoDrop spectrophotometer and the *Dhc* cells were enumerated using qPCR using a SYBR Green method (Attachment 2 [Attachment B.1]).

7.2.2 Results and Discussion

Detailed data are provided in Appendix J. Enumeration data for *Dhc* positive samples is summarized in Figure 7-2, data for *Dhc* negative samples are not shown.

Qualitative *Dhc* results from both the Vortex and the bead beater methods were identical (i.e., all samples that were non-detect [ND] for one method were ND with the other [9 out of 19 samples]). *Dhc* was quantified at a higher concentration using the bead beater method compared to the Vortex method for all 10 *Dhc* positive samples (there were 10 replicates used for statistical analysis). The *Dhc* recovery with the Vortex method ranged from 10% to 78% of the bead beater method with a mean of 32% of the bead beater quantification, indicating that the Vortex method was approximately 1/3 as efficient at extracting *Dhc* compared to the bead beater method. All differences observed between the bead beater and Vortex method was statistically significant at a 5% level of significance using a paired t-test on log transformed data.

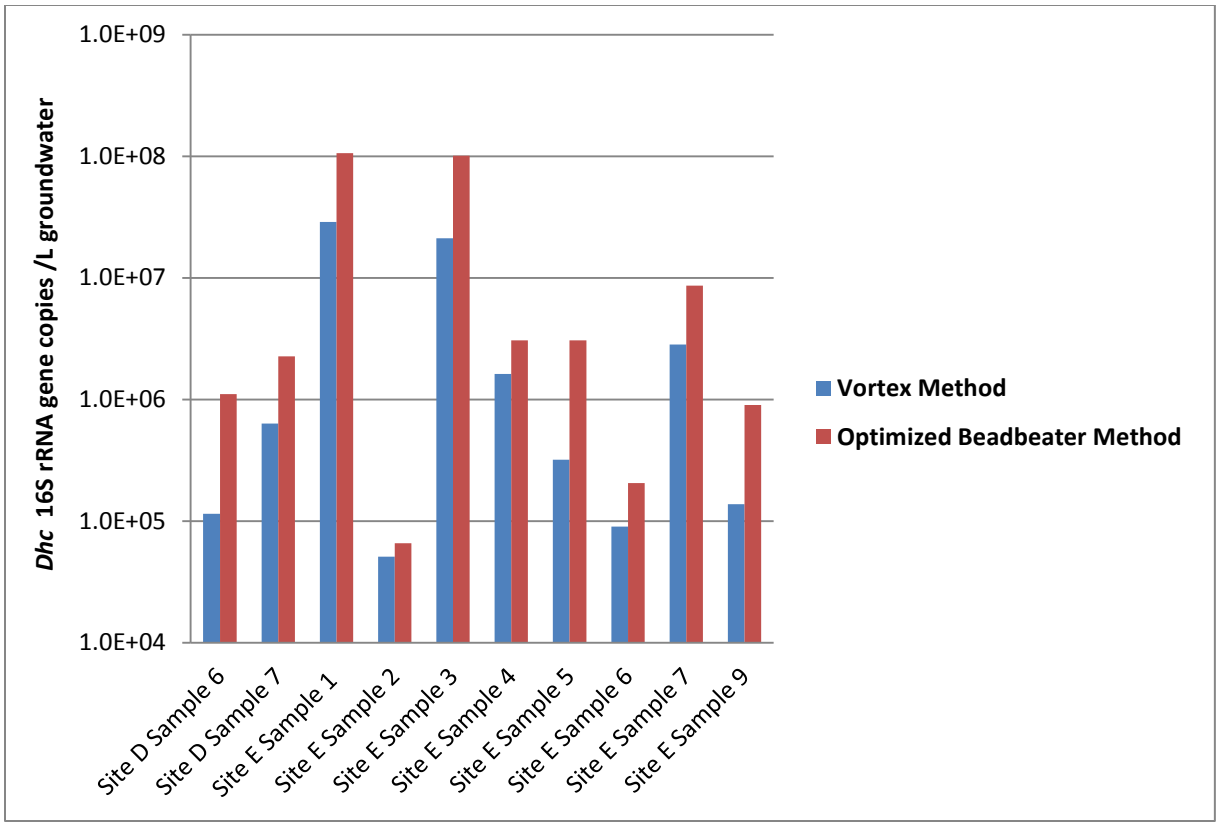

Figure 7-2: Comparison of total *Dhc* enumeration using Vortex versus bead beater method. Groundwater samples were extracted using manufacturer recommended 10 minute Vortex method (MO BIO PowerSoil® DNA Isolation Kit) or 2 minute bead beater method. Results indicated that higher *Dhc* enumeration was obtained in all cases with the bead beater method.

These data suggest that the manufacturer recommended Vortex method may not be optimal for the extraction of *Dhc* DNA from groundwater samples. This relatively inefficient extraction and could lead to an approximate 3-fold underestimation in *Dhc*. It should be considered that optimal bead beating intensity and duration may be sample and microorganism specific and should be determined on a case-by-case basis.

7.2.3 Conclusions

Bead beater duration and intensity may be an important variable in groundwater DNA extraction protocols. In addition, use of a Vortex for bead beating as recommended by a commonly used DNA extraction kit (MO BIO PowerSoil) may not be ideal for recovery of *Dhc* or other potentially difficult to lyse environmental microorganisms. The variability of groundwater samples and microbes contained within them may require optimization of bead beating for particular sample/microbial types.

7.3 PCR Methods

The qPCR reaction is the final step in the analytical process, after biomass collection and nucleic acid extraction. Round Robin 1 indicated that there were statistically significant differences at the level of the qPCR analysis (Section 4.2). Variability could occur in qPCR due to a variety of factors including PCR chemistry and the primers used these factors were assessed in the following subsections.

7.3.1 Impact of qPCR Chemistry:

TaqMan and SYBR Green are commonly used qPCR detection chemistries. The TaqMan assay (also called 5´ nuclease assay) relies both on a specific primer set for amplification of a gene fragment of interest, and a fluorescently-labeled, linear hybridization probe that specifically anneals to the target sequence between the primer binding sites. The TaqMan probe contains a fluorophore on its 5´ end (Figure 7-3) that does not fluoresce in the presence of a quencher molecule attached to the probe's 3´ end. During the extension step of the qPCR assay, the 5´ nuclease activity of Taq polymerase cleaves the probe, annealed between the primer sites, thus releasing the fluorophore from the quencher and resulting in fluorescence light emission. The increase in the intensity of the fluorescence produced is proportional to the amount of target genes in the template DNA.

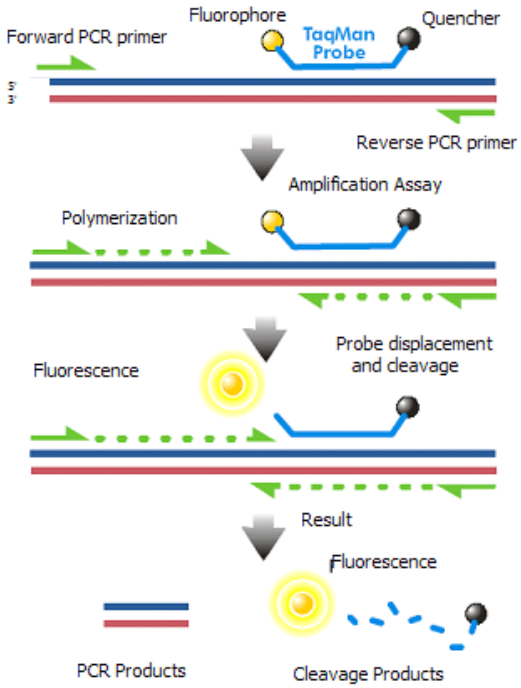

Figure 7-3: Principle of the TaqMan assay. The TaqMan probe binds specifically to the target DNA in between the amplification primers binding sites, making the assay highly target gene-specific. In the presence of template DNA, the probe is cleaved, the quencher released, and fluorescence produced (http://en.wikipedia.org/wiki/File:Taqman.png)

SYBR Green chemistry is slightly less expensive, and method development generally easier than TaqMan assays, because design and purchase of a probe is not required. In contrast to TaqMan methods, SYBR Green detection relies on the SYBR Green dye that binds non-specifically to double stranded DNA. As PCR amplification progresses, the SYBR Green dye binds to each new copy of double stranded DNA produced during each amplification round. The increase in the intensity of the fluorescence produced is therefore proportional to the amount of PCR product (i.e., number of amplicons) produced in the reaction tube.

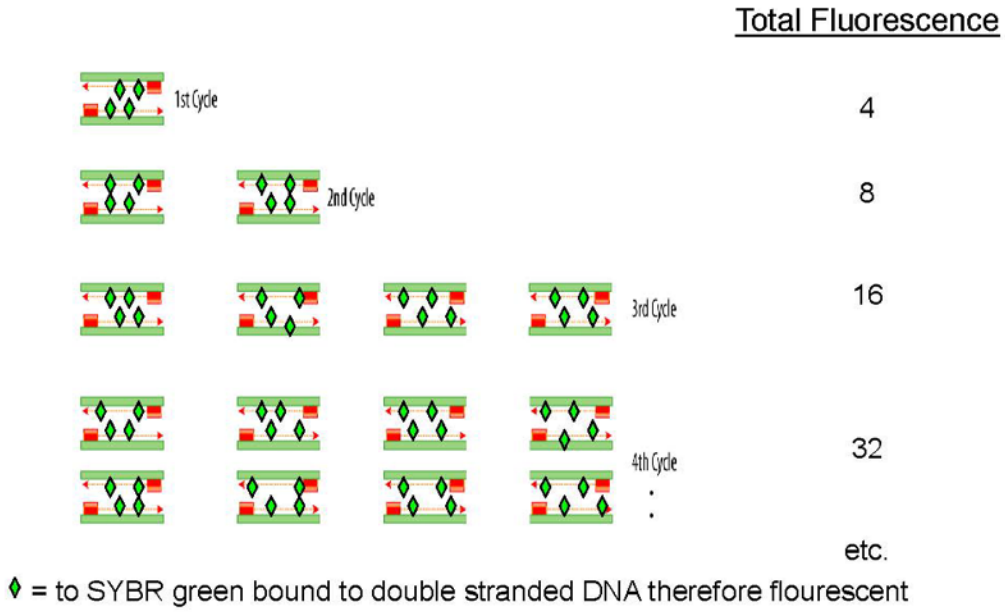

Figure 7-4: Principle of SYBR Green qPCR. SYBR Green (green diamonds) fluoresce maximally when bound to double stranded DNA. As PCR proceeds, the amount of double stranded DNA generally doubles in each PCR cycle and the fluorescent signal increases proportionally. SYBR Green binds to any double stranded DNA including non-specific amplicons and primer dimers. The unspecific binding properties of the SYBR Green dye increases the probability of false positives compared to the TaqMan assay unless appropriate QA/QC measures are implemented.

One drawback of the SYBR Green detection chemistry is that the dye binds to all double stranded DNA, including to primer dimers and non-specific amplification products, and therefore, SYBR Green chemistry is less specific and inclined to yield false positive results than TaqMan-based detection methods.

To determine if the different detection chemistries produce differences in quantification of the *Dhc* 16S rRNA target gene, the same primer set was used to quantify *Dhc* from various laboratory and environmental samples using both TaqMan and SYBR Green chemistries. The

study was carried out using the TaqMan-based primer and probe set (Ritalahti et al., 2006) as well as the same primers (but no probe) in conjunction with the SYBR Green chemistry. These data demonstrated that the TaqMan approach had a 10-fold lower limit of detection/quantification (Hatt and Löffler, 2012). Furthermore, in some samples, the SYBR Green chemistry produced false-positive results or yielded up to 6-fold higher biomarker gene abundances compared to the TaqMan method. Non-specific amplification caused overestimation of the target gene, and melting curve analysis was not sufficient to determine the extent of non-specific amplification (Hatt and Löffler, 2012). Gel electrophoresis and visualization of the amplicons was required to meaningfully interpret the qPCR results obtained with the SYBR Green detection chemistry.

Approach

TaqMan qPCR assays were carried out as described in Appendix K for singleplex qPCR except that 300 nM final concentrations of each forward and reverse primer and the probe were used. A single primer set (*Dhc* 1200F 5'-CTGGAGCTAATCCCCAAAGCT-3' and *Dhc* 1271R 5´-CAACTTCATGCAGGCGGG-3´) (Ritalahti et al*.,* 2006) was used for both TaqMan and SYBR Green qPCR assays. For the TaqMan assay, the probe *Dhc* 1240 5'-6-FAM-TCCTCAGTTCGGATTGCAGGCTGAA-BHQ-3' was also included in the reaction mix (Ritalahti *et al.,* 2006). SYBR Green qPCR assays contained the same concentrations of primer, template and master mix as the TaqMan reactions; however, the master mix was specific for SYBR Green (Applied Biosystems Part Number 4367659) and no probe was added to the assay mix. A final reaction volume of 20 µL was used for both assays.

Results and Discussion

Linear amplification curves were obtained with both TaqMan and SYBR Green detection chemistries over a dynamic range covering 8 orders of magnitude (3.38×10^8 to 3.38×10^0 gene copies) using 10-fold serial dilutions of a plasmid DNA containing the *Dhc* BAV1 16S rRNA gene (data not shown). TaqMan and SYBR Green chemistries resulted in similar amplification efficiencies of 87.0% and 89.5%, respectively. However, there was increased point scatter (i.e., data variability) at the lowest concentration of template DNA for SYBR Green, whereas no increase in scatter was observed with TaqMan detection, indicating the potential for more precise quantification by TaqMan detection chemistry when target genes are present in low abundance.

Quantification of Dhc from Laboratory Samples

Similar amplification efficiencies for TaqMan and SYBR Green detection chemistries (87.6% and 87.0%, respectively) were obtained during qPCR analysis of simulated groundwater samples augmented with the KB-1® consortium at two different dilutions (Figure 7-5A). Quantification of the *Dhc* 16S rRNA genes in *Dhc* strain BAV1 cultures corroborated that both detection chemistries yielded comparable results when template DNA was obtained from defined laboratory cultures (Figure 7-5B). Amplification efficiencies for TaqMan and SYBR Green

detection chemistries for *Dhc* strain BAV1 were 88.6% and 87.0%, respectively, and resulted in similar target gene enumeration.

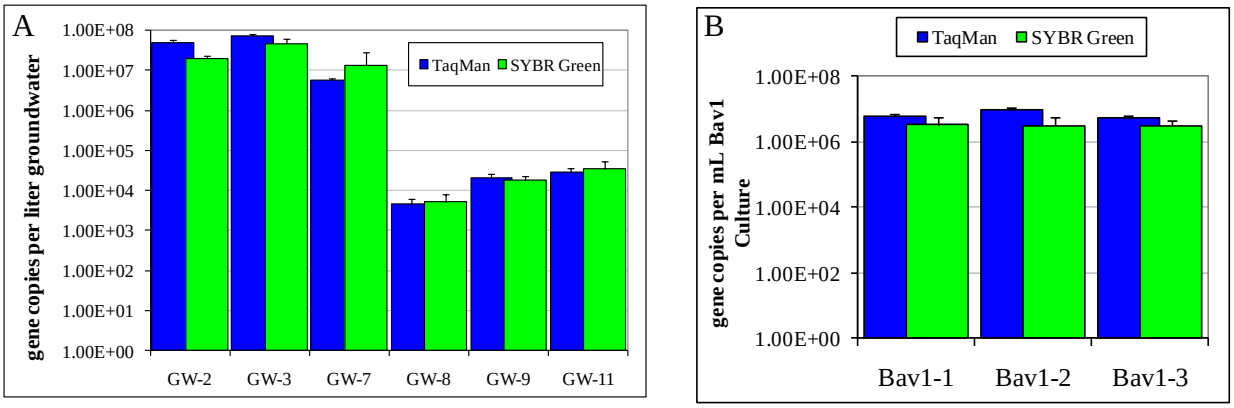

Figure 7-5: Comparison of *Dhc* quantification using TaqMan or SYBR Green detection chemistries using lab samples A: *Dhc* quantification from simulated groundwater augmented with the KB-1®consortium. B: *Dhc* quantification of a pure laboratory culture, *Dhc* strain BAV1.

This experiment indicated that in laboratory samples, SYBR Green and TaqMan assays performed more or less equivalently. To explore the performance of SYBR Green and TaqMan assays using environmental samples harboring diverse microbial communities, additional experiments were performed.

Quantification of Dhc in Environmental Samples Using SYBR Green and TaqMan Detection Chemistries

To test whether environmental samples would produce similar results using both qPCR detection chemistries, environmental samples collected in September 2008 at Fort Dix United States Army Reserve Command (USARC), Trenton, NJ, were assayed in parallel with TaqMan and SYBR Green chemistries. Amplification efficiencies using TaqMan and SYBR Green detection chemistries were 87.9% and 87.0%, respectively. These efficiencies were comparable to previously determined amplification efficiencies using *Dhc*-augmented groundwater samples and the pure culture *Dhc* strain BAV1. When environmental samples were assayed using SYBR Green detection, some variability was observed but the majority of samples produced similar results compared to the TaqMan detection (Figure 7-6: BMW-2, BMW-2x, BMW-4, and BMW-6). One notable exception was well BMW-8. When SYBR Green detection was used, the BMW-8 sample indicated the presence of *Dhc* whereas *Dhc* for this sample was not detected using the TaqMan method.

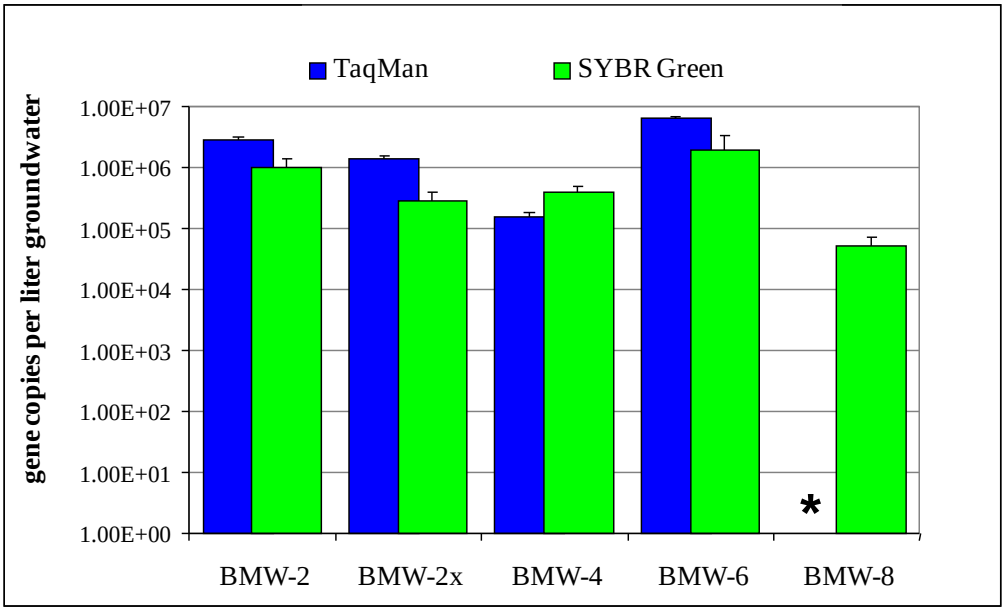

Figure 7-6: Comparison of *Dhc* quantification using TaqMan or SYBR Green detection chemistries for groundwater samples from Fort Dix USRAC. The asterisk indicates that *Dhc* 16S rRNA genes were not detected using the TaqMan detection chemistry for qPCR whereas the green bar indicates SYBR Green chemistry produced a positive result for this sample analysis.

To further examine observed differences between TaqMan and SYBR Green-based target gene quantification, the products of the SYBR Green qPCR reaction were analyzed using melt curve analysis and gel electrophoresis. Representative results are shown in Figure 7-7. For the wells exhibiting slightly lower *Dhc* counts with SYBR Green than with TaqMan (e.g., wells BMW-2 and BMW-2x and BMW-6 in Figure 7-6), a prominent band corresponding to the expected amplicon with a size of 66 base pairs (bp) was observed in the gel analysis. Melting curve analysis of the same samples indicated that a single product, comparable to the amplicon observed for the positive control (plasmid with *Dhc* 16S rRNA gene fragment as template DNA), was generated (Figure 7-7A). This finding indicated that the SYBR Green assay exhibited high specificity for these samples.

For the well BMW-4, that exhibited slightly higher *Dhc* counts with SYBR Green than with TaqMan (Figure 7-6), it is apparent that the high signal obtained with the SYBR Green detection chemistry was largely due to non-specific amplification products. Melting curve analysis for BMW-4 indicated many non-specific products with peaks in addition to the expected peak. Corresponding gel electrophoresis results confirmed this result, with additional bands apparent other than the expected amplicon. Due to the fact that the SYBR Green dye stains all double-stranded (ds) DNA and does not discriminate between target dsDNA amplicons and non-specific dsDNA amplicons or primer dimers, the presence of non-specific dsDNA provides an explanation for the higher fluorescence obtained with SYBR Green detection compared to TaqMan detection chemistry in this sample. The results obtained with samples collected from

well BMW-8 (Figure 7-6 and Figure 7-7C) also suggested that the SYBR Green approach produced inflated signals due to non-specific amplification.

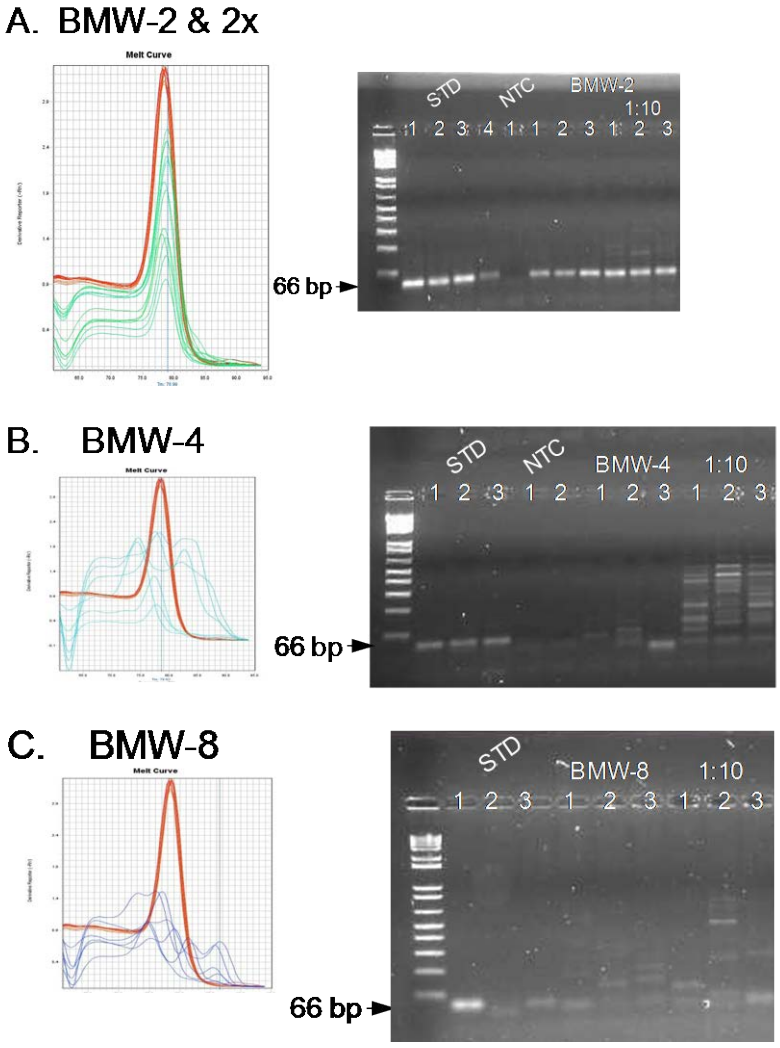

Figure 7-7: Melting curve and gel electrophoretic analyses of the qPCR amplicons with template DNA extracted from environmental groundwater samples using SYBR Green detection chemistry. A: Wells (Fort Dix, September, 2008) BMW-2 and 2x. B: Well BMW-4. C: Well BMW-8. The peak shown in orange represents the amplicon obtained from the positive control (pBAV1 standard plasmid) and corresponds to the lanes marked STD in gel electrophoresis panel. Each template was assayed in triplicate with undiluted sample and a 1:10 dilution. The target amplicon size is indicted by the arrow and marked 66 bp. Results indicated some non-specific amplicons in BMW-4 and BMW-8 with high biased results (BMW-4) and a possible false positive (BMW-8) with SYBR Green compared to TaqMan detection. STD (standard plasmid template [positive control]); NTC (No template control [negative control]).

Conclusions and Recommendations

SYBR Green and TaqMan detection chemistries generated comparable data when template DNA preparations from pure *Dhc* or enriched *Dhc* cultures were used in the analysis. SYBR Green detection has a greater potential than TaqMan to overestimate target genes with template DNA derived from some environmental samples. The reasons for inflated signals obtained with SYBR Green detection chemistry are related to primer dimer formation, and to non-specific amplification of non-target DNA by the primers used, which is more likely to occur when template DNA is obtained from environmental samples harboring diverse microbial communities. The TaqMan approach offers advantages in terms of target specificity for target gene enumeration from groundwater samples. When SYBR Green chemistry is used, it is necessary to include both melt curve analysis and gel electrophoresis of qPCR amplicons to verify that only the desired target DNA (i.e., *Dhc*) is being enumerated, which makes the analysis cumbersome.

7.3.2 Impact of PCR Amplification Primers on *Dhc* Quantification

A variety of different PCR primers have been developed for *Dhc* detection targeting the 16S rRNA gene. Cupples et al. (2008) and Yan et al. (2009) provide a partial summary of primers used for enumeration and detection of *Dhc*. Differences in primer performance can be caused by various factors including: a) specificity for *Dhc* or subpopulations of (different *Dhc* strains) b) amplicon size, and c) region of the 16S rRNA gene targeted. The choice of primers has the potential to impact variability for the quantification of the 16S rRNA gene of *Dhc* in groundwater. For this reason, the amplification efficiency of the main primers sets used by labs participating in this study was compared.

Table 7-2: *Dhc* primer sets used in the project by participating laboratories.

Primer set	Laboratories in this study employing primer set	Amplicon size (base pairs)	Detection chemistry
*Dhc*1200F / *Dhc*1271R	3/5	66	TaqMan
Dhc 1F /*Dhc* 264R	1/5	264	SYBR Green
Dhc-581F/ *Dhc*-1093R	1/5	514	SYBR Green

PCR efficiency is a measure of the effectiveness of the Taq polymerase to copy the template DNA molecules. The doubling of all template molecules in each amplification cycle is defined as 100% efficiency. PCR efficiency has the potential to impact quantification and detection limits of qPCR. Optimal qPCR standard curves are based on assays with 100% efficiency (Bustin,

2004, Bustin et al., 2009); however, PCR efficiencies in the 90 to 110% range are generally considered acceptable.

In general, shorter PCR amplicons lead to higher PCR efficiency, as the entire amplicon is more easily copied during each amplification cycle. This is due to the fact that Taq polymerase has an extension rate of between 30 to 70 bases per second (Bustin, 2004) and shorter amplicons require shorter extension times. Amplicons in the 80 to 250 base pair range (Invitrogen Corp., 2008) are generally preferred for qPCR applications, as longer amplicons may suffer from diminished amplification efficiencies or require additional method optimization in order to achieve desirable efficiency.

Approach

The three different *Dhc* primer sets used by the five participating laboratories in this study were compared using SYBR Green PCR under identical reaction conditions (See Appendix L for detailed methods). The different primers target different regions of the *Dhc* 16S rRNA gene and produce amplicons of varying lengths, from 66 base pairs up to 514 base pairs in length. The goal of this effort was to determine if differences in the performance of these primers were apparent.

Results

Standard curve data summarized in Table 7-3 and detailed in Appendix L indicated that the shorter amplicon primer sets exhibited a higher PCR efficiency than the primer sets yielding longer amplicons.

Table 7-3: Performance of different primer sets targeting the *Dhc* 16S rRNA gene

Primer set	Standard Curve Equation (Plasmid)	Calculated PCR Efficiency
*Dhc*1200F / *Dhc*1271R	Y = -3.18x + 35.98	106%
Dhc 1F /*Dhc* 264R	Y = -3.27x + 34.4	102%
Dhc-581F / *Dhc*-1093R	Y = -3.44x + 36.5	95%

The 1200F/1271R primer set produced a slope of -3.18 (i.e., 106% efficiency). The 1F/264R primer set produced a slope of -3.27 (102 % efficiency) The 581/1093 primer set produced a slope of -3.44 (95 % efficiency). These results confirm an inverse relationship between amplicon length and PCR efficiency. Nevertheless, all primer sets tested demonstrated acceptable efficiency using plasmid DNA as a template indicating that PCR efficiency is not likely a major variable in qPCR analysis between the participating labs.

7.3.3 Conclusions Regarding qPCR Methods

Both SYBR Green and TaqMan qPCR chemistries can produce accurate and comparable quantification results with defined laboratory cultures.

SYBR Green chemistry is more inclined towards false positives than TaqMan, particularly with environmental samples. Greater care is required in interpreting SYBR Green assay results to rule out non-specific amplification, including melting curve analysis and gel electrophoretic confirmation of specific amplification.

Commonly used *Dhc* primer sets with differing amplicon lengths from (66 bp to 514 bp) provided acceptable PCR efficiencies, but shorter amplicons are generally preferred as they are less likely to suffer from PCR inhibition and overall exhibit higher PCR efficiency and fidelity.

7.4 Impact of qPCR Calibration Materials:

Ultimately, the accuracy of qPCR is contingent upon on accurate initial quantification of the DNA (i.e., calibration material) used for standard curve preparation. The literature review , performed at the outset of the project, indicated that qPCR standard curves were produced using a variety of materials including: plasmid DNA containing cloned gene fragments of interest (Koike et al., 2007), PCR-amplified DNA fragments, genomic DNA (Cremonesi et al., 2006; Klerks et al., 2006), viral DNA containing target DNA fragments, artificially synthesized DNA fragments, and whole bacterial cells (McDaniels et al., 2005; Haugland et al., 2005).

This section summarizes experiments to test the impact of DNA quantification methods, primarily spectrophotometry and fluorometry on qPCR calibration and enumeration. This effort was undertaken based on findings in the literature indicated that potential biases could be introduced by DNA quantification methods and the topological form (i.e., supercoiled or non-supercoiled) of DNA used as calibrators (Laghi et al., 2004; Chen et al., 2007, Hou et al., 2010 and Lin et al., 2011).

7.4.1 DNA Quantification

<u>Quantifying DNA using Spectrophotometry</u>

One of the simplest and most common methods for assessing DNA concentrations is using ultraviolet (UV) absorbance at 260 nm (A260). This analysis can be performed using a spectrophotometer, and the readings can be converted to concentrations using extinction coefficients (valid only at neutral pH) specific to the type of DNA being assayed (Mackay, 2007). Spectrophotometric methods can also provide information about the purity of the DNA and generally work best for samples with DNA concentrations greater than 250 ng/mL (Mackay, 2007).

NanoDrop™ spectrophotometers (NanoDrop Technologies, Wilmington, DE) are widely used for quantifying DNA. A NanoDrop spectrophotometer has a dynamic range (2-3700 ng/µL) and requires only 1-2 µL of sample, a key advantage as traditional spectrophotometer cuvettes require volumes (e.g., 100 µl) that can exceed the entire volume of DNA extracted from an environmental sample.

A negative aspect of spectrophotometry is that it cannot distinguish DNA from RNA, and is sensitive to the form of nucleic acid (e.g., single stranded versus double stranded plasmid versus chromosomal etc.). Free nucleotides/nucleosides also absorb at 260 nm, all of which can lead to erroneous overestimates.

Figure 7-8 compares of NanoDrop quantification of DNA in the plasmid stock solution distributed in Round Robin 1 and shows NanoDrop readings obtained in the five analytical laboratories. Each laboratory quantified a DNA stock with an identical quantity of plasmid DNA measure at 19.1 ng μL^{-1} in the source lab. Measured concentrations ranged from 20-25 ng/µL. These results seem to indicate that lab to lab variability for NanoDrop quantification did not exceed 25% for plasmid DNA samples, indicating that DNA quantification was not a major source of inter-laboratory variability.

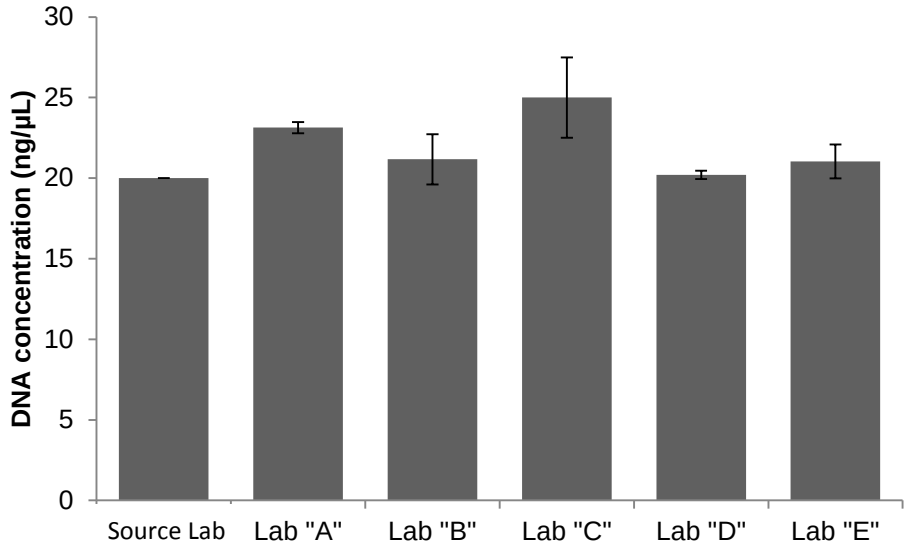

Figure 7-8: Comparison of NanoDrop quantification of plasmid DNA between in 5 laboratories including the source lab in Round Robin 1. The results indicated agreement within 25% between labs.

Quantifying DNA by Fluorometry

Fluorescent dyes such as PicoGreen® and Hoechst dye are widely used to quantify DNA when concentrations are low and for high throughput analyses. Fluorometry methods can be carried out in microtiter plates using small volumes at low nucleic acid concentrations and the dyes have

defined excitation and emission spectra upon binding to nucleic acids. Moreover, dyes have been developed that preferentially bind to certain kinds of nucleic acids, such as double stranded DNA or RNA, imparting greater specificity than spectrophotometry (Wilding et al., 2009). In one study (Wilding et al., 2009) fluorometry was reported as being more reproducible than NanoDrop spectrophotometry with detection at low DNA concentrations (1.5ng/µL) not reproducible with Nano Drop and "eminently reproducible" using a fluorometry method (Quanti-T PicoGreen Assay). The manufacturer reported detection limit for NanoDrop spectrophotometry is 2.0 ng/µL (NanoDrop 1000 Spectrophotometer V.3. User's Manual).

In general, spectrophotometry is less prone to overestimates of DNA concentration due to the presence of contaminants, pH effects, free nucleotides, and RNA (Wilding et al., 2009). Given that spectrophotometry is widely used for quantification of calibration standards, observed differences between spectrophotometry and fluorometry could be an important factor in qPCR calibration. For this reason comparative testing was performed to assess the potential impact of these two commonly used DNA quantification methods.

Comparison of Spectrophotometry and Fluorometry for DNA Quantification

Numerous publications and on line sources suggest quantitative differences occur between NanoDrop spectrophotometry and PicoGreen fluorometry. Genome Quebec Innovation Centre (2013) encourages the use of PicoGreen quantification of DNA samples versus spectrophotometric methods, which "tend to overestimate sample concentration". Wilding et al. (2009) observed 2.6 fold higher quantification with NanoDrop spectrometry versus PicoGreen fluorometry for mosquito genomic DNA, and noted that NanoDrop was especially inclined towards inaccuracy when DNA concentrations were low. A 2.5 fold difference between spectrophotometry and an HPLC method was reported by Lin et al., (2011). Given these findings, a comparison of these methods was performed on a variety of DNA samples from lab cultures and groundwater samples

Approach

A comparison of NanoDrop and PicoGreen quantification was performed for a variety of plasmid samples and genomic DNA samples at varying concentrations. Detailed fluorometry methods are summarized in Appendix I and the data are summarized in Figure 7-5 and Appendix M.

Results

In general parallel DNA quantification with spectrometry versus fluorometry (Figure 7-9) indicated an overall tendency for higher quantification by NanoDrop spectrometry of approximately 2.5-fold compared to fluorometry. In addition, work summarized in Appendix M also noted differences between the two methods, confirming that the spectrophotometric method overestimated the DNA concentrations. A possible reason suggested was the presence of RNA. The data suggest that spectrometry tended to disproportionately inflate genomic DNA quantities, and to a lesser extent plasmid DNA quantities. The difference between spectrophotometry and

fluorometry with plasmid DNA was only a 1.4 fold higher quantification with spectrophotometry, a difference that could be considered negligible. A larger 2.8 fold inflation of DNA concentration was observed when genomic DNA was quantified using spectrophotometry; which could reflect a tendency for genomic DNA preparations to be contaminated with non-DNA compounds that absorb light at 260 nm (e.g., from groundwater samples).

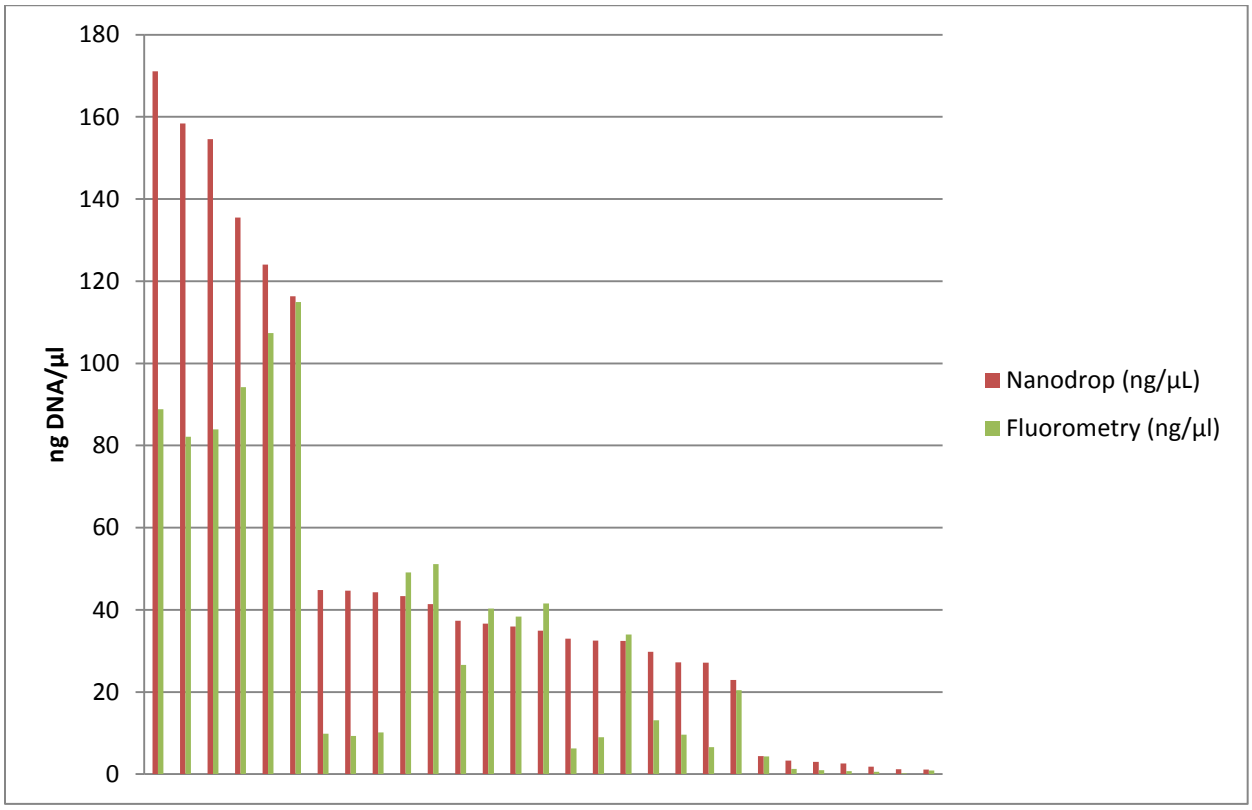

Figure 7-9: Comparison of DNA quantification of plasmid and genomic DNA samples with NanoDrop spectrophotometry and PicoGreen fluorometry. Each bar (red/green) represents a DNA sample quantified by NanoDrop spectrometry (red) or Pico Green Fluorometry (green. DNA quantification averaged 2.5-fold higher with NanoDrop compared with fluorometry, consistent with other studies reported in the literature.

7.4.2 Impact of Plasmid DNA Topology on qPCR Calibration

A number of studies (Laghi et al., 2004; Chen et al., 2007, Hou et al., 2010 and Lin et al., 2011) suggest that different topological forms of plasmid DNA (i.e., supercoiled, nicked, and linear) can affect qPCR enumeration of gene targets. One theory is that supercoiled DNA, which is tightly wound, much like a twisted elastic band (See Figure 7-10), inhibits the denaturing of the DNA in PCR and limits access of the strand to the PCR primers. In effect, this inhibited denaturing decreases the target available for PCR amplification (Lin et al., 2011). This shifts the

standard curve so that it has a similar slope but a higher Y intercept (See Figure 7-11). The altered standard curve overestimates the amount of target in a genomic DNA sample (which is not supercoiled) and is not subject to the same biases as the supercoiled plasmid calibration standards.

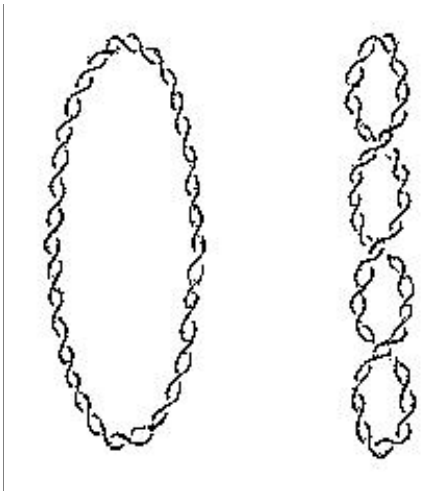

Figure 7-10: Topological forms of plasmid DNA. Left is nicked and in relaxed form, right is supercoiled and more difficult to denature in PCR reactions (http://en.wikipedia.org/wiki/DNA_supercoil)

Differences in supercoiling could impact the accuracy of qPCR where, as is commonly the case, plasmid is the calibration material and genomic DNA is the test target. Chen et al. (2007) reported that overestimates of target quantification of up to 10-fold were attributed to the use of supercoiled plasmid DNA as a calibration material for qPCR. Fortunately, supercoiling of plasmids can be removed by either nicking (cutting one strand with a nick endonuclease) or by linearizing (cutting both strands with a restriction endonuclease). Most extracted bacterial genomic DNA, while also supercoiled when in the cell (Griswold, 2008), is not expected to be supercoiled during qPCR analysis. This is due to the fact that genomic DNA is relatively long, as microbial genomes generally contain more than a million base pairs, and is expected to shear during extraction, thereby releasing the supercoiling.

Given the reported potential for supercoiled plasmid to impact qPCR, experiments comparing the performance of various forms of plasmid (supercoiled, nicked, linear) versus genomic of DNA were performed and are summarized in the following subsections. Two independently performed experiments were performed relevant to determining the impact of supercoiling, or more specifically, comparison of plasmid versus genomic DNA as calibration materials.

Experiment 1

Approach

This study compared standard curves produced by different forms of plasmid and genomic DNA using three different *Dhc* 16S rRNA gene-targeted primer sets. Detailed data and methods for these experiments are provided in Appendix L. Briefly, different sources and forms of DNA were tested including:

1) Supercoiled (i.e., non-nicked/non-linearized) plasmid DNA containing a cloned 16S rRNA gene ;

2) *E. coli* genomic DNA extracted from the MIAC (expected to be non-supercoiled).

3) Nicked plasmid DNA, in which one of two strands is cut (treated with a nicking restriction endonuclease enzyme Nt.Bst 1) to release supercoiling;

4) Linear plasmid DNA (treated with a restriction endonuclease that cuts both strands); and

5) Linear PCR product.

Results

A summary of the results (for plasmid versus genomic DNA) are provided in Table 7-4 below (detailed data provided in Appendix L). The equation of the standard curves and a target quantity for an assumed threshold cycle (C_t of 20) using the respective standard curves is provided.

Table 7-4: Standard curve equations and relative enumeration for three primer sets with plasmid and genomic DNA

Primer Set	Plasmid (presumed supercoiled)		*E. coli* Genomic DNA (non-supercoiled)		
	Equation	Quantification (C_t 20)	Equation	Quantification (C_t of 20)	Fold Change versus Plasmid
**Dhc*1200F /*Dhc*1271R	Y = -3.18x + 36.0	1.1E+05	Y = -3.26x + 35.7	6.6E+04	-1.6
Dhc-581F/ *Dhc*-1093R	Y = -3.44x + 36.5	6.3E+04	Y = -3.69x + 35.9	2.0E+04	-3.1
Dhc 1F /*Dhc* 264R	Y = -3.27x + 34.4	2.5E+04	Y = -3.37x + 34.1	1.5E+04	-1.7

The results indicated that the supercoiled plasmid produced a higher Y intercept than genomic DNA and correspondingly higher *Dhc* enumeration results of 1.6-3.1 fold compared with genomic DNA. This result is consistent with the literature on this topic in that supercoiled DNA produced a higher Y intercept. Other data (Appendix L) indicated that linearized plasmid and the nicked plasmid and PCR products produced even higher Y intercepts (and *Dhc* higher enumeration) compared with supercoiled plasmid and genomic DNA. These results are inconsistent with the literature, which reports that linear or nicked plasmids might be expected to produce a lower Y intercept than supercoiled plasmid (Lin et al., 2011).

The reasons for discrepancies between this study with respect to linear and nicked plasmids and literature findings are unclear and indicate further study is required on the impact of nicking or linearizing plasmids for use as calibration standards. It is notable that nicking of plasmid DNA is a common occurrence and can occur through heat, mechanical sheer and freeze thaw, which are common events in the laboratory (Lin et al., 2011). Therefore the impact of plasmid nicking should be examined further to assess its possible impacts on qPCR calibration, even if nicking is not deliberately performed. Also linear PCR product was not recommended as a calibration standard as large differences were observed between primer sets suggesting possible biases.

Despite the ambiguous results with nicked and linear DNA this study suggested that modestly higher (compared to genomic DNA) qPCR enumeration could occur if non-nicked plasmid (supercoiled) was used as a calibration material. The genomic DNA versus plasmid DNA data suggest supercoiled plasmid increased enumeration over genomic DNA by approximately 1.6 to 3.1 fold, which is consistent with, but lower than, the reported 10-fold overestimate indicated by the literature (Chen et al., 2007). The current study concluded that both supercoiled plasmid and genomic DNA are acceptable calibration materials and that differences between them are likely modest. Nevertheless, modest overestimates related to supercoiled plasmids could be compounded by other upward biases, such as those related to DNA quantification (Section 7.4.1.) These combined impacts were examined in more detail in Experiment 2.

Experiment 2

Approach

In this experiment the performance in standard curves was compared for:

1) Genomic DNA quantified using fluorometry; and

2) Plasmid DNA quantified using NanoDrop spectrophotometry.

Both templates were used to produce a 7-point standard curve with qPCR targeting the *luc* gene present in the genomic (MIAC) or plasmid DNA. Templates were amplified and quantified on the same plate to reduce variability between the treatments. All methods are summarized in Appendix I.

Figure 7-11 demonstrates standard curves produced using plasmid versus genomic DNA and indicates an increase in the Y intercept of 1.8 C_t units for the spectrophotometry quantified plasmid versus fluorometry quantified genomic DNA. The higher enumeration values of the high Y intercept plasmid curve are attributed to the combined impact of different DNA quantitation methods (spectrophotometry versus fluorometry) and the impact of potentially supercoiled plasmid versus non-supercoiled genomic DNA. In a qPCR test the difference in the Y intercept would lead to an approximate and approximate 5-fold quantification difference between the two curves. Some of this difference is due to quantification differences between spectrophotometry and fluorometry (Section 7.4.1) (~2.5-fold) and some is likely due to supercoiling effects (~2-fold). The 2-fold difference due to supercoiling effects is consistent with differences observed between standard curves in Experiment 1 above.

Results

The calibration performance of plasmid DNA (quantified by NanoDrop) and genomic DNA extracted from the MIAC (quantified by fluorometry) were compared. The goal of this experiment was to determine if a commonly used qPCR calibration approach (i.e., non-nicked [presumed to be at least partially supercoiled] plasmid quantified with spectrophotometry) produced inflated qPCR enumeration compared to using an alternate calibration material

(genomic DNA [presumed to be non-supercoiled] quantified by fluorometry). The MIAC was used for this experiment because could also be enumerated by qPCR and also by plate counts. This property allowed comparison of qPCR results to a non-PCR method. It would be expected that the most accurate qPCR method would agree most closely with the plate counts as discussed in Section 6.3.1. This experiment was designed to demonstrate the additive impact of the DNA quantification with spectrophotometry combined with the impact of supercoiling. These approaches combined would increase the Y intercept of the qPCR calibration curve, and together could impart substantial overestimates in gene target enumeration.

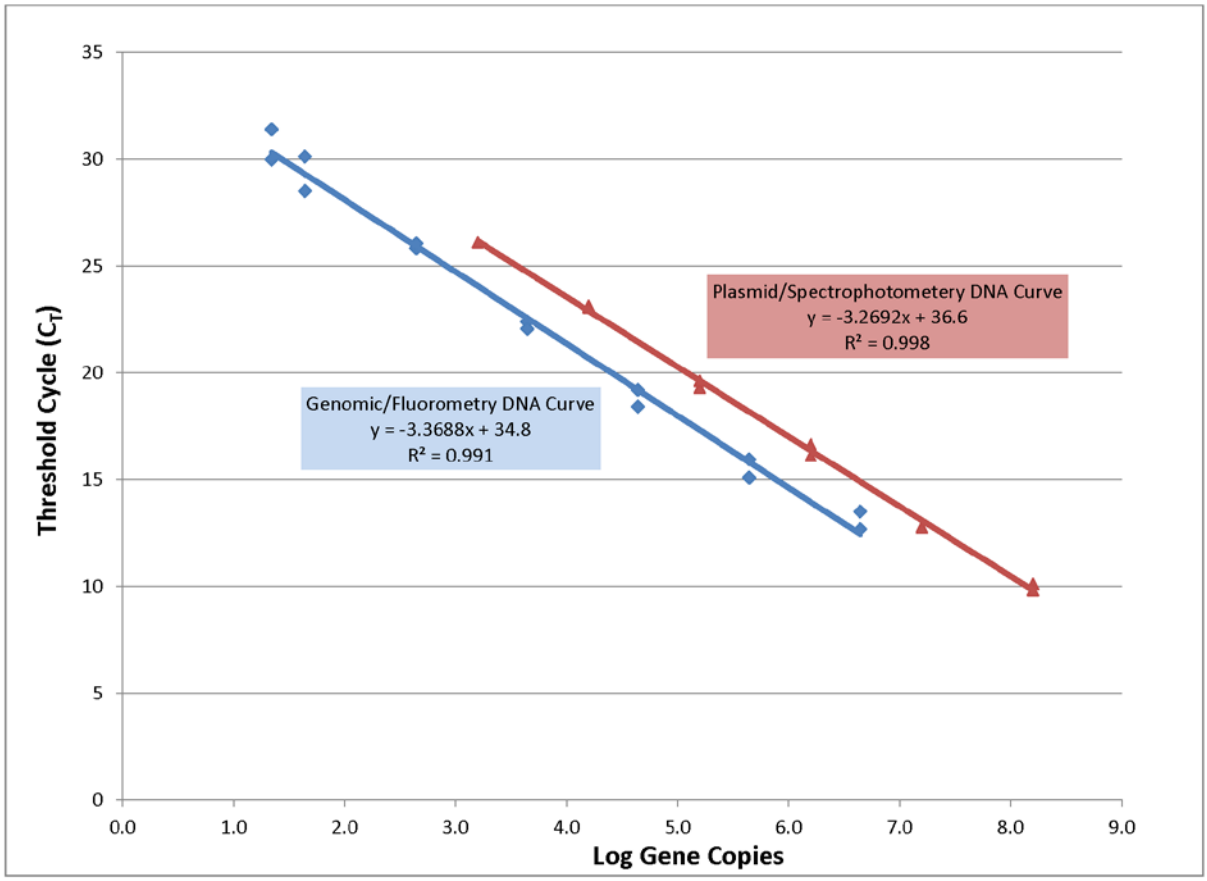

Figure 7-11: Demonstration of differences in standard curves using plasmid DNA-quantified by NanoDrop spectrophotometry- (blue-diamonds) versus genomic DNA -quantified by fluorometry (red-triangles). Differences in the Y intercept value would result in an approximately 5-fold greater quantification in qPCR test results with the plasmid versus the genomic DNA curve (See Table 7-5).

Table 7-5 demonstrates the impact of using the plasmid calibrated and genomic DNA calibrated standard curves for quantifying MIAC frozen aliquots, which were quantified by three methods.

1) qPCR standard curve with luc plasmid quantified with NanoDrop spectrophotometry (red standard curve Figure 7-11);

2) qPCR standard curve with luc genomic DNA quantified by fluorometry (blue standard curve Figure 7-11); and

3) Plate counts.

Table 7-5: Observed differences of qPCR calibrated by plasmid (NanoDrop quantified) compared to genomic DNA (fluorometry quantified) and plate counts

Replicate	qPCR Methods using Different Calibration Approaches		Plate Counts
	Plasmid Standard Curve (quantified by NanoDrop) luc gene copies/mL	Genomic DNA Standard Curve (quantified by fluorometry) luc gene copies/mL	Frozen Cells (cfu/mL)
1	1.55E+08	3.61E+07	2.72E+07
2	1.64E+08	3.61E+07	2.87E+07
3	1.92E+08	4.06E+07	2.55E+07
4	1.99E+08	4.33E+07	3.48E+07
5	1.84E+08	3.93E+07	2.73E+07
6	1.51E+08	3.98E+07	2.51E+07
Average	1.74E+08	3.92E+07	2.81E+07
Percentage of plate counts	620%	140%	100%
Comment	Overestimates more than 6-fold compared to plate counts	Approximately consistent with plate counts	--

The data indicate that the genomic DNA quantified by fluorometry approximated the plate counts (within 40%) whereas the plasmid DNA curve enumeration was 6-fold greater than plate counts (620%). This data suggests that the fluorometry quantified genomic DNA was a more accurate calibration material than spectrophotometry quantified plasmid. Plasmid is more commonly used as a qPCR calibration material due to the ease with which plasmid DNA can be procured. A key challenge in some cases, is obtaining genomic DNA especially where isolated cultures of the target microorganism are not available (e.g., *Dhc* is not easily grown in pure culture). In contrast, plasmid DNA can be readily replicated in *E. coli*. One potential solution, is to use a genomic insertion procedure (as was used with the MIAC) to produce virtually unlimited source of genomic DNA-based targets in *E. coli*. For example, the *Dhc* 16S rRNA gene could be

inserted in the genome of *E. coli* and the *E. coli* genomic DNA could then be used to calibrate qPCR. The use of *E. coli* with genomic insertions to produce calibration materials has the added advantage of qPCR verification through comparisons between qPCR results and plate counts.

7.4.3 Conclusions and Implications for Future Research/Implementation

The literature on the use of DNA quantification and topological form of DNA methods suggest that the both of these factors have the potential to substantially impact qPCR data (i.e., up to 10 – fold). Experiments performed under this project agreed with the literature with respect to modest overestimates of DNA quantity when spectrophotometry was used to quantify genomic DNA, but this effect was less significant with plasmid DNA, which is more commonly used to calibrate qPCR.

Despite several reports in the literature on the impact of supercoiled plasmid on qPCR calibration, these effects are less clear. Experiments carried out in this project seem to suggest a consistent quantitative effect of plasmid versus genomic DNA. In other words, higher quantitative results were obtained with supercoiled plasmid DNA calibration compared to genomic DNA calibration. Nevertheless, this impact was not entirely consistent with the literature. For example, lower quantification was not observed with nicked or linearized plasmid compared to supercoiled in this study. Furthermore, the observed impact of supercoiling was lower in magnitude in this study than suggested by other researchers. Therefore plasmid DNA is still viewed as an acceptable calibration approach by the project team due to the relatively modest impact of these effects.

The following recommendations should be considered when implementing qPCR calibration approaches.

- When using plasmids for calibration, analyze plasmid DNA utilizing gel electrophoresis to ensure that genomic DNA or RNA is not inadvertently co-extracted with the plasmid. The presence of genomic DNA or RNA could lead to an overestimation of gene copies when generating a standard curve.

- Report and record DNA preparation, quantification, storage and use procedures and perform these consistently between batches of calibration materials to reduce the potential for variability between calibration events.

- If possible, verify qPCR results with a non-PCR method, such a plate counts, as was done with the MIAC (Section 6.3). This approach can flag potentially inaccurate qPCR results where they differ from plate counts.

7.5 Overall Conclusions for Analyses and Optimization of Laboratory Methods

The results summarized in this section indicated that several lab procedures in the biomass collection, DNA extraction and qPCR process had the potential to significantly impact the quantitative results obtained for *Dhc*. Key conclusions include.

- Filtration procedures applied for biomass collection affect *Dhc* recovery. Low pressure vacuum or positive pressure filtration using membrane filters yielded highest *Dhc* recovery, therefore the minimum practical vacuum or positive pressure should be used to prevent *Dhc* losses.

- Choice of filter membrane material also had substantial quantification impacts and should be optimized for the specific application.

- Cell lysis is a critical step in DNA extraction protocols and beat beating protocols should be optimized for the organism(s) of interest to ensure effective cell lysis.

- TaqMan chemistry offers higher sensitivity and specificity than SYBR Green qPCR when environmental samples are analyzed.

- For qPCR standard curve preparation, DNA quantification of calibration materials is a critical step and the accuracy of DNA quantification by spectrophotometry or fluorometry must be verified. Since different DNA templates can affect calibration results the quality and consistency of the standard (e.g., plasmid DNA, genomic DNA) must be monitored.

In general, the experiments carried out in this section indicated that the current laboratory approaches to biomass concentration, DNA extraction and qPCR can be accurate and consistent. Nevertheless it is also clear that method optimization, organism specific customization, and attention to ongoing quality control and verification is essential for obtaining the most accurate results. For example, SYBR green chemistry is generally accurate but requires extra vigilance in order to rule out false positives. Also qPCR calibration using *E. coli* with chromosomal transgene targets allows verification of qPCR by plate counts. Further experience with a variety of organisms, in addition to *Dhc* and *E. col*i, will aid in determining the best individual approaches for cell concentration and nucleic acid extraction for a variety of microorganisms.

8 ASSESSMENT OF GROUNDWATER SAMPLING, PRESERVATION AND STORAGE METHODS

Groundwater sampling is feasible at most remediation sites through existing infrastructure (e.g., monitoring wells) whereas soil/sediment samples are often more difficult to obtain. When compared to soil/sediment samples, groundwater collected through monitoring well screens may also provide a more representative sample because the groundwater sample integrates a greater aquifer volume than a solid sample. Groundwater samples may also be less inclined to matrix inhibition compared to soil /sediment as the concentrations of humic compounds are generally lower in groundwater (see Section 6.5). For these reasons, groundwater is the most commonly matrix used for bioremediation focused qPCR analysis.

For accurate quantification of microorganisms in groundwater, a representative groundwater sample must be obtained, contained, stored and preserved such that biomarkers (DNA, RNA, lipids etc.) can be accurately quantified upon arrival in the laboratory. There are various factors that can affect sampling and stability of samples and therefore the representativeness of the sample.

Ultimately, recommended field sampling techniques and preservation protocols will be required to develop standardized methods for quantifying microorganisms of interest in groundwater including:

- Filtration approaches (e.g., on site versus laboratory filtration) (Section 8.1);
- Well sampling methods (high purge versus low purge) (Section 8.2); and
- Storage and preservation of groundwater samples, collected biomass and extracted DNA (Section 8.4).

8.1 Site Description

Plumes A and B at the Bachman Road Site (Site) in Oscoda, Michigan were selected as the study sites for evaluating filtration approaches and well sampling methods. Source area bioremediation activities, employing extraction and reinjection of groundwater amended with electron donor and a bioaugmentation culture, were conducted at the site. The site was well-instrumented and demonstrated evidence that stable reductive dechlorination conditions were established. Stable site conditions are important to differentiate variation associated with field sampling methods rather than simply variation of *Dhc* abundance in the aquifer (i.e., fluctuations in target cell abundances are not expected to occur over the sampling period).

Groundwater sampling to establish baseline parameters was conducted at the Bachman Road site on November 23, 2011. Standard operating procedures (SOPs) and results of *Dhc* and VOC analyses are provided in Attachment 4. Based on these data, the following three (3) monitoring locations were selected for the field variability assessment:

1) ML-3 (22.3 feet [ft]) – source area A multi-level sample point with high (i.e., ~10^7/L) *Dhc* titers; and

2) A-ML-3 (10 ft) – plume B multi-level sample point with low (i.e., ~10^4/L) *Dhc* titers. Note: A-ML-3 was damaged during the final sampling event of this study.

3) Nearby multi-level A-ML-25 was used as an alternate sample point.

8.2 On Site versus Laboratory Filtration of Groundwater

One of the initial decisions when designing a field sampling plan for qPCR testing is whether sufficient biomass from groundwater samples can be obtained by either:

1) Collecting groundwater in bottles and shipping water to the testing laboratory where filtration is performed prior to DNA extraction ("lab filtration"); or

2) Sterivex cartridge filtration of groundwater in the field (i.e., on site) and shipping the cartridges to the testing laboratory ("on site filtration").

While shipping of groundwater to labs has traditionally been used, on site filtration reduces shipping costs as well as contaminated water disposal costs for the analytical laboratory. The use of Sterivex™ cartridges (originally designed to filter-sterilize liquid samples) have found increasing use as a means of collecting biomass from water bodies.

The recent development of commercial protocols (e.g., PowerWater® Sterivex™ DNA Isolation Kit) has decreased the difficulties related to removing biomass from these filters, which previously required opening the filter with a tube cutter and removal of the filter membrane using a scalpel. Ritalahti et al. (2010) demonstrated that Sterivex™ filtration yielded precise and accurate results with laboratory samples spiked with *Dhc* and from groundwater samples. The goal of this experiment was to test if consistency in terms of *Dhc* enumeration could be demonstrated in multiple labs using on site versus off site lab filtration.

8.2.1 Approach

The comparison of "on site filtration" and "lab filtration" was carried out as part of Round Robin 4, a five laboratory round robin conducted in February 2012 on groundwater samples obtained from the Bachman Road site in Oscoda, MI (for detailed methods/overview see Attachment 4). Sampling was performed using low flow methods and groundwater was purged from wells ML-3 and A-ML-3 at a flow rate of 100 to 200 mL/min. Approximately 40 L of groundwater were collected from each monitoring location into a clean, sealable polyethylene drum. The polyethylene container was sealed and the groundwater was homogenized by turning the container end to end several times and stirring with a sterilized paddle during aliquoting. Using low flow pumping, groundwater samples were transferred into 1-L plastic sample bottles for subsequent lab filtration. The remaining groundwater in the drum was pumped through

Sterivex™ cartridges (1 L per cartridge). Groundwater samples and cartridges were shipped in coolers with ice packs to the five participating laboratories by express air freight, and efforts were made to coordinate storage times prior to sample processing so that losses of *Dhc* biomarkers due to storage times were minimized. The samples were processed by standard methods for both on site filtered and lab filtered samples (Attachment 2). It should be noted that Sterivex cartridges used for onsite filtration were used incorrectly (without end caps), which could have led to some biomass loss into the secondary containment tube provided with the filter during shipping. To the extent possible, labs attempted to recover biomass, which may have leaked from the cartridges during shipping prior to extraction.

8.2.2 Results and Discussion

Figure 8-1 demonstrates mean *Dhc* enumeration results for five lab-filtered groundwater samples and five on site filtrations with Sterivex™ cartridges. The results indicated that the *Dhc* enumeration results within labs were statistically similar between the methods within a factor of 2 at a 95% confidence level. Overall, the results suggested that the field filtration netted slightly higher *Dhc* numbers per liter. This result is consistent with a previous study (Ritalahti *et al.*, 2010) that compared the relative efficiency of field filtration and lab filtration. In the prior study, both filtration procedures were determined to be approximately equal in terms of total DNA recovery upon optimization of the biomass recovery from the Sterivex membranes. Furthermore, it was determined that on site filtration could increase the yield of *Dhc* by up to 5-fold versus laboratory vacuum filtration using membrane filters (MO BIO Laboratories Carlsbad, CA) (Ritalahti *et al.*, 2010). This finding suggests that on site filtration may be superior, in particular for efficiently harvesting relatively small *Dhc* cells, which may be lost in laboratory vacuum filtration protocols as was observed in the experiments reported in Section 7.1.2.

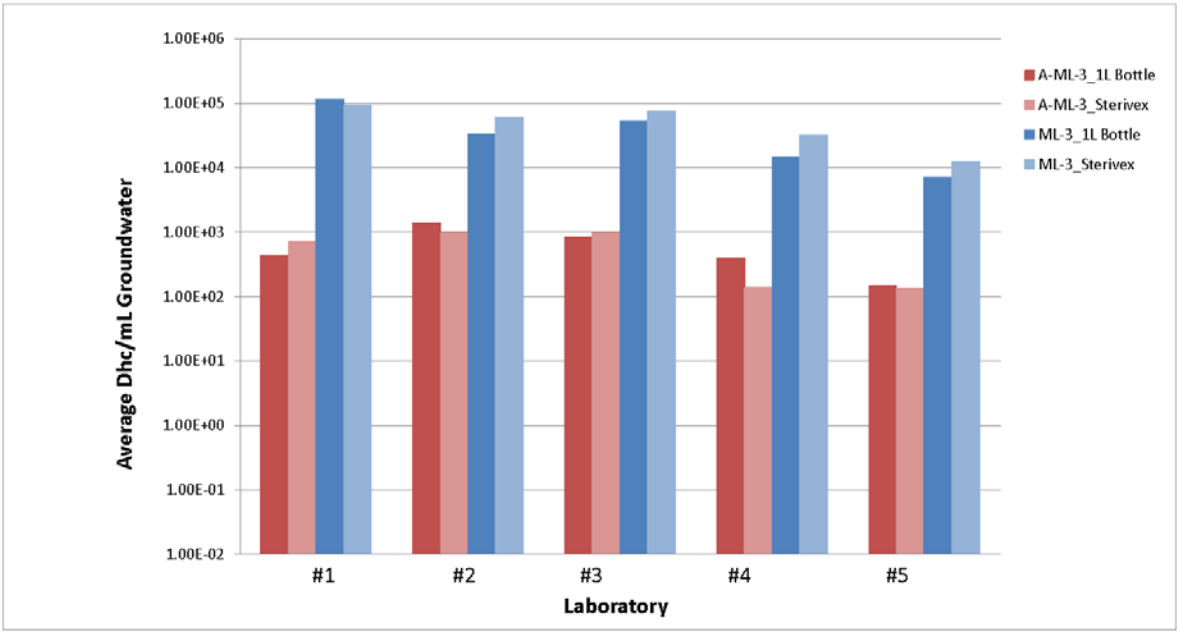

Figure 8-1: Comparison of lab filtration versus on site filtration with Sterivex cartridges. Groundwater was filtered in the lab or on site for wells A-ML-3 and ML-3 and *Dhc* 16S rRNA genes were quantified in five independent labs. All labs reported similar results (within 2-fold) between lab filtration and on site filtration methods for both wells, suggesting that the two approaches were similar terms of biomass recovery.

8.2.3 Conclusions and Implications for Future Research / Implementation

The results obtained in this study corroborated those described by Ritalahti et al. (2010) and indicated that on site filtration is a comparable, or possibly superior method, in terms of total biomass and *Dhc* cell recovery as compared to laboratory vacuum filtration procedures.

Given the performance advantages, shipping cost savings and the fact that contaminated groundwater need not be shipped and discarded, on site filtration is a practical and viable approach for collecting biomass for MBT analysis in groundwater.

8.3 Low Flow versus High Flow Sampling Methods

The goal of this task was to assess variability in *Dhc* quantification when using low flow and high flow well sampling methods. *Dhc* enumeration data collected using low and high flow purging methods were compared to evaluate the impact of the sampling procedure. These comparisons are needed to assess the effect of sampling-induced turbidity changes and re-suspension of particles residing in the well on *Dhc* quantification.

8.3.1 Approach

Groundwater sampling was carried out at the Bachman Road site (Oscoda, MI) at wells A-ML-25 and MW-2A in May, 2012. Further details regarding this test are provided in Attachment 5. On site filtered biomass samples were collected using Sterivex™ cartridges in 20-minute intervals, following purging and stabilization of geochemical parameters.

The development of field sampling standard operating procedures (SOPs) was conducted as part of ESTCP project ER-0518. SOPs for groundwater sampling from ER-0518 were followed and are included in Attachment 5. Negative controls (i.e., field blanks) were shipped to the field, returned to the lab and processed in the same manner as a cartridge used for groundwater sampling.

Low Flow Sample Collection

Groundwater was purged from the well using low-flow methods (i.e., 100 to 200 mL/min). A consistent flow rate was maintained throughout the purging and sampling process.

1) Geochemical conditions were recorded during purging using a hand-held water quality meter.

2) Purging continued until geochemical conditions stabilized and aquifer-representative groundwater was evident.

3) 1 L of groundwater was collected from each monitoring location and passed through a Sterivex™ cartridge. Both the inlet and outlet of each cartridge were capped.

4) Step 4 was repeated in 20-minute intervals between sampling (i.e., time = 0, 20, 40, and 60 minutes) for a total of four samples per monitoring location.

High Flow Sample Collection

Groundwater was purged from the monitoring locations identified in Section 7.1 using high flow methods (e.g., 1,000 to 2,000 mL/min). A consistent flow rate was maintained throughout the purging and sampling process.

1) Using a hand-held water quality meter, geochemical conditions were recorded during purging.

2) Purging continued until three well casing volumes were purged for MW-2A and five gallons were purged for ML-3 and A-ML-25.

3) Groundwater was collected from each monitoring location into a clean polyethylene carboy.

4) The polyethylene carboy was capped and the groundwater homogenized by turning the container end to end several times.

5) Five 1 L aliquoted samples were passed through Sterivex™ filters. Cartridges were capped.

6) Sterivex™ cartridge samples were shipped on ice packs by express courier to the testing laboratory.

In the analytical laboratory, DNA was extracted from Sterivex membranes using the PowerWater® Sterivex™ DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) and MIAC from previously frozen aliquots (Section 6.4.1) was added to the DNA extraction kit bead tube during extraction.

Statistical evaluations using a t-test were conducted to investigate whether there were significant differences in average *Dhc*/mL groundwater (unadjusted and MIAC adjusted average *Dhc*) and percent *luc* recovery between high- and low-flow methods for samples collected at wells A-ML-25 and MW-2A.

8.3.2 Results and Discussion

The recovery of the MIAC in these experiments was relatively low, ranging from 13-17% for samples from well MW-2A and 7% -15% for samples from well A-ML-25. This is somewhat lower than the previously observed recovery for the MIAC for lab-filtered samples from this site, which were typically closer to 30%. Nevertheless, these lower MIAC recoveries may have been due to real losses occurring during sample processing as the *Dhc* numbers were also lower than previously observed at these locations. Lower MIAC quantification may simply indicate the MIAC is functioning as intended.

Table 8-1 presents the results of the t-tests conducted for the comparisons between high-and low-purge methods for both *Dhc* and percent *luc* recovery. The results of the t-tests for samples collected at A-ML-25 suggest no statistical difference within a factor of 1 (i.e., the results are statistically identical) between high and low purge methods for *Dhc* (both unadjusted and adjusted). The results of t-tests for samples collected at MW-2A suggest no statistical difference within a factor of 2 between the means of the high and low purge methods for unadjusted *Dhc* and no statistical difference for high and low flow methods for the adjusted data.

Table 8-1: Summary of means and results of the t-tests conducted to compare means between low-flow and high-flow sampling methods.

Parameter	Well	Mean Low	Mean High	Statistical Difference?
Average *Dhc*/ mL groundwater	A-ML-25	2.67	2.58	No difference within a factor of 1
Average *Dhc*/ mL groundwater	MW-2A	958	1921	No difference within a factor of 2
Percent luc recovery	A-ML-25	0.17	0.13	No difference within a factor of 1
Percent luc recovery	MW-2A	0.07	0.15	No difference within a factor of 2
Adjusted Average *Dhc*/ mL groundwater	A-ML-25	15.73	16.10	No difference within a factor of 1
Adjusted Average *Dhc*/ mL groundwater	MW-2A	14912	12724	No difference within a factor of 1

Note: "no difference within a factor of 1" indicates the values are statistically the same.

Mean values obtained from averaging up to five replicate samples and test results from both high- and low-flow sampling were used in the data analysis to compare the two sampling approaches. Figures 8-2 and 8-3 display the plotted means of unadjusted *Dhc* enumeration and MIAC adjusted *Dhc* enumeration, respectively. Results obtained were very similar between the two sampling regimes.

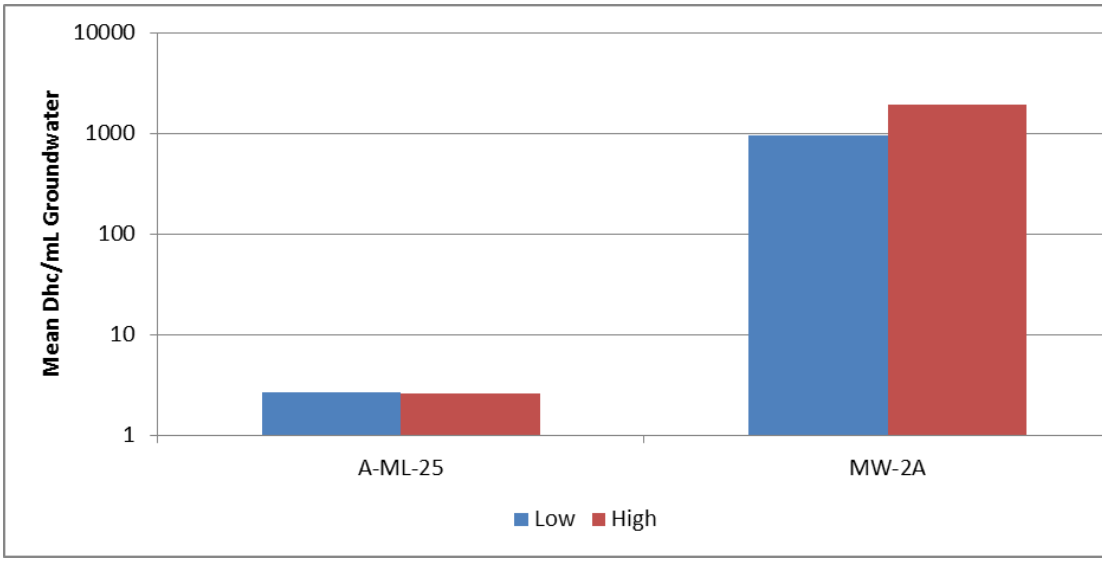

Figure 8-2: Comparison of high flow and low flow groundwater sampling methods (unadjusted for MIAC recovery). Mean *Dhc*/mL for four groundwater samples collected at A-ML-25 and

MW-2A using low flow and high-flow methods indicated no significant difference (within factor of 1 i.e., the same) between the sampling methods for well A-ML-25 and statistically within a factor of 2 for well MW-2A (See Table 8-1).

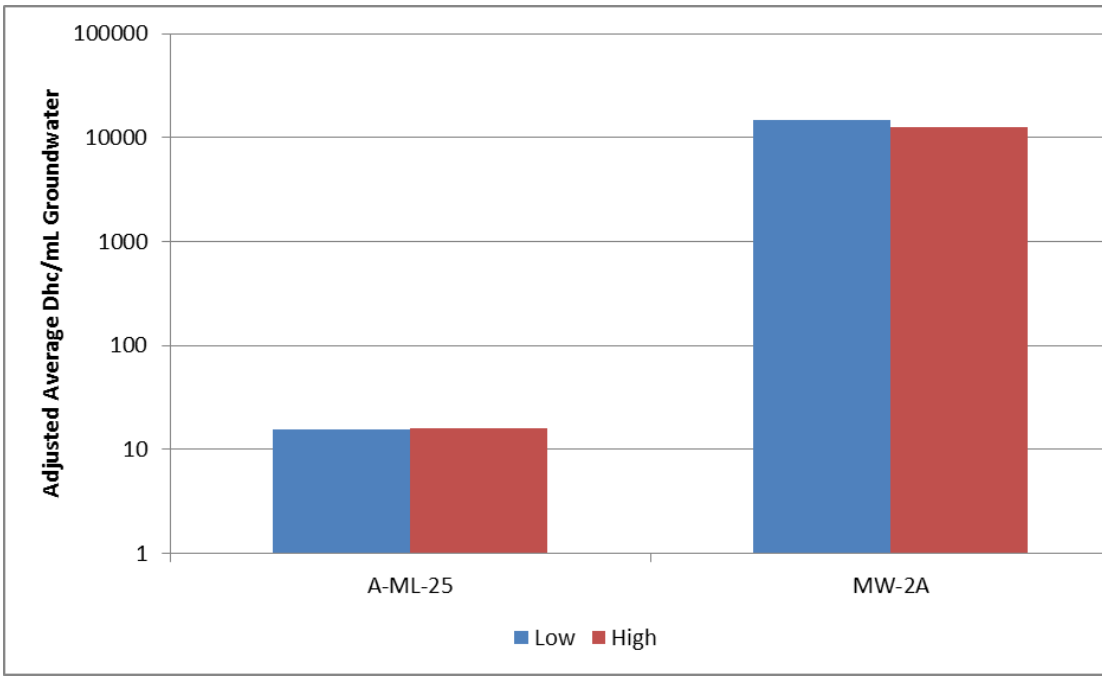

Figure 8-3: Comparison of high flow and low flow groundwater sampling methods (adjusted for MIAC recovery). Mean MIAC adjusted *Dhc*/mL groundwater for samples collected at A-ML-25 and MW-2A using low flow (blue bars) and high-flow methods (red bars). The adjusted results indicated no statistically significant difference between high and low flow sampling methods for both wells.

8.3.3 Conclusions and Implications for Future Research / Implementation

Based on the above data, there were only minor differences observed between the high and low flow sampling regimes at the two wells at the Bachman Road site. Adjustment with the MIAC reduced any observed differences to within a factor of 1, indicating that the high and low flow sampling methods were essentially identical. Further study is required to determine if similarities between high and low flow sampling methods are applicable to other sites, nevertheless, these data suggest sampling flow rate was not a major factor in *Dhc* testing variability. Further research is required to determine if high and low flow sampling methods would be similar in other wells and at other sites.

8.4 Minimizing Data Variability between Sampling Events

The ability to obtain consistent qPCR enumeration results over time in the same well where the *Dhc* population is stable would provide indications that *Dhc* quantification is not unduly impacted by sampling or analytical variability between sampling events. *Dhc* populations can vary widely at sites where geochemical and microbiological conditions are in flux. Nevertheless, at the Bachman Road site, where plume conditions are stable and the *Dhc* population is well established, abrupt changes in *Dhc* abundance would not be expected.

8.4.1 Approach

A preliminary examination of the ability to obtain consistent results from the same well is summarized below and is based on samples obtained in Round Robin 4 (Attachment 4). A follow-on analysis carried out in one participating lab, approximately 3 months later (Attachment 5), was used to determine if similar results could be obtained between sampling events.

The Bachman Road site was chosen as a test site in part because it likely has a "stable" plume. The site is well-instrumented and relatively "stable" reductive dechlorination conditions have been observed. Stable site conditions are important for assessing variation in quantification results associated with sampling and analysis effects, not due to active growth of *Dhc*.

8.4.2 Results

Figure 8-4 and 8-5 summarize unadjusted and adjusted data for well ML-3 in February and May 2012. Figure 8-4 indicates an approximate one order of magnitude difference between sampling events (10-fold) for unadjusted data, but MIAC (*luc*) recovery was correspondingly low for samples analyzed in May. Low MIAC recovery suggested that losses in the analytical process may have occurred.

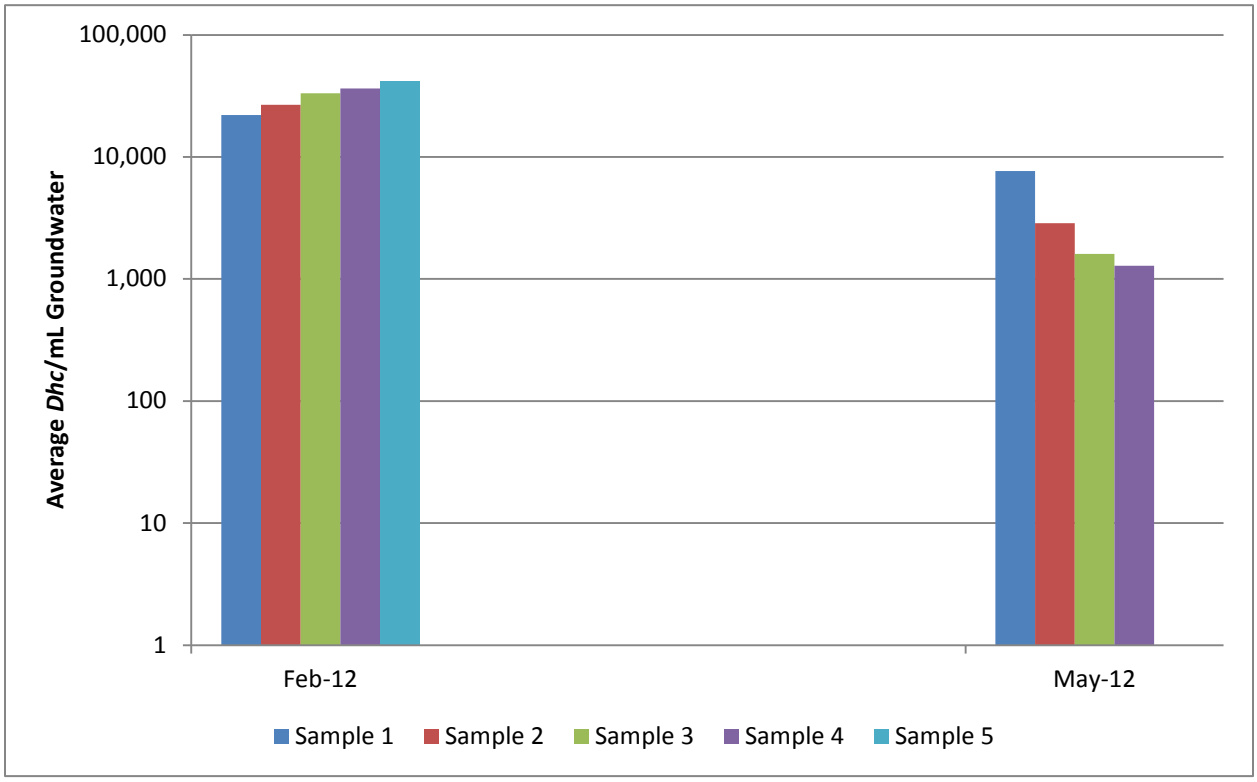

Figure 8-4: *Dhc* quantification for Bachman Road well ML-3 in February and March 2012 unadjusted for MIAC. The unadjusted May, 2012 data are approximately an order of magnitude lower than the results for February, 2012, however the recovery of the MIAC was correspondingly low, suggesting that losses of biomarkers may have occurred during extraction or analysis.

Despite the relatively large differences observed in unadjusted data between groundwater sampled and analyzed in the February and May sampling events, data adjusted for recovery of the MIAC indicates much more consistency. When a t-test was performed on these data, no statistically significant differences were observed between the means of adjusted *Dhc* counts from the February and May sampling events. This is compelling illustration of the ability for the MIAC to improve data interpretation.

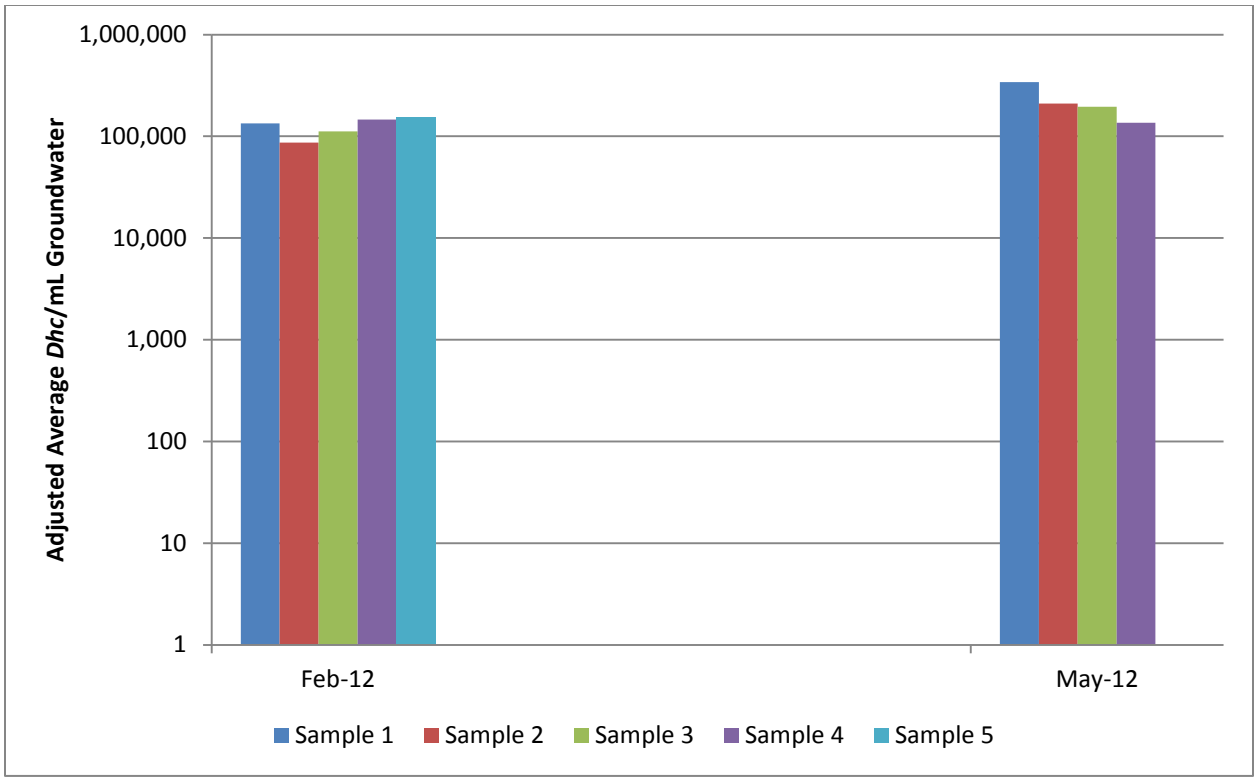

Figure 8-5: *Dhc* quantification for Bachman Road well ML-3 in February and March 2012 (adjusted using MIAC recovery). After adjustment for *luc* recovery the *Dhc* enumeration results for February and May were statistically identical indicating that with the use of MIAC reduced variability between sample events.

The ability to obtain consistent data between sampling events may be improved by the integration of an MIAC which can correct for changes in recovery between sampling and testing events. Variation in recovery over time could be due to a variety of factors including, changes in lab personnel, performance of DNA extraction kits, qPCR reagents, and possibly changes to groundwater composition. Further testing of the ability of the MIAC to improve consistency over time at different sites and time frames would be a worthwhile area for further research that would also improve our understanding of changes in *Dhc* populations in the longer term.

8.5 Preservation Methods for Groundwater, Biomass and Extracted DNA Samples

After groundwater sampling, samples are handled on site prior to shipping to the analytical laboratory where storage prior to processing may occur. After processing, the membrane filters with the biomass may be stored prior to DNA extraction. Furthermore, the extracted DNA may be stored prior to qPCR analysis and then archived for long periods for potential future testing. Therefore, understanding the conditions under which biomarkers are stable is important for establishing maximum hold times and determining acceptable storage conditions for various sample types.

The impacts of air exposure, temperature fluctuations, and other perturbations may vary depending on the type of microorganism being quantified and the biomarker target (e.g., RNA vs. DNA). While RNA is generally less stable than DNA, DNA can also undergo degradation. Nevertheless, it has been reported that DNA can be stored with minimal losses at 4°C for several weeks, at -20°C for up to a year and at -80°C for several years (De Paoli, 2005). For long-term storage, DNA should be dissolved in Tris-EDTA buffer to maintain a constant pH which decreases the tendency for DNA to degrade. The impact of sample type (Sterivex cartridge or groundwater), storage temperature, and storage time on quantification of *Dhc* biomarker genes was assessed in the experiments detailed in Appendix N and summarized in Table 8-2.

8.5.1 Approach

Biomass in unprocessed groundwater and biomass collected with Sterivex cartridges was exposed to varying storage temperatures and incubation times, after which DNA was extracted and *Dhc* 16S rRNA genes were enumerated using qPCR methods. Further details regarding the methods used in these experiments are provided in Appendix N.

8.5.2 Results

Table 8-2 summarizes *Dhc* recovery for a variety of conditions including storage of unprocessed groundwater and concentrated biomass on membrane filters. Recovery is presented as a percentage of initial *Dhc* enumeration prior to storage (100%).

Table 8-2: Summary of *Dhc* Recovery Percentage for Different Preservation Scenarios

Storage Condition	Storage Duration	Recovery After Incubation	
		Groundwater	Biomass On Filter
Immediate Extraction	0 days	100%	100%
4°C	1 day	100%	80-100%
22°C	5 days	0.1%	Not Tested
4°C	14 days	50-80%	0.7%-3.0%
-20 °C	14 days	0%	2-30%
-80 °C	14 days	0%	100%

The results of these experiments indicated that storage for 1 day at 4°C for biomass suspended in groundwater did not significantly impact recovery which was in the 80-100% range. Storage of groundwater samples at 4°C for 14 days indicated decreases to 50-80% of original *Dhc* cell abundance. In contrast, losses for biomass on filters stored under the same conditions were high with only 0.7-3% recovery. In fact, biomass on filters was not stable even at -20 °C and required storage at -80 °C for high recovery after 14 days. Groundwater was sensitive to storage at room temperature with up to a 1,000-fold decrease (0.1%) in *Dhc* enumeration after 5 days of incubation.

8.5.3 Conclusions

Preservation experiments suggest that storage conditions of groundwater samples impact biomarker quantification. The magnitude of storage effects could in some cases far exceed other variables that quantitatively affect the analysis. Temperature in particular appears to have a significant effect on biomarker stability with groundwater samples stored at room temperature exhibiting significant (i.e., up to 1,000-fold) loss of biomarkers compared with refrigerated samples.

After sampling, the groundwater and/or Sterivex cartridges are shipped to the analytical laboratory, which typically requires 24-48 hours, and logistics (e.g., sample queues) may require sample storage for a period of time prior to analysis. To the extent possible, storage prior to analysis should be minimized. The activities listed below are also recommended to maximize the stability of the target biomarkers.

- Groundwater samples stored at 4ºC were stable (i.e., no substantial biomarker losses) for at least 24 hours, 50%-80% biomarker losses were observed with 2 weeks of incubation. Current maximum hold times for qPCR analysis are as high as 10 days, shorter hold times could improve data quality. Furthermore samples that arrive in the laboratory above 4°C should be flagged to allow better assessment of possible biomarker losses.

- On site filtered samples may be less stable than groundwater samples with major losses observed when stored for 2 weeks at 4°C, therefore immediate sample processing or freezing at -80°C upon arrival in the lab is recommended. Shorter maximum hold times for on site filtration samples should be considered.

- Exposure of samples to temperatures above 4°C should be avoided by minimizing shipping times and use of sufficient ice/cold packs.

- Longer term storage of filtered biomass samples should occur at -80 ºC

In practice, it is not unusual for samples to arrive at commercial laboratories at temperatures above 4°C due to warming after shipping delays and melting of ice/icepacks, particularly in summer. Protocols that could improve stability of on site filtered sampels include use of preservatives such as Bacteria Protect™ or the use of dry ice, which is commonly used for shipping heat-labile reagents and other sensitive samples. Future research could focus on the efficacy and practicality of these preservation strategies for both on site filtered samples and groundwater samples.

8.6 Conclusions

The following key conclusions were made regarding sampling methods and preservation:

- On site filtration is a comparable or possibly superior method in terms of total biomass and *Dhc* cell recovery as compared to laboratory vacuum filtration procedures.

- High and low flow sampling approaches produced similar results.

- The developed MIAC was successfully used to reduce variability associated with repeated analysis of the same well in sampling events 3 months apart. The ability to normalize *Dhc* qPCR data to the recovery of the MIAC between sampling events improves data interpretability.

- Biomarker loss in groundwater and on site filtered samples can reduce the quantitative results of *Dhc* analysis and exposure to elevated temperatures should be avoided.

9 MULTI-LAB VARIABILITY ASSESSMENT AFTER METHOD OPTIMIZATION

After baseline variability assessment (Section 4) it was determined that variation between labs and sample to sample variability within some labs was significant (i.e., greater than 3-fold) as indicated in the multi-lab Round Robin 1 (DNA) and Round Robin 2 (whole *Dhc* cells in simulated groundwater [RR2]). Based on these findings, efforts were made to improve the consistency of the analysis both within and between labs. This optimization included the following modifications in the protocols used in various labs:

- Use of genomic DNA to calibrate qPCR instead of plasmid DNA;
- DNA quantification of calibration materials using fluorometry instead of spectrophotometry;
- Optimization of bead beating methods in the DNA extraction process;
- Freezing of biomass collected on filters at -80°C instead of -20°C;
- Using robot-controlled pipetting for qPCR reaction assembly in one participating lab previously employing manual pipetting;
- Use of MIAC recovery percentage to adjust *Dhc* data; and
- Use of Sterivex cartridges for onsite biomass collection protocols.

Two additional multi-lab round robins were performed after method optimization efforts. Round Robin 3 (RR3) that replicated RR2 and Round Robin 4 (RR4) in which site groundwater samples and on site filtration using Sterivex cartridges was compared. Both of these tests included the MIAC to assess recovery and to adjust data to determine if these protocols could reduce inter-lab variability.

9.1 Round Robin 3: *Dhc* qPCR Enumeration in Simulated Groundwater Integrating the MIAC

To assess baseline variability a multi-lab round robin, RR2 was carried out (Section 4.3) and indicated greater than 3-fold difference in *Dhc* enumeration between some participating labs. Method optimization in specific labs and use of the MIAC was performed with the goal of decreasing inter-lab variability. RR3was in many respects a repeat of RR2 to determine if the consistency of data improved due to optimization efforts.

9.1.1 Approach

RR3 was essentially identical to RR2 with the exception that the MIAC was added to the simulated *Dhc* spiked groundwater samples and the *Dhc* spikes used were approximately 10-times more concentrated in RR3. A higher concentration of *Dhc* was used in RR3 so that the low concentration samples would not be close to the method detection limit, which might have increased variability in RR2. Production and distribution of spiked *Dhc* aliquots was identical to RR2 (Section 4.3.1.) detailed methods for RR3 are outlined in Attachment 3. Briefly, *Dhc* spiked simulated groundwater samples were prepared and the five participating labs were provided with 12 bottles, each containing 500 mL. These 12 samples included 5 replicate samples at high *Dhc* abundance, 5 replicate samples at low *Dhc* abundance, and 2 samples with no *Dhc* (blanks). The five receiving laboratories were not informed of the expected *Dhc* titer i.e., it was a blind study). Upon sample receipt, each laboratory added a predetermined spike of the MIAC to the simulated groundwater samples and processed the samples according to the lab's standard or optimized protocols. After DNA extraction, qPCR was performed for the *Dhc* 16S rRNA gene and the *luc* gene to determine recovery efficiency of the MIAC.

Statistical analysis using t-tests, conducted at a significance level of 5% (i.e., $\alpha = 0.05$), were used to investigate whether there was a significant difference in average *Dhc*/mL groundwater (unadjusted and adjusted average *Dhc* [adjusted for MIAC recovery]) for samples collected at wells A-ML-25 and MW-2A within a factor of 3. The project team decided that method variability of up to 3-fold (i.e., a factor of three) was reasonable and would not unduly impact the interpretation of *Dhc* data. In part, 3-fold variability was considered reasonable, given the large data range of *Dhc* encountered in site samples, of 6-7 orders of magnitude or 1-10 million-fold. A factor of three was chosen to represent a guideline of "the same for practical purposes" for *Dhc* enumeration results.

9.1.2 Results

No false positives or false negatives were reported by the participating laboratories in this experiment. Overall the variability was lower than that observed in RR2. This may be due to the method optimization efforts and possibly due to the higher *Dhc* titers used, which decreases the likelihood of false negatives and overall data variability. Figure 9-1 summarizes the results obtained for RR3.

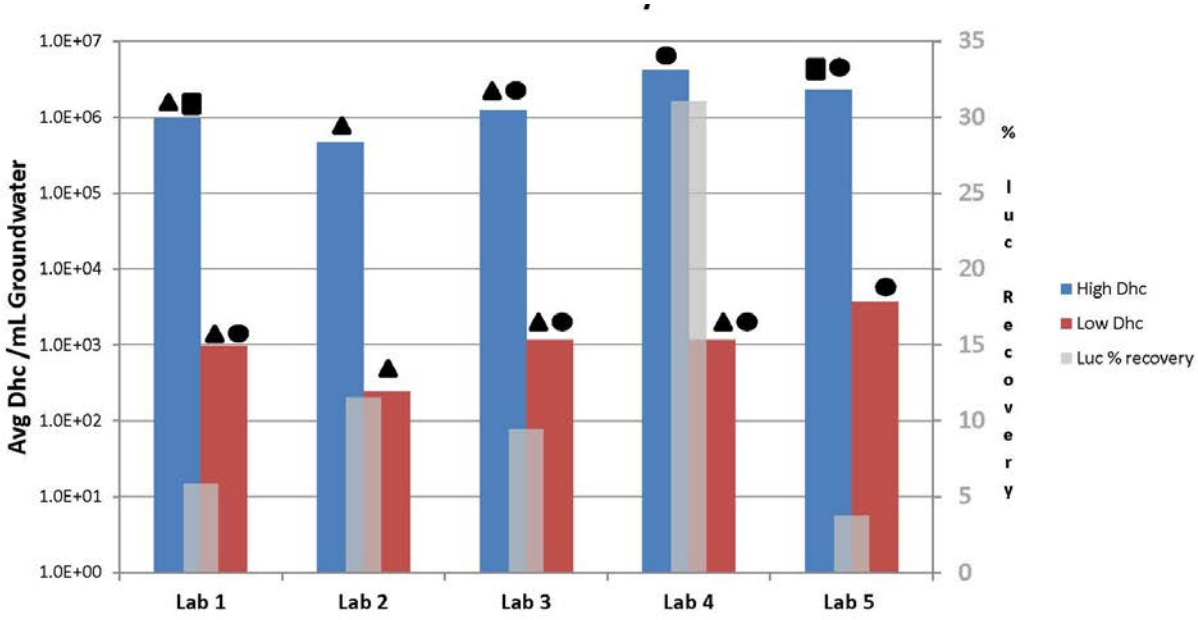

Figure 9-1: Summary of *Dhc* quantification results from five labs in RR3 with simulated groundwater. RR3 was essentially identical to RR2 with the exception the MIAC was added to the simulated groundwater samples. The blue and red bars represent the mean of 5 replicates for high and low *Dhc* spikes quantified by 5 independent laboratories. The grey bars represent the mean % *luc* recovery for the MIAC (in the combined 10 samples), which ranged from ~4% to 30%. Labs that returned results for *Dhc* within 3-fold of each other are indicated with identical geometric symbols. Three out of five labs returned results within 3-fold for the high *Dhc* spike and four out of five labs for low *Dhc* spike. Maximum differences were 9-fold for the high *Dhc* spike (Lab 4 versus Lab 2) and 15-fold for the low *Dhc* spike (Lab 2 versus Lab 5). In RR2 (Figure 4-4) more substantial differences of 12-fold (high *Dhc*) and 40-fold (low *Dhc*) were observed.

In RR3, for high *Dhc* abundances, three out of five labs returned results statistically within 3-fold of each other. For the low *Dhc* abundance samples, four out of five labs were within 3-fold of each other. In RR3 15-fold was the largest difference between means for *Dhc* quantification (Lab 2 versus Lab 5). In RR2 (Figure 4-4) more substantial (up to 40-fold) differences between *Dhc* mean quantification were observed. Therefore, the results of RR3 represent a quantitative improvement in between laboratory variability over RR2.

In RR3, the ability of multiple labs to use the MIAC was also demonstrated. Recovery of the MIAC (% *luc* recovery) ranged from 4% to just over 30 % (grey bars Figure 9-1). The MIAC was included in this round robin with the singular goal of testing its use and recovery percentage in multiple labs. The MIAC (*luc*) recovery was not used to adjust *Dhc* data. MIAC recovery could be used to improve data consistency between labs by adjusting for target gene losses.

This approach was used in RR4 which was carried out with groundwater obtained from a contaminated site.

9.2 Round Robin 4: *Dhc* Enumeration in Site Groundwater Integrating the MIAC

To demonstrate the value of the MIAC approach with site groundwater, Round Robin 4(RR4) was performed. RR4 assessed the ability of the five participating labs to quantify *Dhc* in site groundwater using the optimized analytical procedures and integrated the MIAC for data adjustment to account for biomarker gene loss.

9.2.1 Approach

RR4 was performed using groundwater from the Bachman Road site located in Oscoda, Michigan. Groundwater was obtained from wells with "low' *Dhc* abundance of 10^4/L (A-ML-3) and another well with "high" *Dhc* abundance of 10^7/L (ML-3). The wells were sampled according to the detailed protocols outlined in the work plan (Attachment 5). Groundwater samples were shipped to the lab for biomass collection and Sterivex cartridges were used for on-site biomass collection.

9.2.2 Results

Lab Filtration Methods

The results of bulk groundwater shipped to five independent labs and using lab filtration using optimized methods and integrating the MIAC are presented in Figure 9-2. Figure 9-2 presents RR4 data with *Dhc* enumeration unadjusted for MIAC recovery. MIAC recovery could be used to adjust for differences in extraction efficiency that may be responsible for some of the observed between lab variability. Figure 9-3 presents the *Dhc* enumeration data for RR4 adjusted for the MIAC (*luc*) recovery.

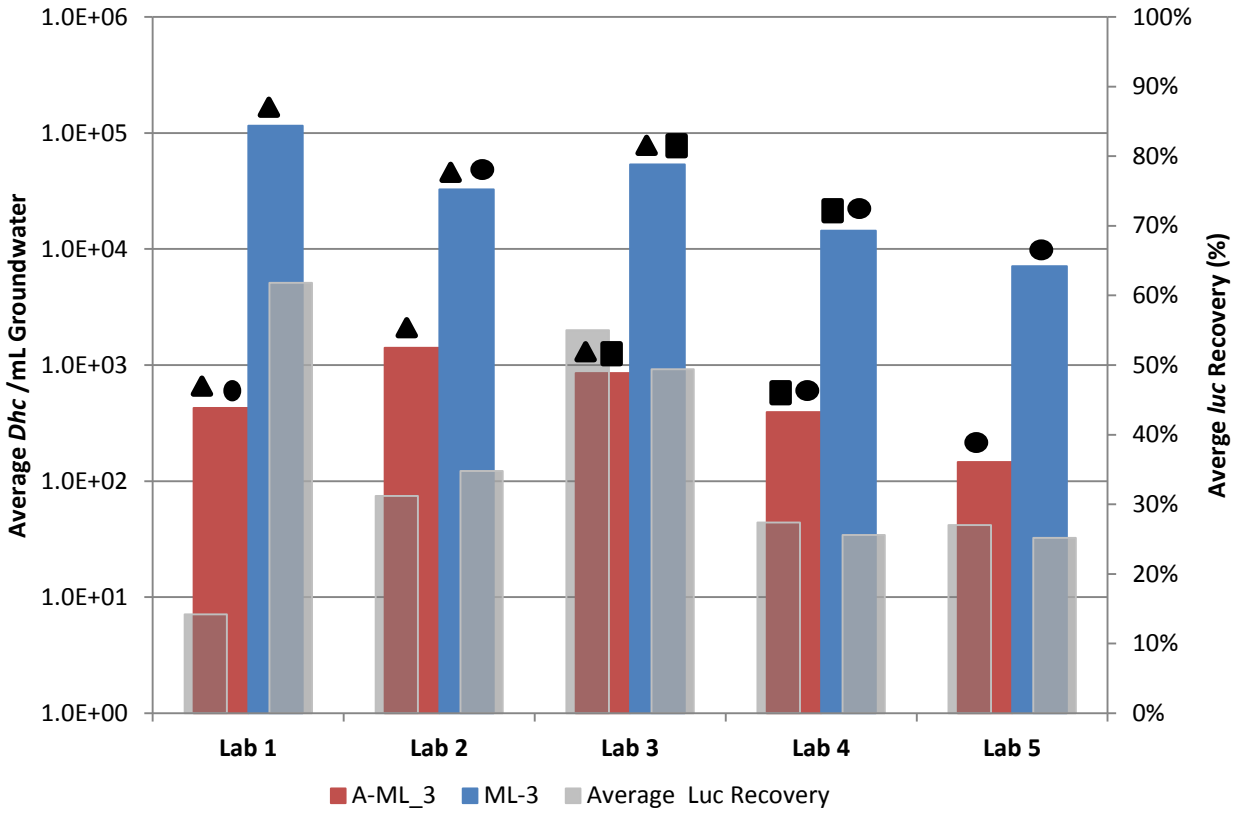

Figure 9-2: Lab filtered Bachman Road site groundwater samples. Bars are average *Dhc* enumeration of 5 replicates, well A-ML-3 (red bars) and ML-3 (blue bars) % *luc* recovery (grey bars). The mean % *luc* recovery for the MIAC ranged from 14% (Lab 1: well A-ML-3) to 62% (Lab 1: well ML-3). With the exception of Lab 1, most labs obtained relatively consistent MIAC recovery between the two wells. Average MIAC recovery was substantially higher compared with RR3. Mean *Dhc* quantification varied by up to 16 -fold for well ML-3 (Lab 1 vs. Lab 5) and 10-fold for well A-ML-3 (Labs 2 vs. Lab5). This level of variability is consistent with observations from RR3 (simulated groundwater). Identical symbols indicate labs with no statistically significant differences (exceeding a maximum 3-fold difference) in mean *Dhc* quantification at a 95% level of confidence in t-tests. Using this criterion, three of five labs were statistically within 3-fold for both well ML-3 and well A-ML-3. Note *Dhc* data are not adjusted for *luc* recovery.

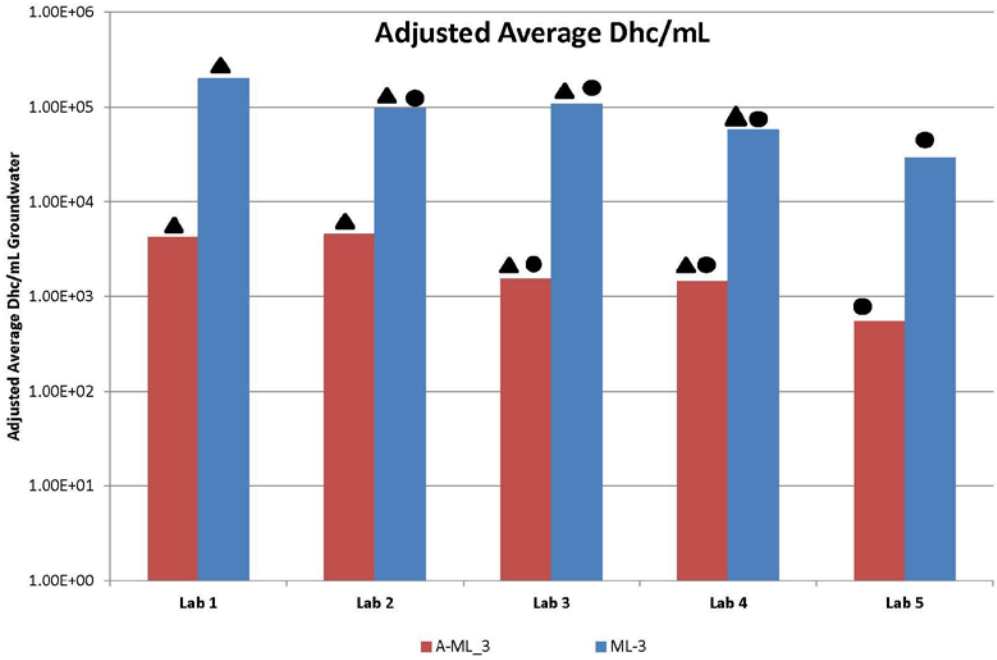

Figure 9-3: *Dhc* enumeration of lab filtered groundwater samples adjusted for MIAC (*luc*) recovery. After data adjustment between lab data variability was decreased to maximum 7-fold for well ML-3 (Labs 1 vs. Lab 5) and maximum 8- fold for well A-ML-3 (Labs 2 vs. Lab 5). Data variability was decreased compared to unadjusted data (Figure 9-2 [16-fold and 10-fold]) demonstrating the utility of the MIAC to reduce between-lab data variability. Identical symbols indicate labs with no statistically significant differences (exceeding a maximum 3-fold difference) in mean *Dhc* quantification at a 95% level of confidence in t-tests. Using this criterion, four out of five labs were within 3-fold of each other for both wells.

On-site Filtration of Groundwater

On-site filtration has been observed to be as good, or better, than lab filtration in terms of biomass recovery as detailed in Section 8.1) in this study, on-site filtration was able to reduce between laboratory variability. Figure 9-4 presents unadjusted data for the on-site filtration and indicates lower variability compared with laboratory filtration.

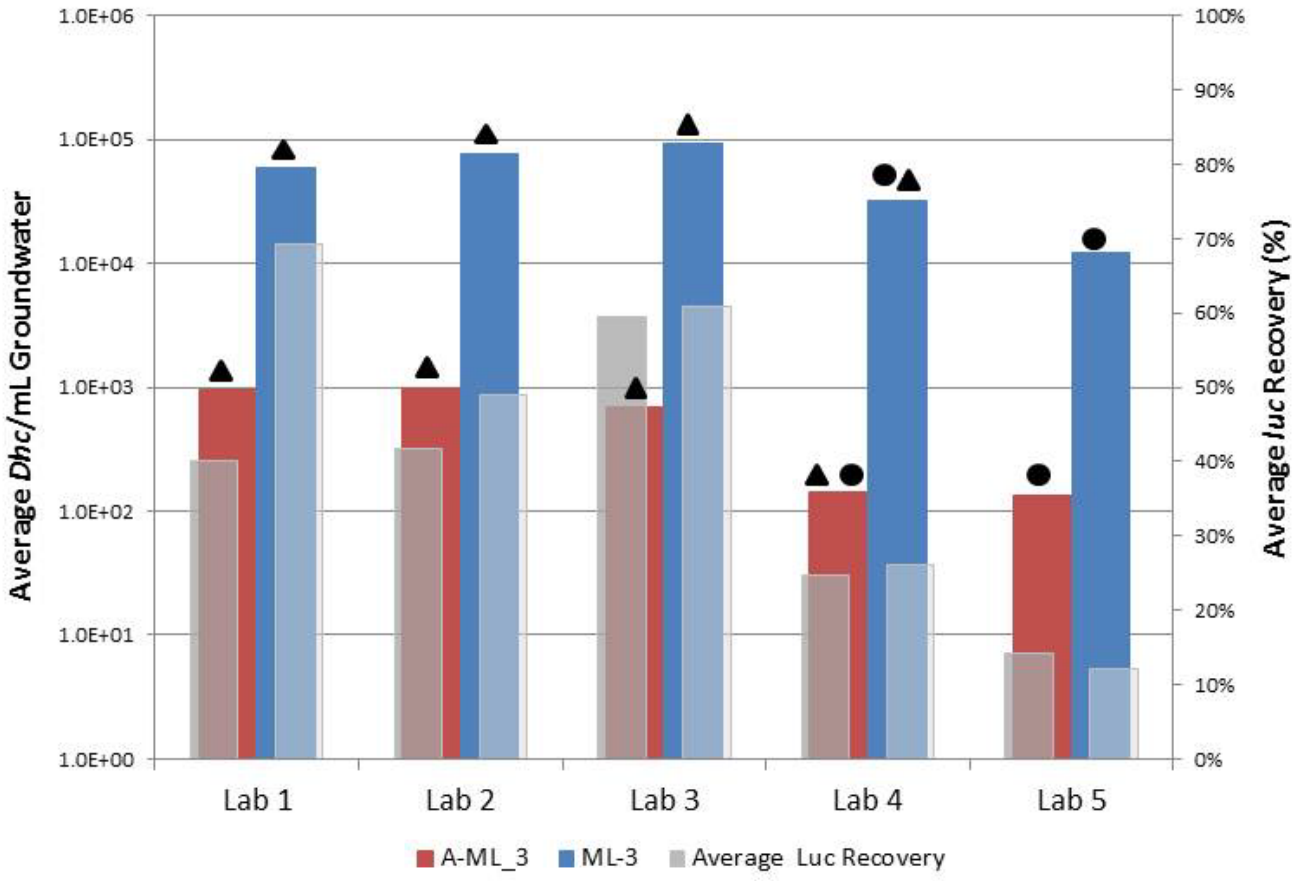

Figure 9-4: On-site filtration of Bachman Road groundwater samples. Mean results (5 replicates) are unadjusted *Dhc* enumeration results for 5 independent labs, *luc* recovery % (grey bars) ranged from approximately 25-70%. Maximum inter-lab variability was approximately 8-fold for ML-3 (Lab 1 vs. Lab 5) and approximately 7-fold for well A-ML-3 (Lab 2 vs. Lab 4). Compared to higher (16-fold) variability observed for lab filtration of groundwater (Figure 9-2). Identical geometric symbols indicate results between labs that were statistically similar within 3 fold at a 95% level of confidence in t-tests; four out of five labs were statistically similar for both wells using this criterion.

Further reductions in between lab data variability were obtained by using data adjustment with the MIAC percent recovery, combined with the on-site filtration. The results presented in Figure 9-5 represent the lowest inter-lab variability achieved in the project. Adjusted data for well ML-3 ("high *Dhc*") are virtually identical between the five labs with a 1.1 fold (i.e., only 10% variability) between labs. Adjusted data for well A-ML-3 with "low *Dhc*" abundance variability was somewhat higher but still fell statistically within the 3-fold "the same for practical purposes" guideline discussed at the beginning of Section 9. These results suggest that the MIAC is a useful tool to adjust data for biomarker gene losses and combined with on-site filtration and can markedly reduce data variability between labs.

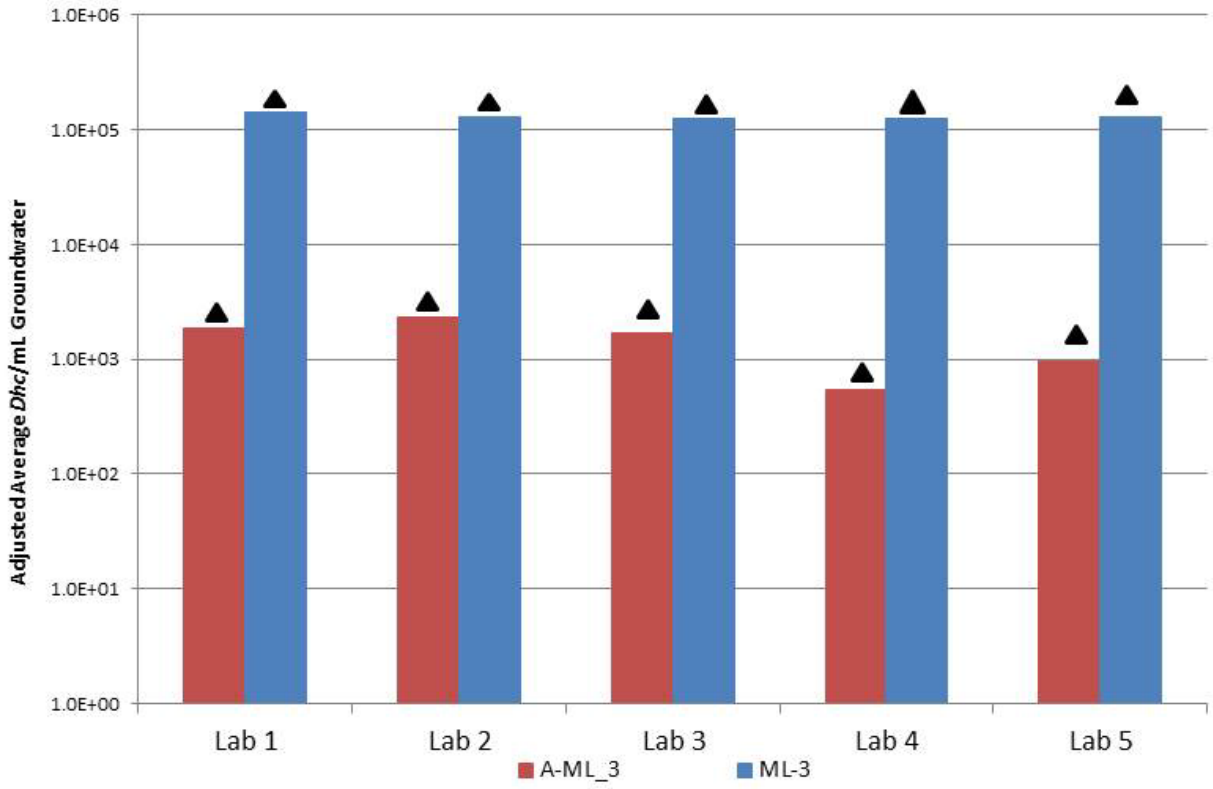

Figure 9-5: Mean *Dhc* enumeration results for 5 labs using onsite filtration for sample collection and adjusted for MIAC recovery. Maximum between lab variability was 1.1-fold for ML-3 (Lab 1 vs. Lab 3) and 4.4-fold for well A-ML-3 (Lab 2 vs. Lab 4). Identical symbols indicate labs with no statistically significant differences (exceeding a factor of 3 [i.e., a maximum 3-fold difference]) in mean *Dhc* quantification at a 95% level of confidence in t-tests. All labs (five out of five) labs were within 3- fold of each other for both wells using these criteria. These results are an improvement on the unadjusted *Dhc* enumeration for lab filtered groundwater (Figure 9-4) and are the lowest inter-lab variability observed in the project.

9.3 Conclusions

The inter-lab data progressing from Round Robin 2 to Round Robin 4 chronicles a trend of decreasing inter-lab variability achieved through method optimization and use of the MIAC. Table 9-1 provides a summary of the improvements in the spread of the mean data and the number of labs reporting results statistically within 3-fold. In the initial whole cell round robin (Round Robin 2), up to 40-fold variability between the means of highest and lowest reporting labs was observed. In RR3 without MIAC data adjustment, this had decreased to between 9-fold and 15-fold. This level of variability was essentially replicated with site groundwater in RR4 (10-16 fold variability) without MIAC data adjustment. Further improvements in data consistency were obtained by adjusting the *Dhc* qPCR data using the percent recovery of the

MIAC. In RR4, following the adjustment of *Dhc* cell numbers for *luc* % recovery, the variability was reduced by up to half to 7-8.4 fold for the high and low *Dhc* cell titers, respectively. Further increases in data consistency were obtained by use of on-site filtration protocols. In this case the unadjusted data had a maximum variability of 7.2-7.6 fold versus 10-16-fold for lab filtered samples. This suggests that a substantial portion of the inter-lab variability may have been due to lab specific filtration effects and removing this variable made the results more consistent. The adjustment of the data obtained on-site filtration with the Sterivex cartridges for MIAC percent recovery lead to the most consistent data with maximum variability of 4.3- fold (low *Dhc* titer) and only 1.1- fold for the samples with high *Dhc* titer. This indicates an substantial 10 times improvement in fold variability over the course of the project between highest and lowest reporting labs and indicates that with optimized methods integrating a MIAC to adjust for biomarker loss consistent results were obtained in multiple labs.

Table 9-1: Summary of Between Lab Variability with Progressing Method Optimization

Round Robin	Description of Round Robin	Maximum between Lab Variability (High *Dhc*)	Maximum between Lab Variability (Low *Dhc*)	Number of Labs Reporting Results within 3-fold (High *Dhc*)	Number of Labs Reporting Results within 3-fold (Low *Dhc*)	Comment	Figure
#2	*Dhc* spike simulated groundwater	12-fold	40-fold	4/5	3/5	High inter-lab variability in first whole cell round robin	4-4
#3	*Dhc* spike simulated groundwater after method optimization efforts	9-fold	15-fold	3/5	3/5	Reduction in maximum variability Compared to round robin 2	9-1
#4	Site groundwater (lab filtration)	16-fold	10-fold	4/5	4/5	Maximum variability similar with real versus simulated groundwater	9-2
#4	Site groundwater (lab filtration) - adjusted for MIAC recovery	7-fold	8.4-fold	4/5	4/5	Data adjustment with MIAC decreased maximum variability by ~ ½	9-3
#4	Site groundwater (on site filtration)	7.2 fold	7.6 fold	4/5	4/5	Onsite filtration (unadjusted) had lower variability compared to lab filtration	9-4
#4	Site groundwater (on site filtration) adjusted for MIAC rec*overy*	1.1 fold	4.3 fold	5/5	5/5	All labs reporting results defined as “the same for practical purposes”	9-5

10 SOLIDS GROUNDWATER PARTITIONING OF DECHLORINATORS

Microbial monitoring of aquifers relies on groundwater samples, and it is assumed that the non-attached ("planktonic") cells represent, or at least correlate, with the true cell abundance in the aquifer formation regardless of the organism(s) of interest, the specific environmental conditions, and the type of porous medium (e.g., low versus high organic matter content). Obviously, partitioning of cells of interest (e.g., *Dhc*) between solid and aqueous phases has implications for the interpretation of qPCR results generated with groundwater samples. To address this question, a series of one-dimensional columns inoculated with Bio-Dechlor INOCULUM (BDI), a PCE-to-ethene-dechlorinating bacterial consortium containing *Dhc* strains and *Geobacter lovleyi* strain SZ (strain SZ) (Amos et al., 2009), were conducted to quantify the distribution of bacterial cells between the resident solid and the aqueous phases. Also included in these experiments was *Anaeromyxobacter dehalogenans* strain W, a versatile Deltaproteobacterium that uses a variety of electron acceptors, including chlorinated phenols (Thomas et al., 2008).

10.1 Background

Phase distribution studies demonstrated that bacterial cells exhibit both preferential attachment to aquifer solids, with only a small fraction existing in a non-attached state (Balkwill and Ghiorse, 1985; Hazen et al., 1991), and preferential distribution in the aqueous phase, with negligible cell numbers associated with the solids (Godsy et al., 1992; Bekins et al., 1999; Fennell et al., 2001; Schaefer et al., 2009). Studies at sites undergoing reductive dechlorination of PCE to ethene have demonstrated that *Dhc* cells are present in groundwater, but are also attached to the surfaces of porous aquifer media, and possibly exist in biofilms (Lendvay et al., 2003; Schaefer et al., 2009). Little is known about the distribution of *Dhc* cells between the aqueous phase (i.e., non-attached cells) and the aquifer solids (i.e., attached cells). Hence, it is unclear how *Dhc* cell titers measured in groundwater (i.e., non-attached cells) correlate with the total abundance of *Dhc* cells in the aquifer (i.e., non-attached plus attached cells). The assessment of the fraction of *Dhc* attached to aquifer solids is challenging because collecting aquifer solids is costly, not always feasible, and the attached *Dhc* cells are heterogeneously distributed within the aquifer formation requiring multiple sampling events (Lendvay et al., 2003). Therefore, the analysis of aquifer solids is not practical, and *Dhc* monitoring regimes rely on groundwater samples collected from wells reaching the contaminant plume. Presumably, groundwater analysis avoids biases due to the heterogeneous distribution of *Dhc* cells within the formation because the collection of groundwater integrates target cell abundance over a larger aquifer pore volume (PV). Contemporary approaches correlate increases of *Dhc* cell titers in groundwater as a line of evidence that a microbial remedy is working and that dechlorination to ethene is feasible. However, the limited knowledge of how *Dhc* cell titers in groundwater correlate with the attached (or total) *Dhc* abundance, and whether the *Dhc* phase distribution differs by soil/sediment type, renders data interpretation ambiguous, and thus limits the value of the information generated by groundwater analysis.

10.2 Research Objectives

The major aim was to develop a basic understanding of the distribution of *Dhc* cells between aqueous and solid phases within an aquifer. The transition from attached to non-attached state is controlled by numerous parameters and is a complex, dynamic process. Hence, the distribution of cells between solid and aqueous phases will likely vary over spatial and temporal scales. The goal of this effort was not to elucidate the specific environmental controls that govern this dynamic process, but rather to provide a basic understanding of the distribution of key dechlorinating bacteria (i.e., *Dhc*) between groundwater (i.e., non-attached cells) and the aquifer matrix (attached cells). Contemporary groundwater monitoring programs rely on groundwater sampling to estimate cell abundances of relevant microbes such as *Dhc*; however, it is unclear how cell abundances determined in groundwater correlate with the true abundance in the aquifer formation. The hypothesis tested is that non-attached *Dhc* cells quantified in groundwater serve as a reliable measure of the total *Dhc* abundance (i.e., non-attached plus surface-attached cells). This work measured the distribution of *Dhc* cells between aqueous and solid phases in two distinct porous media (i.e., Federal Fine Ottawa sand and Appling soil) in the presence/absence of substrates to determine how soil properties (e.g., organic matter content) and growth affect the distribution of *Dhc* cells between the aqueous and solid phases. To explore the effects of the microbe, two other microbial species relevant for bioremediation, *Geobacter lovleyi* strain SZ and *Anaeromyxobacter dehalogenans* strain W, were included in this analysis. This evaluation has been accomplished through three major efforts: (i) measurement of cell retention and distribution in continuous flow column experiments under non-growth conditions; (ii) measurement of cell retention and distribution in continuous flow column experiments in the presence of growth substrates; and (iv) assessment cell-characteristic physical properties that correlate with surface attachment behavior.

10.3 Approach

10.3.1 Porous Media

Porous media column studies

Federal Fine Ottawa sand (30-140 mesh) with low organic carbon content (< 0.01 mg/g) and translucent color was used in the column studies (U.S. Silica Company, Berkeley Spring, WV). The Federal Fine Ottawa sand had a mean grain size (d50) of 0.32 mm and an intrinsic permeability of 4.2×10^{-11} square meters (m^2) (Suchomel et al., 2007). As a contrasting solid phase, Appling soil with 0.75% organic carbon by weight was used to represent a well-characterized field soil with a d50 of 0.22 mm (Wang et al., 2010).

10.3.2 Medium Preparation and Bacterial Cultures

Reduced mineral salts medium was prepared as described (Sung et al., 2003; Amos et al., 2008; Amos et al., 2009). Bio-Dechlor INOCULUM (BDI), a non-methanogenic, PCE-to-ethene dechlorinating consortium that contains multiple dechlorinators, including three *Dhc* strains (FL2, GT and BAV1), and *Geobacter lovleyi* strain SZ (Ritalahti et al., 2005; Ritalahti et al., 2006; Sung et al., 2006b; Sung et al., 2006a; Amos et al., 2007b; Amos et al., 2008; Amos et al., 2009) was used as the inoculum. The well-characterized consortium was maintained by periodic additions of 0.33 mM PCE as electron acceptor and 20 mM lactate as electron donor. Triplicate samples were collected from the seed consortium for DNA extraction and qPCR enumeration of dechlorinator 16S rRNA genes (Ritalahti et al., 2006). The BDI consortium used for inoculation contained $9.8 \pm 0.2 \times 10^7$ *Dhc* and $3.7 \pm 1.2 \times 10^7$ strain SZ cells per mL.

Strain W was grown in the same mineral salts medium at 30°C without shaking in 60 mL (nominal capacity) glass serum bottles (Wheaton, Millville, NJ). Each bottle contained 40 mL of reduced, bicarbonate-buffered (30 mM) medium with a nitrogen gas (N_2) and carbon dioxide (CO_2) headspace (80:20, vol/vol) (Löffler et al., 1996; Sanford et al., 2002; Thomas et al., 2010). Acetate (5 mM) (Sigma-Aldrich, St. Louis, MO) was provided as an electron donor, and 10 mM fumarate (Sigma-Aldrich) served as an electron acceptor. The strain W culture used for inoculation contained $1.42 \pm 0.01 \times 10^8$ cells per mL.

10.3.3 Cell Abundances and Distribution Following Inoculum Delivery

Modified glass columns (2.5 cm diameter, 10 cm length) were used to evaluate aquifer matrix (i.e., Federal Fine Ottawa sand versus Appling soil) filtration effects on the distribution of *Dhc*, strain SZ and strain W cells (Figure 10-1). Column packing was accomplished in 2 cm lifts with sterilized, dry Federal Fine sand and autoclaved, moist (deionized water) Appling soil. Columns were then purged with sterile CO_2 gas to remove other gases. Up-flow injection of autoclaved degassed water was then used to saturate the column material for a minimum of 10 PVs. A bromide tracer (1,000 mg/L) dissolved in reduced mineral salts medium was introduced (1 mL/min) prior to inoculation to determine the hydraulic flow field conditions. Samples were continuously collected using a fraction collector (Figure 10-1) in approximately 4 mL increments (five samples per PV) and analyzed for bromide using an ion selective probe. The PVs were calculated to be approximately 20 to 21.6 mL for the Appling soil and Federal Fine sand columns, respectively, based on the bromide tracer effluent measurement. The column was then flushed with reduced mineral salts medium to remove the bromide tracer and prepare the columns for inoculation. These columns were operated at a flow rate of 0.06 mL/min (50 cm/day) resulting in a residence time of 9.5 hours.

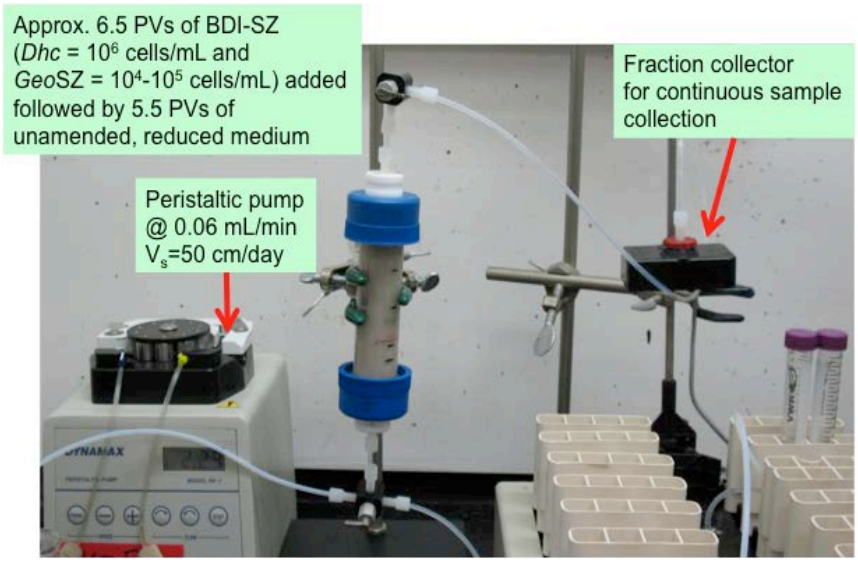

Figure 100-1: Experimental setup of 10 cm glass columns evaluating culture delivery and retention.

In the first set of column experiments, the BDI inoculum was diluted with reduced mineral salts medium to yield $7.4 \pm 1.2 \times 10^5$ *Dhc* and $1.9 \pm 2.1 \times 10^5$ strain SZ cells mL^{-1}. After flushing the column with one PV of reduced medium, approximately 7 PVs (150 mL) of the inoculum were introduced. A separate set of column experiments were conducted with the strain W culture. In these experiments, 3 PVs of reduced medium and then approximately 8 PVs (170 mL) of the inoculum containing $7.8 \pm 1.6 \times 10^5$ strain W cells mL^{-1} were introduced into the column. All column experiments concluded with the introduction of 5 PVs of reduced medium. Effluent samples were collected at a frequency of four 5-mL samples per PV in 15 mL sterile plastic tubes using a CF-2 fraction collector (Spectrum Labs; Rancho Dominguez, CA). Analysis of effluent samples with a Carl Zeiss Primo Star microscope (Göttingen, Germany) verified cell breakthrough. At the termination of each column experiment, the column end plates were removed and soil was excavated in increments of 1.2 cm near the inlet (first quarter of the column) and 2.5 cm for the remainder of the column, to give a total of five segments, which were transferred into sterile 50-mL plastic tubes.

10.3.4 Aqueous and Solid Phase Cell Distribution under Growth Conditions

To examine the distribution of *Dhc* and strain SZ cells between the resident solid phase and the aqueous phase in the presence of growth substrates, two distinct solid media were used: a low organic content sandy soil (Federal Fine Ottawa sand) and a soil with high organic matter content (Appling soil). Commercially available glass columns (2.5 cm diameter, 15 cm length) were retrofitted with three sampling ports (Figure 10-2). Prior to initiating the experiments, column pieces were either autoclaved or washed in a 70% ethanol solution, and the solid media were autoclaved. These columns were packed under saturated conditions (2 cm lifts) with the different porous media using a suspension of the BDI consortium as the resident aqueous phase to ensure a uniform initial distribution of microorganisms. In order to maintain anoxic

conditions, packing was performed inside an anoxic glove box. Following packing, the columns were kept in the glove box for approximately 24 hours in an anoxic environment.

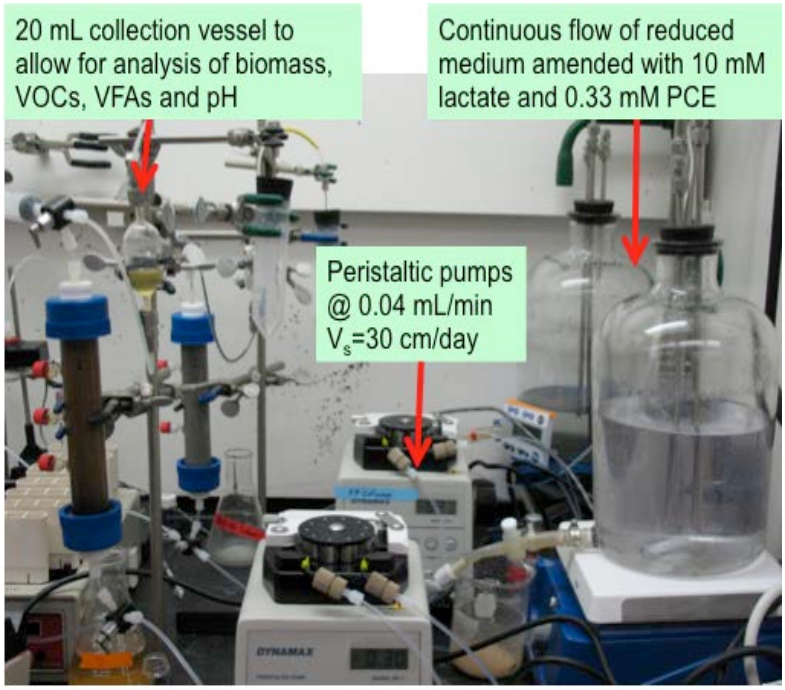

Figure 100-2: Experimental setup of 15 cm columns evaluating the distribution of cells between the aqueous and solid phases.

The columns were operated at a nominal flow rate of approximately 0.04 mL/min (equivalent to a pore-water seepage velocity of 30 cm/day with a 1 day residence time) with a continuous influent feed of reduced mineral salts medium amended with 0.33 mM PCE and 10 mM lactate. The influent system to maintain anoxic conditions for medium delivery included a glass column filled with powdered ferrous iron and an oxygen trap (Figure 10-2).

Aqueous samples from the side ports and effluent were collected daily and analyzed for chlorinated ethenes, pH, and organic acids. Biomass was collected from each sample for subsequent molecular analysis to quantify *Dhc* and strain SZ 16S rRNA genes (Amos et al., 2009). Once steady-state effluent chlorinated ethene concentrations were measured, columns were destructively sampled to measure the attached and non-attached *Dhc* and strain SZ cells. Columns were divided into five sections including the inlet, the outlet and ± 0.5 cm around each sampling port.

10.3.5 Assessment of Physical Surface Deposition Characteristics

Effect of surface charge on attachment behavior

Physical surface deposition characteristics were determined and compared between the BDI consortium, strain SZ, strain W, *Dhc* strain FL2, *Dhc* strain GT and *Dhc* strain BAV1. The mean diameter, size distribution and electrophoretic mobility of these six bacterial cultures in aqueous suspension were characterized by dynamic light scattering (DLS) using a Zetasizer Nano ZS Analyzer (Malvern Instruments Ltd., Southborough, MA) operated in non-invasive back scattering (NIBS®) mode at an angle of 173°. Approximately 1 mL of each undiluted cell suspension was loaded into a disposable cuvette (DTS0012, Malvern Instruments Ltd., Southborough, MA) and analyzed using a green laser at a wavelength of 532 nm. All particle size and electrophoretic mobility measurements were performed in at least triplicate, and the operation of the analytical system was verified using a monodisperse suspension of polystyrene spheres (3100A, Nanosphere Size Standards, Duke Scientific Corp., Palo Alto, CA) with a mean diameter of 97 ± 3 nm and a zeta potential transfer standard of -68 ± 6.8 mV (DTS1050, Malvern Instruments Ltd., Southborough, MA). The zeta potential of bacterial cells was calculated from electrophoretic mobility using the Smoluchowski equation (Hiemenz and Rajagopalan, 1997).

Calculations of interaction energy

According to Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, the interaction energy (E_i) between a particle and a plane (surface) is composed of the electrical double layer repulsion energy (E_{EDL}) and the van der Waals attraction energy (E_{VDW}). The value E_{EDL} can be calculated according to the equation of Bayoudh (Bayoudh *et al.*, 2009):

$$E_{EDL} = \pi\varepsilon_r\varepsilon_0 a\left[2\psi_b\psi_s \ln\left(\frac{1+e^{(-\kappa d)}}{1-e^{(-\kappa d)}}\right) + (\psi_b^2+\psi_s^2)\ln(1-e^{(-2\kappa d)})\right] \quad (1)$$

where ε0 is the permittivity of a vacuum, εr is the relative dielectric constant, a is the radius of bacterium, and ψb and ψs are the surface potentials of the bacterium and sand surface, respectively, and d is the surface to surface distance. The Debye-Huckel reciprocal length parameter (κ) can be calculated as $\kappa = \left(\frac{2e^2 N_A I_c}{\varepsilon_0\varepsilon_r kT}\right)^{1/2}$, where *e* is the electron charge, T is the absolute temperature, k is Boltzmann constant, NA is Avogadro's number, and Ic is the ionic strength. Under low ionic strengths, the thickness of particle stern layer is relatively small and therefore, the surface potential is approximately equal to the zeta potential. The value of EV was computed as (Bayoudh et al., 2009):

$$E_V = -\frac{Aa}{6d} \quad (2)$$

The Hamaker constant (A) of "bacterium-water-sand" is 2.4×10^{-21} J, which was calculated based on (Hiemenz and Rajagopalan, 1997):

$$A_{BWS} = (A_B^{1/2} - A_W^{1/2}) \times (A_S^{1/2} - A_W^{1/2}) \quad (3)$$

where the Hamaker constants of bacterium (A_B), quartz sand (A_S), and water (A_W) are 6.12×10^{-20} J (Rijnaarts et al., 1995), 4.14×10^{-20} J (Hiemenz and Rajagopalan, 1997), and 3.70×10^{-20} J (Israelachvili, 1992), respectively. Additional information about zeta potential and aggregate size measurements are available (Wang et al., 2008).

10.3.6 Sample Preparation and DNA Extraction

Effluent biomass was collected from 5 to 15 mL aqueous column samples by centrifugation. Side port samples (1 to 1.25 mL) were collected using a 2.5 mL glass syringe (Hamilton Co., Reno, NV) and centrifuged at 13,200 rpm at room temperature for 15 minutes. After removing the supernatant, the cell pellets were stored at -20°C until genomic DNA was extracted using the DNeasy® Blood and Tissue Kit (Qiagen, Valencia, CA) according the bacterial protocol, with modifications previously described (Ritalahti et al., 2006). DNA was obtained in final volumes of 400 µL buffer AE (provided with the DNeasy® Blood and Tissue Kit) and stored at -80°C until qPCR analysis.

Solid samples were collected through destructive sampling as described above. The saturation of solids was maintained during sampling to maintain the distribution of attached and planktonic cells. Between 0.25 to 0.35 g of solids was collected with sterile spatula, transferred to a MO BIO PowerSoil® bead tube (MO BIO Laboratories, Inc., Carlsbad, CA), and prepared for DNA extraction according to established protocols (Cápiro et al., 2008). Following homogenization, DNA was extracted from 0.25 to 0.35 g (wet weight) solid material using the MO BIO PowerSoil® DNA Isolation Kit in accordance with manufacturer procedures. DNA was obtained in a final volume of 100 µL in solution C6 (provided with the PowerSoil® Isolation Kit) and stored at -80°C until qPCR analysis.

10.3.7 Quantitative Real-Time PCR (qPCR) Analysis

Dhc, *Geobacter lovleyi* strain SZ and *Anaeromyxobacter dehalogenans* strain W cell numbers were quantified in triplicate qPCR reactions targeting the 16S rRNA genes with an ABI 7500 Fast Real-Time PCR or ABI 7300 Real-Time PCR System (Applied Biosystems, Foster, CA) under the standard 7500 or 7300 operating mode, respectively. DNA concentrations were determined by spectrophotometry at 260 nm. Primers and probes were obtained from IDTdna Technologies (Coralville, IA). Standard curves using a 10-fold dilution series of plasmid DNA were generated and *Dhc* target gene abundances were determined (Ritalahti et al., 2006). Quantification of strain SZ and strain W 16S rRNA genes was performed as described (Amos et al., 2009; Thomas et al., 2009) using the SYBR Green detection chemistry. Cell number

estimates were made by dividing the qPCR-derived gene copy numbers by the chromosomal gene copy numbers for each of the target genes in the host organism genome. *Dhc* strains carry a single copy of the 16S rRNA gene (Kube et al., 2005; Seshadri et al., 2005) while *Geobacter lovelyi* strain SZ and *Anaeromyxobacter dehalogenans* strain W each possess two copies of this phylogenetic marker (Thomas et al., 2008). PVs were determined with the bromide tracer for the Appling soil and the Federal Fine sand. Using the procedure outlined by Amos, total retained cell numbers were reported per 4.98 and 5.54 grams of wet porous medium (attached cells plus cells retained in the pore water), which corresponds to the amount of porous medium associated with a pore volume of 1 mL in Appling soil or Federal Fine sand, respectively, to allow for direct comparison to aqueous samples (quantified per mL).

10.3.8 Analytical Methods

Aqueous-phase samples (1 mL) were analyzed for chlorinated ethenes as described (Amos et al., 2007a) with a Hewlett-Packard (HP) 7694 headspace autosampler connected to a HP 6890 gas chromatograph (GC) equipped with an HP-624 column (60 m by 0.32 mm inner diameter; film thickness, 1.8 μm) and a flame ionization detector (FID). Standard curves for chlorinated ethenes were prepared as described (Gossett, 1987; Löffler et al., 1997). The pH of column effluent was measured using a VWR Model 8000 pH meter (VWR Scientific, West Chester, PA) equipped with an Accumet gel-filled pH combination electrode (Fisher Scientific). Organic acids were quantified using a High Performance Liquid Chromatography (HPLC) system (Waters, Corp., Milford, Massachusetts) equipped with a Waters 2487 dual-wavelength absorbance detector set at 210 nm and a Waters 717 plus autosampler (He et al., 2003).

10.4 Results and Discussion

10.4.1 Distribution of Cells Following Inoculation without Substrates

Following the introduction of the BDI consortium, *Dhc* cells were detected in the column effluent within 1 PV in both the Federal Fine and Appling columns. *Dhc* breakthrough at early time (PVs 0-2) through the column was comparable to that of the conservative tracer in both the sand and the soil matrices. *Dhc* titers ranging from 8.5×10^2 to 1.8×10^6 cells mL^{-1} were persistent for an additional ca. 7 PVs (i.e., the pulse width) in the Federal Fine column until returning to background titers (10^2 cells mL^{-1}) (Figure 10-4). Following the introduction of medium, the cell titers decreased in the Federal Fine sand column, dropping below the detection limit of 10^2 cells per mL after 12 PVs (Figure 10-4). Within the Appling soil column, cell titers ranged from 9.6×10^3 to 8.3×10^5 cells mL^{-1}, and remained above 10^4 cells mL^{-1} following flushing with nearly 7 PVs of reduced medium (Figure 10-4). In contrast to the Federal Fine sand column, the number of *Dhc* cells eluting from Appling soil declined more gradually following introduction of medium, with approximately 4×10^4 cells mL^{-1} detected in the column effluent at the conclusion of the experiment (11.7 PV, Figure 10-3). The observed tailing of the *Dhc* breakthrough curve (BTC) obtained for Appling soil suggests that cells experienced rate-

limited release (i.e., progressively slower detachment of cells over time) from the soil matrix. At the termination of the experiment, *Dhc* cells were retained (total, aqueous + solid phase) over the entire length of each column, with measured average abundances of 1.7 ± 0.05 x 10^5 cells per 5.45 g of wet Federal Fine sand and 2.4± 0.06 x 10^5 cells per 4.98 g of wet Appling soil (Figure 10-4). Overall, these findings indicated that a majority of *Dhc* cells under non-growth conditions were associated with the aqueous phase in both porous media (Table 10-1).

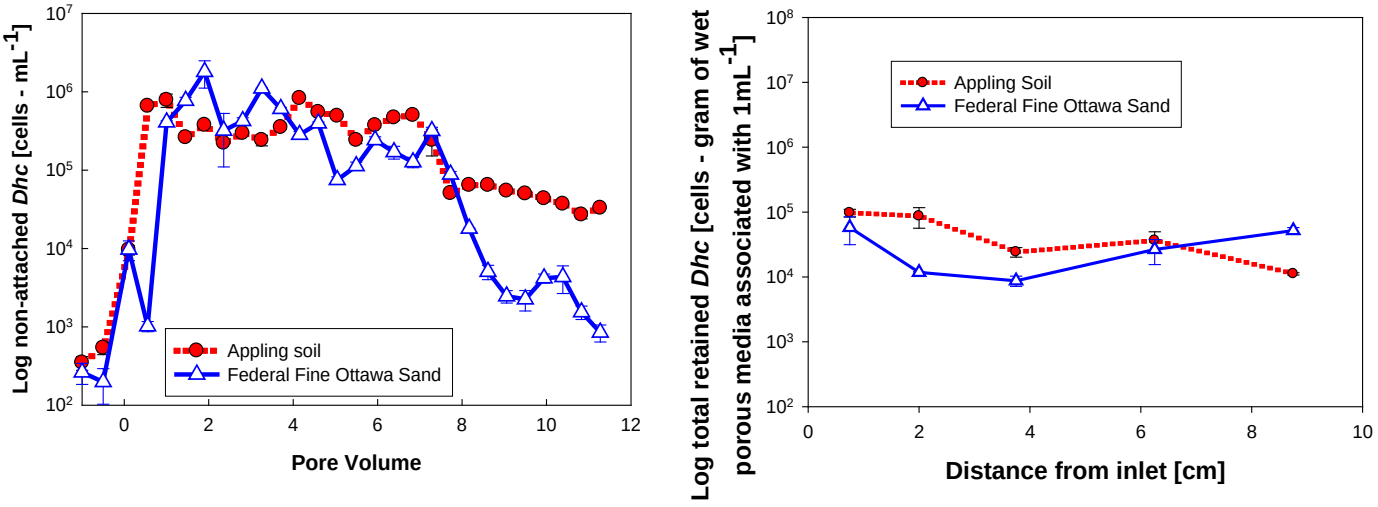

Figure 100-3: *Dhc* distribution in Federal Fine Ottawa sand and Appling soil in the aqueous effluent (right) and solid (left) phases in the absence of growth substrates.

Strain SZ cells were also quantified in both column effluents within 1 PV following the injection of consortium BDI (Figure 10-5). In the Federal Fine column effluent, strain SZ cell numbers ranged from 2.7 x 10^2 to 7.7 x 10^4 cells mL^{-1}, and dropped below the detection limit of 10^2 cells mL^{-1} following flushing with 1 PV of medium (Figure 10-5). Measurements strain SZ cells retained in the solid phase following destructive sampling indicated that strain SZ showed limited attachment to the Federal Fine sand. Strain SZ cell were only detected in one transect (4.4 ± 2.3 x 10^3 cells per 5.45 g of wet sand) (Figure 10-5). This result coincides with the column studies described in Section 6.4.2, that indicated that metabolically inactive (i.e., no growth substrates provided) strain SZ cells are primarily associated with the aqueous phase in low-organic content sand columns. In contrast, in the Appling soil column effluent, strain SZ cells recovered in column effluent exceeded the injected cell abundance by an order-of-magnitude (Table 10-1), indicating that Appling soil provided substrates for growth (Figure 10-5). Prior work has demonstrated that iron-reducing *Geobacter* spp. mineralize a variety of organic compounds coupled to ferric iron reduction (Lovley, 1987), and elemental analysis of the Appling soil indicated a total iron content of 7.2 g kg^{-1}. Strain SZ cells were retained in the solid phase throughout the length of the Appling soil column at an average abundance of 6.4 ± 0.08 x 10^6 cells per 4.98 g of wet soil.

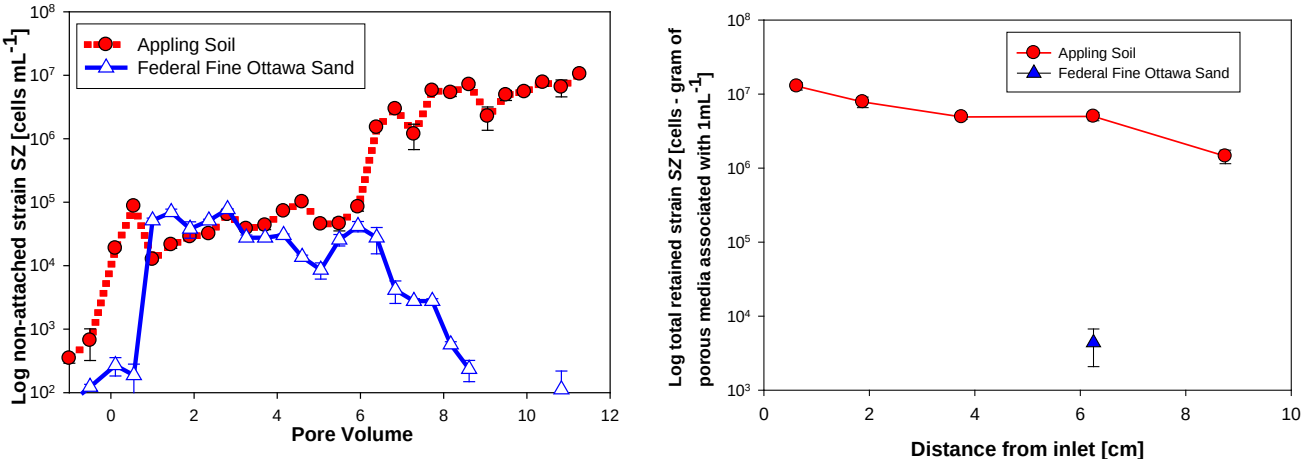

Figure 100-4: Strain SZ distribution in Federal Fine Ottawa sand and Appling soil in the aqueous effluent (right) and solid (left) phases in the absence of growth substrates.

Replicate column experiments with *Anaeromyxobacter dehalogenans* strain W revealed breakthrough within 1 PV, and cell numbers stayed relatively constant with an average of 1.4 ± 0.09 x 10^5 cells per mL and 3.8 ± 0.2 x 10^5 cells per mL eluting from the Federal Fine and Appling columns, respectively, over the course of 12 PVs. Strain W cells attached uniformly throughout the length of both columns with averages of 2.5 ± 0.2 x 10^5 cells per 5.45 g of wet sand and 1.1± 0.1 x 10^6 cells per 4.98 g of wet soil for Federal Fine and Appling soil, respectively (Figure 10-6). Tailing in the BTCs was observed in both porous media, suggesting that the cells experienced rate-limited detachment from the solid matrices, similar to what was observed with *Dhc* and strain SZ in the Appling soil column experiments. The total number of strain W cells recovered in the Appling column exceeded the number of cells injected into the column (Table 10-1), which again suggested that cell growth occurred, presumably due to organics and ferric iron associated with the Appling soil.

The results of the column studies conducted without substrate additions demonstrated that cell retention depends on the characteristics of the porous medium and the organisms. In the absence of substrates, *Dhc* cells were predominately associated with the aqueous phase, and their attachment was comparable between the two porous medium types. Strain SZ cells were not detected throughout most of the column packed with Federal Fine sand, yet strain SZ retention was three-times greater than that of *Dhc* in the Appling soil column. The greater elution of strain SZ cells from the Federal Fine sand column (> 99%) was likely due to a combination of low organic carbon content and low specific surface area of the solid phase. Further, the propensity for non-growing (non-dechlorinating) strain SZ cells to be associated with the aqueous phase is consistent with observations made by Amos et al. (2009) in a column packed with Federal Fine

sand performed with the same BDI-SZ consortium. Overall, in the absence of substrates, all three bacterial species were predominately detected in the aqueous phase (Table 10-1).

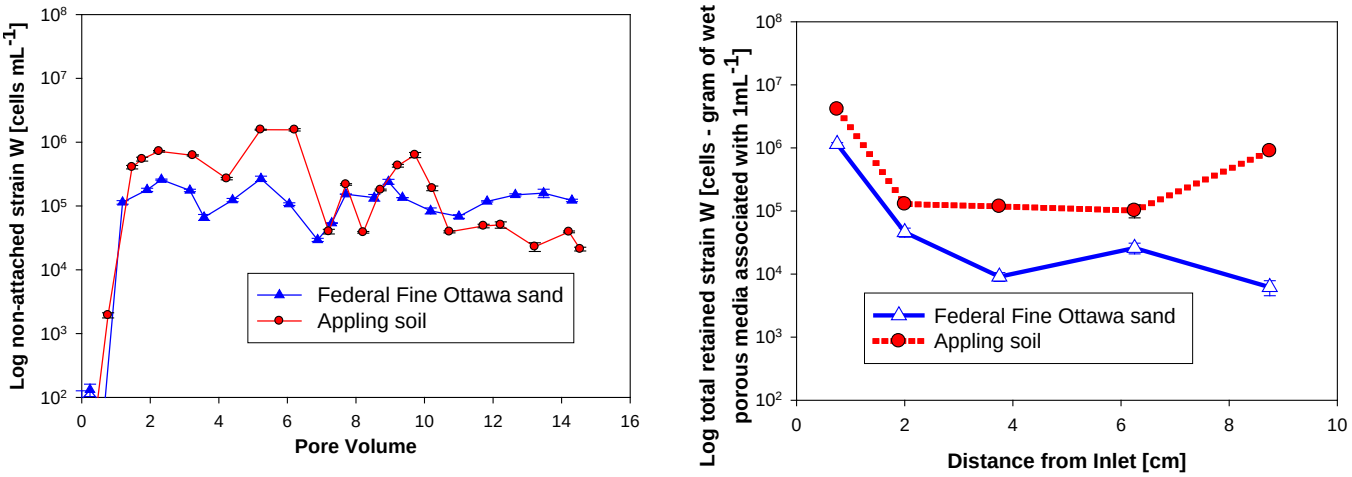

Figure 100-5: Strain W distribution in Federal Fine Ottawa sand and Appling soil in the aqueous effluent (right) and solid (left) phases in the absence of growth substrates.

Table 10-1: Recovery of *Dhc*, strain SZ and strain W cells from the aqueous and solid phases following inoculation to saturated columns packed with Federal Fine Ottawa sand (FF) and Appling soil (AP).

	FF *Dhc*	**AP *Dhc***	**FF strain SZ**	**AP strain SZ**	**FF strain W**	**AP strain W**
Total cells in inoculum	8.76E+07	9.80E+07	5.15E+06	5.36E+07	1.07E+08	1.51E+08
Aqueous phase cell recovery	7.09E+07	7.07E+07	4.88E+06	5.43E+08	4.39E+07	1.15E+08
Solid phase cell recovery	3.49E+06	4.55E+06	1.82E+04	1.28E+08	8.48E+06	4.32E+07
Total cell recovery	7.44E+07	7.53E+07	4.90E+06	6.71E+08	5.24E+07	1.58E+08
Total fraction recovered	85%	77%	95%	1252%	49%	104%
Fraction in aqueous phase	95%	94%	99.5%	81%	84%	73%

10.4.2 Distribution of Bacterial Cells between Aqueous and Solid Phases in the Presence of Growth Substrates

The analysis of target cell abundances in side port and effluent aqueous samples, as well as in solid phase transect samples revealed that *Dhc* and strain SZ were predominantly attached to the solids in both the Federal Fine sand and Appling soil columns (Table 10-2). The mean distribution of *Dhc* cells along the length of the Federal Fine sand packed column was 4.6 ± 2.3 x 10^5 cells per mL in the aqueous phase and 3.4 ± 1.2 x 10^6 cells per 5.54 g of wet sand (Figure 10-7). By comparison, within the Appling soil column, on average 5.9 ± 4.4 x 10^4 cells per mL were present in the aqueous phase and 2.6 ± 0.7 x 10^6 cells were retained per 4.98 g of wet soil (Figure 10-7). Within both porous media, the numbers of cells associated with the solid phase consistently exceeded those of non-attached (i.e., aqueous phase) cells by at least a factor of approximately 10 (i.e., an order-of-magnitude) through the length of the columns, and up to 2 orders-of-magnitude in the Appling soil column.

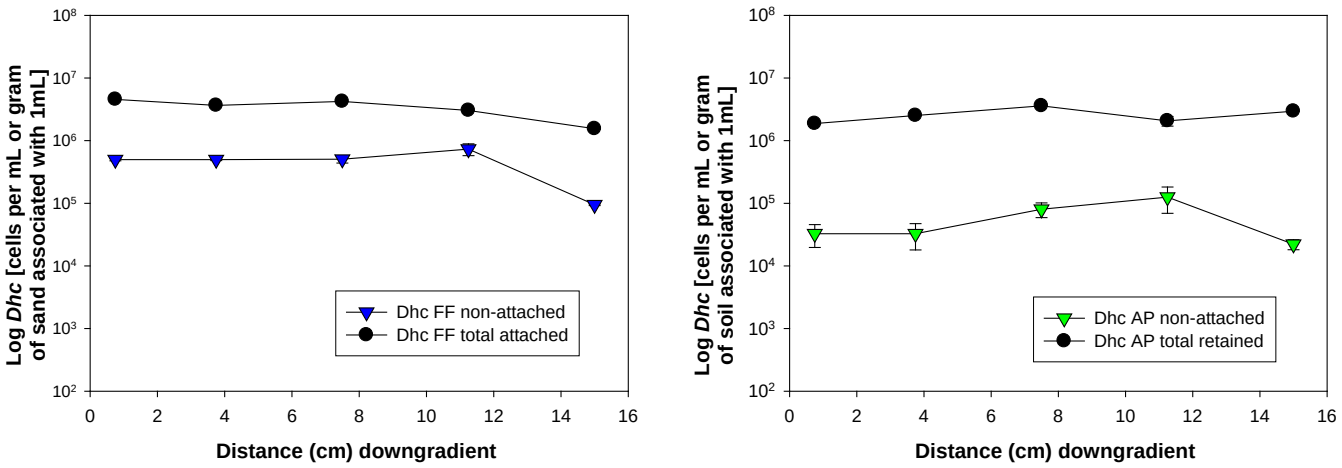

Figure 100-6: *Dhc* cell distribution in the aqueous and solid phases in columns packed with Federal Fine Ottawa sand (left) and Appling soil (right). The columns were inoculated with consortium BDI and received mineral salts medium amended with 10 mM lactate and 0.33 mM PCE.

Strain SZ cells revealed a different behavior and their distribution between the aqueous and solid phases ranged from nearly equal at some locations in the Federal Fine sand column to up to four orders-of-magnitude greater abundance in solids-attached cells in the Appling soil column. The average numbers of strain SZ cells in the Federal Fine sand column was 1.3 ± 0.5 × 10^5 cells mL^{-1} in the aqueous phase (side-port samples) and 2.8 ± 3.8 x 10^6 cells-5.54 g of saturated $sand^{-1}$ retained in the solid phase (Figure 10-8). Cell numbers in the Appling soil column averaged 4.2

± 5.6 × 10^4 cells mL^{-1} in the aqueous phase and 1.8 ± 2.0 × 10^7 cells-4.98 g of saturated $soil^{-1}$ attached to the solid phase (Figure 10-8).

Access to growth substrates influenced cell attachment in both porous media, and cells were measured predominately attached at all sample locations. In the Appling soil column, the fraction of *Dhc* and strain SZ cells measured in groundwater samples was consistently less than 2% (with one exception at 12%). Similar to columns performed without the addition of growth substrates, medium of moderate organic carbon content also influence the phase distribution of *Dhc* and strain SZ cells (Table 10-2).

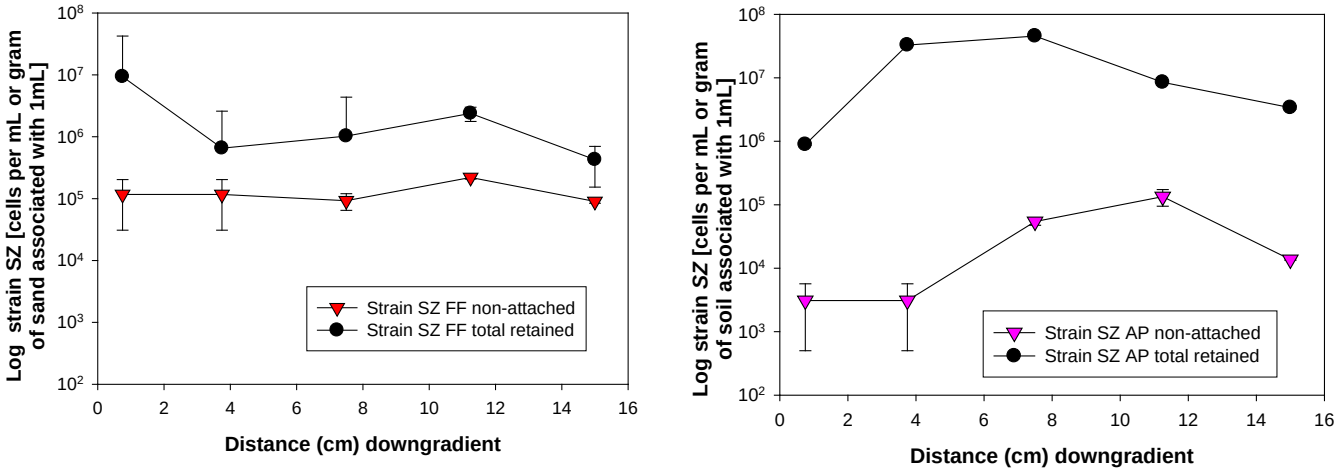

Figure 100-7: Strain SZ distribution in the aqueous and solid phases within columns packed with Federal Fine Ottawa sand (FF, left) and Appling soil (AP, right). The columns were inoculated with consortium BDI and received mineral salts medium amended with 10 mM lactate and 0.33 mM PCE.

Table 100-2: Fraction of total *Dhc* and strain SZ in the aqueous phase measured in Federal Fine Ottawa sand (FF) and Appling soil (AP) columns under growth conditions.

Location	Distance from the Inlet (cm)	Fraction of Cells in the Aqueous Phase			
		***Dhc* FF**	***Dhc* AP**	**Strain SZ FF**	**Strain SZ AP**
Inlet	0.75	11%	2%	1%	0.3%
Port A	3.75	14.0%	1%	18%	< 0.1%
Port B	7.5	12%	2%	9%	0.1%
Port C	11.25	24%	21%	9%	1.6%
Outlet	15.0	26%	1%	21%	0.4%

10.4.3 Physical Properties Support Surface Attachment

The dynamic light scattering properties of cells in consortium BDI and the five isolates are shown in Table 10-3. The average hydrodynamic diameter of cells in these cultures ranged from 0.59 to 2.68 μm. The measured average sizes of strain SZ and *Dhc* strain GT isolates were 1.06 and 0.84 μm, respectively, which are in reasonable agreement with previously reported size data acquired using electron microscopy (He et al., 2005; Sung et al., 2006b; Sung et al., 2006a). The polydispersity indexes (PDI) of all cultures were higher than 0.29, indicating the cells were not homogeneously dispersed in the aqueous phase. The inhomogeneity of cell size distribution among populations can be explained by the formation of different sized aggregates. All cells were negatively charged, with zeta potentials ranging from -10.5 to -33.6 mV (Table 10-3).

Table 100-3: The mean hydrodynamic diameter and zeta potential of cells used in this study.

Name	d_H (μm)	PDI	ZP(*mV*)
BDI	0.96 ± 0.03	0.29	-23.0 ± 1.0
Strain W	0.59 ± 0.01	0.51	-20.6 ± 1.2
Strain SZ	0.84 ± 0.12	0.59	-10.5 ± 1.3
Dhc-FL2	2.57 ± 1.28	1.00	-33.6 ± 0.9
Dhc-GT	1.06 ± 0.43	0.70	-32.6 ± 0.7
Dhc-BAV1	2.68 ± 1.71	0.88	-22.0 ± 0.6

d_H – average hydrodynamic diameter determined using dynamic light scattering. PDI – polydispersity index. ZP – zeta potential based on the Smoluchowski model (Hiemenz and Rajagopalan, 1997). The standard deviation of each sample was calculated based on at least three replicate measurements.

The cultures were maintained in medium with an ionic strength of 60 mM.

The interaction energies between bacteria and Federal Fine sand grains and Appling soil particles at an ionic strength of 60 mM are presented in Figures 10-8 and 10-9, respectively. Negative values represent a net attractive force, while positive values correspond to a net repulsive force. In Federal Fine sand, the energy profile of all six cultures indicated an energy barrier (i.e., a positive peak in Figure 10-9) and unfavorable conditions for attachment. The energy barriers of all three *Dhc* strains were greater than 500 kT, which were more than nine fold higher than those observed with strain SZ and strain W. This finding is consistent with the behavior observed in the column experiments demonstrating that the fractions of *Dhc* in the aqueous phase was greater than those of strain SZ and strain W. In contrast to the Federal Fine system, interaction forces between bacteria and Appling soil all displayed low energy barriers (Figure 10-9), suggesting that bacterial cells were more likely to attach to the higher organic content Appling soil. This analysis based on DLVO theory provides another line of evidence demonstrating that the characteristics of the microbe itself and the properties of the porous media (e.g., surface charge) influence bacterial attachment.

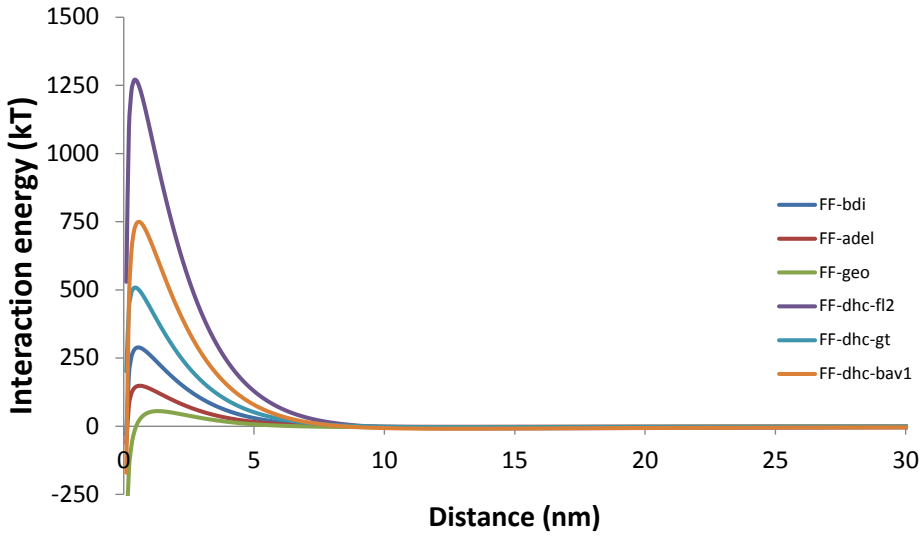

Figure 100-8: The interaction energy between bacteria and Federal Fine sand, calculated using DLVO theory.

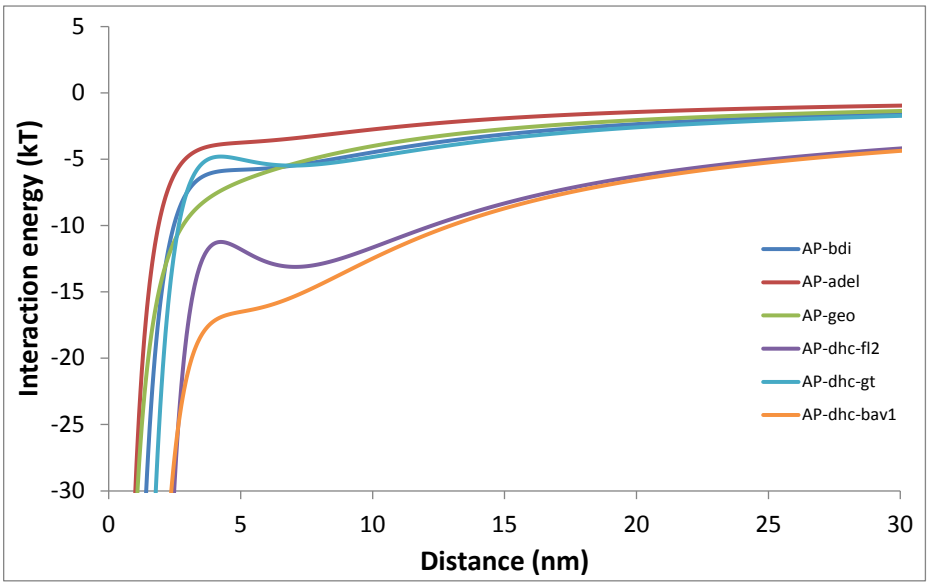

Figure 100-9: The interaction energy between bacteria and Appling soil, calculated using DLVO theory.

10.5 Conclusions and Implications for site Assessment and Bioremediation Monitoring

- The distribution of bacterial cells between the resident solid and aqueous phases varies with organismal characteristics, the specific environmental conditions (e.g., growth substrates present/absent), and the properties of the solid matrix (e.g., organic carbon content).

- When no growth substrates were provided, microbial cells were predominately associated with the aqueous phase; however, the organic content of the solid phase influenced the distribution of different microbes between the aqueous and solid phases.

- Under conditions favoring growth (i.e., in the presence of growth substrates), the attached *Dhc* cells exceeded the non-attached cells by one and two orders-of-magnitude in the Federal Fine sand and Appling soil and columns, respectively. Similarly, under growth-promoting conditions, attached *Geobacter lovleyi* strain SZ cells exceeded non-attached cells by one and four orders-of-magnitude in columns packed with Federal Fine sand and Appling soil, respectively.

- Zeta potential measurements and calculations based on DLVO theory corroborate organism-specific attachment behavior, and strain SZ and *Anaeromyxobacter dehalogenans* strain W cells demonstrated a higher propensity for attachment compared to *Dhc* cells.

- The DLVO calculations support that *Dhc* strains, strain SZ, and strain W do attach more readily to the Appling soil matrix than to the Federal Fine sand.

- When *Dhc* cell titers in groundwater exceed 10^6 cells L^{-1}, the possible underestimation of the true *Dhc* abundance will not affect qPCR data interpretation, and ethene formation is predicted to occur.

- At sites with intermediate (10^3-10^5 cells L^{-1}) or low ($<10^3$ cells L^{-1}) *Dhc* cell abundances in groundwater, the qPCR data interpretation in terms of reductive dechlorination potential is more challenging because the attached *Dhc* population size can vary and exceed the planktonic *Dhc* cell titers.

10.5.1 Implications for Site Assessment and Bioremediation Monitoring

Groundwater analysis will “not see” the fraction of cells of interest (e.g., *Dhc* cells) attached to the solids. In the absence of growth substrates, the majority of *Dhc* cells are found in the aqueous phase; therefore, groundwater samples alone are sufficient to assess the total microbial population. In contrast, groundwater analysis may underestimate the true *Dhc* cell abundance in

the aquifer during growth-promoting conditions. In sandy aquifers with low organic carbon content and growth substrates available, the fraction of attached cells can exceed the number of non-attached cells by around one order of magnitude. A greater fraction of *Dhc* cells will associate with the solids as the organic carbon content increases, and *Dhc* cells attached to Appling soil exceeded the non-attached cells by up to two orders of magnitude in the presence of growth substrates.

Recognizing when this underestimation of *Dhc* cells in groundwater samples might occur, has implications for bioremediation practice because site management decisions are normally based on the abundance of *Dhc* cells in groundwater. Complete dechlorination to ethene is generally observed when *Dhc* abundances in groundwater exceed 10^6 *Dhc* cells L^{-1}. Therefore, when groundwater *Dhc* titers exceed 10^6 cells-L^{-1}, qPCR data interpretation will not be affected and ethene formation is expected to occur regardless of the extent of *Dhc* cell abundance underestimation. Differences or variations in dechlorination rates at sites where comparable planktonic cells numbers $>10^6$ cells-L^{-1} are detected may be attributed to the undetermined fraction of attached cells. At sites with intermediate *Dhc* cell abundances (i.e., 10^3-10^5 cells-L^{-1}) in groundwater samples, the qPCR data interpretation in terms of reductive dechlorination potential is more challenging because the attached *Dhc* population size can vary and exceed the planktonic cell populations by several orders-of-magnitude depending on the solid matrix and electron donor availability. Similarly, the actual *Dhc* abundance at sites where *Dhc* biomarker genes are barely detected in groundwater samples may actually harbor a substantial *Dhc* population with ethene formation potential associated with the solids and may have potential for ethene formation. Thus, failure to detect sufficient *Dhc* (i.e., $\geq 10^6$ cells-L^{-1}) in groundwater may not prove that ethene formation will not occur in the aquifer formation, and additional analyses (e.g., qPCR using nucleic acids extracted from solid samples) are required to determine the aquifer's natural attenuation potential. Therefore, in aquifer formations that measure *Dhc* less than 10^6 cells-L^{-1} in groundwater samples, but reductive dechlorination daughter products are present, additional analysis of aquifer solids can help to evaluate the potential for MNA or whether more aggressive treatments (e.g., biostimulation, bioaugmentation) are necessary to achieve remedial goals.

11 KEY CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH/IMPLEMENTATION

SERDP project ER-1561, *Standardized Procedures for Use of Nucleic Acid-Based Tools for Microbial Monitoring,* focused on standardizing methods for quantitative real-time polymerase chain reaction (qPCR) enumeration of microbes in groundwater samples with the goal of understanding and minimizing variability caused by analytical methods and sampling procedures.

This study indicated that fundamentally qPCR methods applied to remediation samples can be accurate and consistent. The goals set out in the statement of need were accomplished along with clarification of future research needs, which are summarized in Section 11.4. This project examined a wide range of techniques and challenges with the overarching goal of increasing our ability to confidently quantify microorganisms, and in particular *Dhc*, in groundwater samples. The goal was to achieve high precision and accuracy using qPCR, and to better understand and identify cases where test results can be biased. The two keys to accomplishing these goals were: 1) step-wise testing of critical points in the sampling and analysis stream to better understand major sources of errors and possible biases, and 2) development and testing of a microbial internal amplification control (MIAC) to account for matrix interference and biomass losses.

The project has achieved several significant milestones that will improve qPCR methods, data interpretation and, by extension, increase confidence in these results, including:

- Agreement of qPCR methods with non-molecular methods such as plate counts and microscopy, that speak to the fundamental accuracy of qPCR;
- A better understanding of where in the analysis variability is most likely to occur;
- The development of an effective microbial internal amplification control (MIAC) with the ability to quantify biomass losses and inhibition and flag suspect samples/analyses;
- Obtaining sample-to-sample consistency within labs and between sampling events in the same well;
- The ability for five independent labs to return similar *Dhc* enumeration results for identical groundwater samples;
- A better understanding of the distribution of *Dhc* between aquifer solids and groundwater.

The following subsections summarize the key conclusions and recommendations of the project from groundwater sampling, sample shipping and storage, biomass concentration, DNA extraction, qPCR and reporting. Figure 11-1 provides a summary of sample and analysis flow

with selected recommendations/findings at key steps. These recommendations have the potential to increase precision, accuracy and confidence in environmental qPCR methods.

11.1 Conclusions and Recommendations for Sampling, Extraction and Analysis

Project findings and recommendations span the sampling, shipping, extraction and data interpretation steps. Key findings related to these areas are summarized in the subsections below and in Figure 11-1.

Sampling of Groundwater

The first step in the analysis chain for qPCR testing is obtaining a groundwater sample from a well. A key variable tested was the impact of low flow versus high flow sampling. A second sampling variable tested was the impact of on site filtration using Sterivex™ cartridges shipped to the lab (i.e., field filtration) versus shipping of groundwater to the laboratory where it is subsequently filtered (i.e., laboratory filtration). Key observations from the sampling study included:

- There was no significant difference observed in terms of *Dhc* quantification (less than 2-fold) between high and low flow sampling methods performed at two different wells.
- On site filtration is at least as efficient as laboratory filtration in terms of total biomass recovery and appears to be efficient at collecting *Dhc*.
- On site filtration reduced inter-lab variability compared to groundwater filtration in the lab.

In addition, based on initial round robin testing, replicate groundwater samples must be collected to characterize variability in qPCR data. The use of "pseudoreplicates" (multiple aliquots from the same DNA extract removed immediately before qPCR measurement) will not allow assessment of field variability.

Shipping and Preservation of Samples

After a site groundwater sample, or a filter cartridge with concentrated biomass, is collected, the sample is shipped to the testing laboratory, which typically requires 1-2 days, and logistics (e.g., sample queues) may require sample storage for a period of time prior to analysis. Storage of samples at temperatures above 4°C led to substantial reductions in the enumeration of *Dhc*. To the extent possible, storage time prior to analysis should be minimized and exposure to temperatures above 4°C avoided. Instability of biomass was especially apparent with biomass concentrated on filters. Therefore shorter hold times for biomass collected on onsite (e.g., Sterivex) filters should be considered compared to hold times for unfiltered groundwater, which may exhibit somewhat higher stability.

Collection of Biomass by Filtration

Whether filtration is performed in the field or in the laboratory, choices have to be made regarding the filter type, pore size and vacuum (or pumping) strength. The following observations were made during laboratory filtration tests using 0.22 µm pore size filters:

- On site filtration reduced between lab variability, suggesting that a portion of between lab variability was related to differences in filtration.

- For DNA extraction combined with laboratory vacuum filtration, the choice of membrane may impact recovery of biomarkers. For example, cellulose nitrate membranes exhibited superior performance to polyethersulfone in terms of nucleic acid recoveries for lab filtration.

- High vacuum strength was associated with increased *Dhc* losses, including complete loss of detection. Therefore, excessive vacuum strength should be avoided during groundwater filtration.

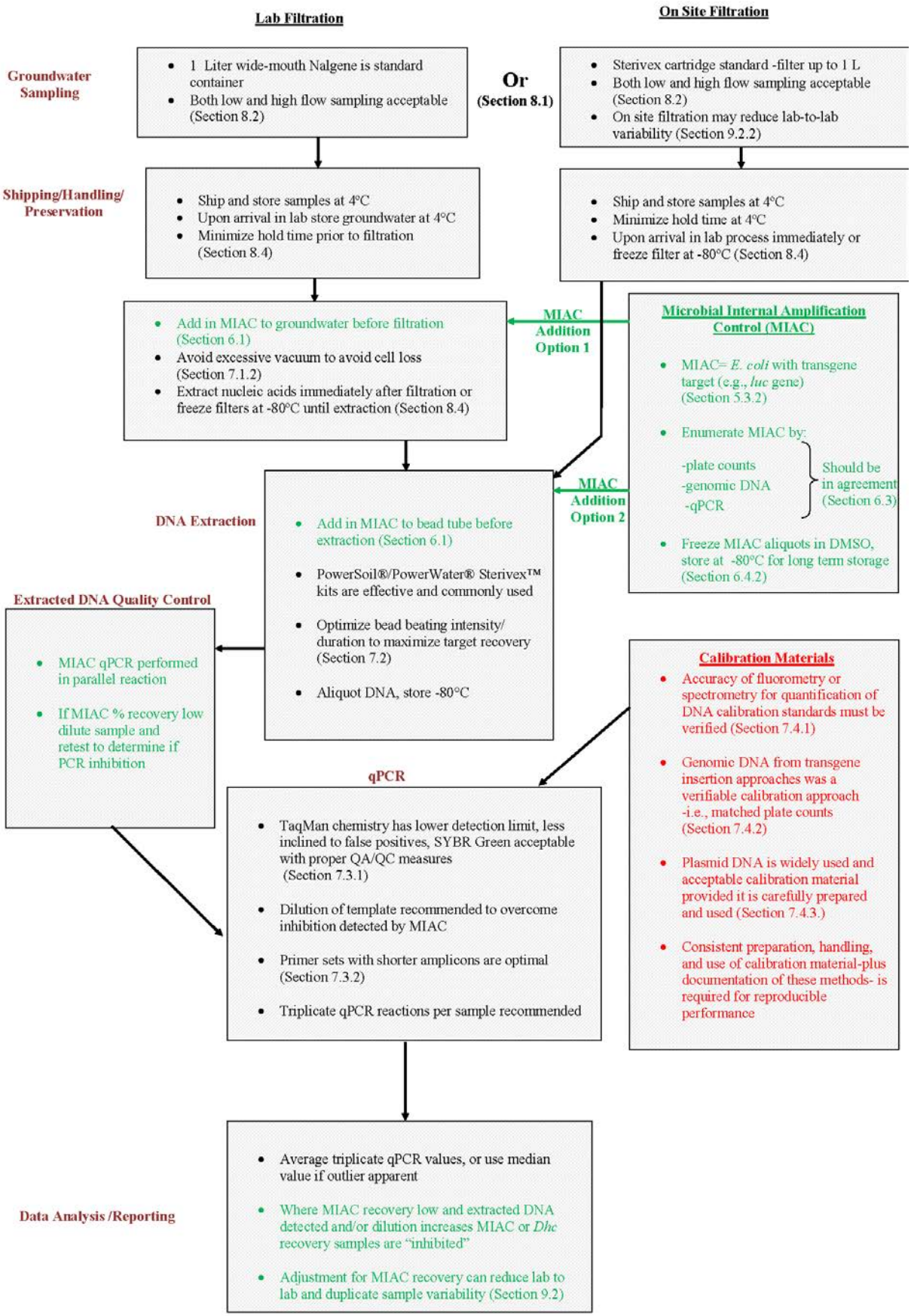

Figure 11-1: Key project findings and recommendations for improving qPCR enumeration in groundwater samples

qPCR

The final step in the analytical process is quantification of the genes of interest in a qPCR assay. Based on the findings of this project, this step may actually be a relatively minor source of variation when compared to the potential bias introduced through sample handling, filtration, DNA extraction and quantification of calibration materials. Nevertheless, observations were made that may improve the accuracy of qPCR analysis including:

- Both SYBR Green and TaqMan qPCR chemistries can produce accurate and comparable quantification results. Nevertheless, SYBR Green chemistry is more likely to produce false positives than TaqMan, therefore, greater care is required in interpreting SYBR Green assays to rule out non-specific amplification. The use of melting curve analysis and agarose gel electrophoresis confirmation target gene-specific amplification is recommended for this purpose.

- Commonly used *Dhc* primer sets with differing amplicon lengths (from 66 bp to 514 bp) provided accurate enumeration and acceptable PCR efficiencies, nevertheless, shorter amplicons are preferred as they are less likely to suffer from PCR inhibition and exhibit higher PCR fidelity.

Calibration materials used for qPCR must be suitable templates for PCR and accurate quantification of these materials is also essential for achieving accurate qPCR results. The most commonly used qPCR calibration material is plasmid DNA with a cloned target gene, which is quantified by spectrophotometry and/or fluorometry. Data from this project and the literature suggested that:

- Spectrophotometry is likely more inclined than fluorometry to high biases due to non-specific absorbance by non-DNA molecules, for this reason fluorometry with DNA specific dyes may offer improved accuracy for quantifying DNA.

- Due to supercoiling, plasmids may be less efficient templates for PCR reactions than genomic DNA and have the potential to introduce high biases in qPCR enumeration. For calibration purposes genomic DNA, may offer advantages to supercoiled plasmids.

11.2 Establishing Confidence in Environmental qPCR Methods

A major project goal reflected in the Statement of Need was to improve the remediation community's confidence in MBTs. This goal was accomplished by:

1) Verifying and optimizing previously used sampling preservation, extraction and qPCR methods; and

2) Improving data interpretation through the development and deployment of an effective microbial internal amplification control (MIAC). The MIAC provides a way to adjust the qPCR data for biomarker loss, which can result in comparable data between labs.

The combination of method optimization and the MIAC tool has markedly improved the consistency results obtained over the course of the study and will lay the groundwork for the establishment of standardized methods for qPCR testing of groundwater.

Conclusions Regarding the Developed MIAC

One of the primary achievements of the project was the development of an effective MIAC, which could be used for qPCR quality control (i.e., ongoing method validation for reagents, laboratory personnel/equipment), method optimization, detection of inhibition and potentially data adjustment. The developed MIAC, an *E. coli* mutant with a chromosomal *luc* gene insertion, can be quantified by plate counts as well as qPCR and was demonstrated to be effective for all of these purposes.

The following key observations were made regarding the effectiveness of the MIAC:

- Plate counts were useful to determine the cell titer of the MIAC.

- The MIAC was successfully used to recognize inhibition due to the presence of PCR inhibitors such as humic acids. This ability could be used to flag samples, which could generate underestimates or false negatives. The MIAC in combination with sample dilution approaches provides an effective approach to screen for and quantify inhibition.

- Low MIAC recovery was also demonstrated as a feasible tool to flag technician error or other possible failures of the testing procedure (e.g., extraction inefficiencies etc.) decreasing the possibility of reporting inaccurate results.

- The MIAC quantification can be used to quantify biomarker gene loss so that target gene quantification can be adjusted accordingly.

- Data adjustment using a MIAC could improve the consistency of target biomarker results between sampling events which improves data interpretability and confidence in the results.

Conclusions Regarding Lab-to-Lab Variability after Method Optimization/Integration of the MIAC

The combination of improved methodologies and the integration of the MIAC to correct for biomarker losses improved precision and accuracy of *Dhc* 16S rRNA gene qPCR enumeration.

The following conclusions regarding data variability were made.

- False positives were generally not reported by any of the labs in the round robins and errors were typically false negatives, or underestimates; indicating that qPCR analysis of groundwater tends to being conservative, i.e., false negatives are more likely than false positives.

- The MIAC-adjusted results of the final round robin demonstrated inter-lab variability that agreed as closely as 1.1 fold. Decreases in lab-to-lab variability of approximately 10-fold were achieved over the course of this study.

11.3 Aquifer Matrix/Groundwater Partitioning of Microbial Cells

The attachment behavior of cells (i.e., attached to aquifer solids or non-attached) has implications for estimating the total number of target cells in an aquifer (i.e., non-attached cells plus attached cells) based on groundwater analysis. It is obviously important to understand if differences in the attached versus non-attached cell ratios occur (i) between soils with different properties, (ii) under varying geochemical conditions, and (iii) in response to organismal characteristics of the microbe(s) of interest to better interpret groundwater MBT results.

The following conclusions can be drawn from the column studies (Section 10).

- The distribution of bacterial cells between the solid and aqueous phases varied by the organism characteristics, the specific environmental conditions (e.g., growth substrates present/absent), and the properties of the solid matrix (e.g., organic carbon content).

- When no growth substrates were provided, the microbial cells tested were predominately associated with the aqueous phase (i.e., planktonic); however, the organic content of the solid phase influenced the distribution of different microbes between the aqueous and solid phases.

- Under conditions favoring growth (i.e., in the presence of growth substrates), attached *Dhc* cells exceeded the non-attached cells by up to two orders-of-magnitude.

One of the implications of the findings is that the application of MBTs to groundwater samples may result in a significant (up to 100-fold) underestimation of the total number of *Dhc* cells in aquifers that support *Dhc* growth. Empirical data correlate the *Dhc* abundance in groundwater with reductive dechlorination activity and end points. For example, ethene formation is likely to occur when the *Dhc* cell titers exceed 10^6 cells L^{-1}. The results of ER-1561 do not change this interpretation even though the true *Dhc* abundance in the aquifer is one or two orders of magnitude higher. At sites with intermediate (10^3-10^5 cells L^{-1}) or low ($<10^3$ cells L^{-1}) *Dhc* cell abundances in groundwater, the interpretation qPCR data in terms of reductive dechlorination potential and end points is more challenging because the attached *Dhc* population size can vary and exceed the planktonic *Dhc* cell titers. Under such scenarios, additional analyses, such as determining soil organic matter content and qPCR analysis of solid materials, may be warranted.

11.4 Future Research Needs

The following were identified by the project team as areas for further research that would build on the accomplishments of project ER-1561.

MIAC

Evaluating the performance of the MIAC in a variety of field samples over a period of time would be informative for assessing biases and matrix inhibition. Inclusion of the MIAC into the analysis stream of an active (e.g., a commercial) laboratory would be a practical way to determine the "real world" performance of the MIAC tool. Evaluation of the performance of the MIAC in soil (aquifer solids) samples, which are generally more prone to PCR inhibition than groundwater samples, would also be informative. Application of the MIAC to groundwater samples on site would be useful to assess possible biomarker losses during shipping and storage. One way of field testing the MIAC would be the addition of lyophilized (freeze dried) MIAC cells to groundwater samples in the field, and then assessing their recovery. This approach would integrate the performance of the MIAC throughout the entire analysis sequence, from shipping and storage through DNA extraction analysis.

Standards

While the *Dhc* qPCR measurement method has been verified by comparing qPCR measurements to microscopic and plate count techniques, independently prepared and verified microbial standards are needed to verify the accuracy of measurements for each sample set. This type of QA/QC sample is routine for chemical methods and would provide greater confidence in *Dhc* measurements. This type of standard is particularly important for labs which seek to demonstrate capability with MBT measurements. Currently we are not aware of a source of a verified *Dhc* reference standard.

Sampling/Preservation Methods

Testing sampling methods (e.g., high and low floe methods) at additional wells and at a variety of sites with varying hydrogeology and geochemistry would be useful to determine if these methods are equivalent at sites with varying geology/hydrogeology. Determining potential benefits of dry ice shipping of filter samples in terms of biomass stability would be of interest. The addition of preservatives to filters (e.g., Bacteria Protect™) for extension of filter holding times warrants further study.

Calibration Materials

Further testing of the impact of plasmid topological forms (i.e., nicked/linear/supercoiled), with remediation relevant qPCR targets, to replicate findings in the literature and to confirm the magnitude of the effect of supercoiling on qPCR calibration. In addition, development of standardized methods for producing, storing, and using plasmid and genomic DNA in qPCR protocols would provide needed practical guidelines for this essential step. The development of the above approaches could increase the reproducibility of qPCR results by making calibration more consistent.

Aquifer Solids/Groundwater Partitioning of *Dhc*

The examination of attached/planktonic cell partitioning of *Dhc* between solid and aqueous phases with a variety of geologic materials (i.e., sands, clays, gravel, etc.) and geochemical conditions would be informative as to the expected remediation performance based on *Dhc* enumeration in groundwater samples alone. Understanding the effects of the geology (properties of the solid matrix) and the groundwater geochemistry (e.g., is growth supported?) will lead to more accurate estimates of *Dhc* partitioning and would support data interpretation.

Data Interpretation

Comparison of groundwater enumeration results using optimized methods with performance criteria (e.g., generation of ethene) in different geologies/geochemical conditions (e.g., different groundwater temperatures) to determine if different *Dhc* groundwater threshold concentrations should be considered as a barometer of success under different conditions.

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SUPPLEMENTAL INFORMATION

LIST OF APPENDICES:

LIST OF ATTACHMENTS:

Attachment 1: Round Robin 1 (DNA) Results and Summary

Attachment 2: Quality Assurance Project Plan Round Robin (2) Simulated Groundwater (Round Robin 2)

Attachment 3: Quality Assurance Project Plan "Round Robin" *Dehalococcoides* Analysis Including Microbial Internal Standard (Round Robin 3)

Attachment 4: Work Plan Assessment of Molecular Biological Tools Lab Variability with Field Samples (Round Robin 4)

Attachment 5: Work Plan Assessment of Molecular Biological Tools Field Variability (Round Robin 5)

Appendix A

Methods Used to Compare QPCR to Microscopy and Plate Counts

Dhc Enumeration Methods

Direct *Dhc* counts were evaluated at Georgia Tech (GT) using two Acridine Orange (AO) stained filters, 20 fields each, and at University of Delaware (UDel), using SYBR Gold direct counts performed on four independent filters, 10 fields each. 0.2 or 0.4 milliliter (mL) of *Dhc* sp. strain BAV1 culture was combined with a 0.1% solution of AO stain, and incubated for 20 minutes in the dark. The sample was vacuum filtered to a 25 mm, Irgalan Black stained polycarbonate filter with 0.22 µm pore diameter (Millipore) and cells were counted under fluorescein isothiocyanate (FITC) excitation with a 1000x phase contrast, oil immersion microscope. The number of bacteria in 20 fields per slide was counted within a visual field of a 25,000 μm^2 area grid. An automated image analysis system was used by the UDel group to count SYBR Gold stained cells.

Dhc enumeration with qPCR

Six 5-mL samples taken from a pure BAV1 culture were filtered through Sterivex™-GP filters and DNA prepared as described in Appendix A with either *Dhc* 1200F/1271R primers and *Dhc*-probe or with 300 nM of both *bvcA925F*/1017R primers and *bvcA* probe (Ritalahti *et al.*, 2006). Since all *Dhc* strains characterized to date contain only a single 16S rRNA gene and only a single *bvcA* reductive dehalogenase gene is found in strain BAV1, enumeration of the 16S rRNA gene or the *bvcA* gene provides the cell count of *Dhc mccartyi* strain BAV1.

E. coli Enumeration Methods

E. coli cells were directly counted using 4',6-diamidino-2-phenylindole (DAPI) staining and epifluorescence microscopy. *E. coli* cultures used for DNA extractions also were serially diluted in 1×PBS and plated on LB agar. Several dilutions were plated in triplicate and the plates incubated overnight at 37°C. Colony forming units (CFU) were counted and the CFU per mL calculated. For *E. coli* cell enumeration, five independently inoculated *E. coli* strain TOP10 attTn7::luc cultures were grown at 37°C with shaking for 15 hours. 2 mL of each were used for DNA extraction as described above for qPCR assay. Both a single copy of the *luc* gene inserted into the *E. coli* genome and the single-copy *E. coli* gene *dxs* were used as targets for qPCR assay (for detailed methods See Attachment 2 [Attachment B.5]) Since both of these qPCR targets occur as a single copy on the *E. coli* chromosome, the gene copy estimates correspond to cell enumeration.

Appendix B

Detailed Methods for DNA Round Robin 1

Materials and Methods

Template DNA of *Dhc* strain BAV1 was desiccated and shipped dry to all participating laboratories. The nearly complete *Dhc* sp. strain BAV1 16S rRNA gene was cloned into the pCR2.1 TOPO-TA vector (Invitrogen) and Top10 electrocompetent *E. coli* cells (Invitrogen) were transformed. The *E. coli* clone was grown in liquid YPD (Yeast extract/Bacto peptone/Dextrose) medium with 50 μg mL^{-1} ampicillin. Plasmid DNA was extracted with a Qiagen Plasmid Midi Kit and purity was assessed by gel electrophoresis. The size of the plasmid was 3,931 base pairs (bp) and the insert size was 1,420 bp. The plasmid solution was diluted with water to yield a final DNA concentration of 19.1 ng μL^{-1} (quantified by NanoDrop spectrophotometry). Fifteen 100-μl aliquots were distributed into UV-treated, 1.5 mL screw cap plastic tubes. In addition, the plasmid DNA stock solution was diluted to a concentration of 1 ng μL^{-1} using the formula C1xV1=C2xV2. The 1 ng μL^{-1} solution was used to generate a series of 10-fold dilutions, and the tubes containing $1.69x10^7$, $1.69x10^5$, and $1.69x10^3$gene copies μL^{-1} were designated M1, M2, and M3, respectively. Aliquots (100 μL) were removed from the M1, M2, and M3 dilutions and transferred to sterile, UV-treated, 1.5 mL screw cap plastic tubes. Water was removed from all tubes using a SpeedVac vacuum concentrator (3 hours at 60°C).

All tubes were shipped FedEx (1 to 2 day delivery) to the participating laboratories on May 12, 2008. Replicate M1, M2, and M3 samples were maintained at ambient room temperature for 1 day prior to analysis. The DNA concentration in the tubes was not disclosed to the recipients. Each laboratory received three tubes of the undiluted (19.1 ng μL^{-1}) plasmid DNA, one each of the M1, M2, and M3 samples, and 1 mL of sterile 10 mM Tris buffer (pH 7) for rehydrating the DNA. Prior to analysis, the dried DNA samples M1, M2, and M3 were rehydrated by adding 100 μL of 10 mM Tris buffer and incubated on a heating block at 65°C for 20 minutes. The solutions served as template DNA for *Dhc* 16S rRNA gene-targeted qPCR. Each laboratory used their standard operating procedures (SOPs) to quantify *Dhc* 16S rRNA gene copy numbers. The results were reported as gene copies μL^{-1} of template DNA solution.

Standard Curves

Four of the five participating laboratories used the undiluted plasmid DNA (19.1 ng μL^{-1}) to prepare qPCR standard curves. One laboratory used an existing standard curve and did not generate a standard curve with the DNA sample provided. To generate standard curves, the DNA was quantified by a NanoDrop spectrophotometer. The DNA solution was diluted with PCR-grade water to generate 100 μL of 1 ng μL^{-1} plasmid solution using the formula C1xV1=C2xV2. Three series of eight 10-fold dilutions were prepared to yield a total of 27 tubes spanning a concentration range from $1.69x10^8$ to $1.69x10^0$copies μL^{-1}. The *Dhc* 16S rRNA gene copies in each dilution were quantified on a qPCR plate using the absolute quantification mode of the AB 7500 fast instrument, with the standard curve providing the quantification of each target gene. Three independent dilution series were generated and each dilution was analyzed in triplicate qPCR reactions. A total of 12 standard curves were generated in four laboratories, but only 11 were included in the analysis because one standard curve was discarded due to a dilution

error. Calculating gene copy numbers per reaction of the standard curve was performed as described (Ritalahti et al., 2006). Using the equation, [(DNA ng μl^{-1}) x (0.001 ml μl^{-1}) x (3 µl rxn^{-1}) x ($6.023*10^{23}$)] / [(5.5×10^{3}) x (660) x (10^{6})] a one µL aliquot of a 1 ng μL^{-1} DNA solution consisting of a 5,351 kb plasmid contained 1.69×10^{8} *Dhc* 16S rRNA gene copies.

Reference

Gietz, R. D., R. H. Schiestl, A. R. Willems, and R. A. Woods. 1995. Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. *Yeast* 11:355–360)

Appendix C

Data Analysis of Round Robin 1 and Round Robin 2

Statistics Summary for Naked DNA and Whole Cell Round Robins - ER1561

Overview

This document provides a summary of the statistical analysis of two experiments for SERDP Project ER1561, the naked DNA (Round Robin 1) and whole cell (Round Robin 2) experiments. The naked DNA round robin experiment involved distributing samples of plasmid DNA, or naked DNA, at three concentrations to each lab. In the whole cell round robin experiment, artificial groundwater was spiked with a *Dehalococcoides* culture (*Dhc*). Two concentrations of spiked groundwater, 5 replicates of each, were distributed to the participating labs.

The participating labs for these experiments were:

Georgia Institute of Technology (Georgia Tech or GT, Frank Löffler)

Microbial Insights (MI, Dora Ogles)

Savannah River National Laboratory (SNRL, Chris Yeager)

SiREM (Phil Dennis)

University of Toronto (UT, Elizabeth Edwards)

Conclusions

True replicates, rather than pseudoreplicates (i.e. qPCR replicates), are needed to characterize the range of values observed for a sample. True replicates should include all sample processing steps in the lab.

Five replicates are sufficient to observe differences based on current data from the labs. If variability changes within the labs, the number of replicates needed may also change.

Lab is a significant source of variation in both experiments but for different reasons. See discussion of individual experiments for more information.

Whole Cell Round Robin (Round Robin 2)

For the whole cell round robin, the experiment plan is described in a Quality Assurance Project Plan entitled “Round Robin Simulated Ground Water *Dehalococcoides* Analysis”. The experiment was conducted largely as planned with two exceptions. In the first exception, the University of Toronto used two different methods to generate data. For the purposes of statistical analysis, the data was treated as if two labs had analyzed samples. In the second exception, a miscommunication may affect experiment results. Lab 5 received notice to refrigerate samples about 2 days late. When they removed their samples from the cooler, the cooler was approximately at room temperature. In a subsequent experiment, substantially lower *Dhc* abundances were observed in samples held at room temperature, compared to 4°C, for 5 days. The longer holding time and elevated temperature may affect the concentrations

determined by Lab 5. This difference was not accounted for in the statistical interpretation of the data.

The data set included a few non-detects in the low *Dhc* abundance results. For the purposes of statistical analysis, non-detects were assigned as the lowest values reported from that lab. To meet assumptions of analysis of variance (ANOVA), the data was log transformed.

Results were analyzed using a 3-way ANOVA considering lab, concentration, and replication/pseudoreplication. Since replicates are not distinguishable in a systematic way, replication/pseudoreplication was treated as a random effect. Initially, an interaction term for concentration and lab was included in the ANOVA. However, the interaction was not significant and was dropped from the final ANOVA. Results from the final ANOVA are shown in Table C1. In this experiment, all factors are statistically significant. The data are shown in Figure C1.

Table C1: Results from ANOVA for the whole cell round robin.

Factor	Degrees of Freedom	F statistic	p-value
Lab	5	3.103	0.016
Abundance	1	213.163	<0.001
Replication/pseudoreplication	1	321.6 [a]	<0.001

a – Statistical determination based on a likelihood ratio test for the difference between ANOVA models that include and do not include the replication terms and using chi squared statistic rather than F-statistic.

Gene abundances and lab will be discussed first. One would expect different values at differing starting abundances; however, the group was unsure if the data reported by different labs was different. This experiment showed that some labs reported statistically different results. In Figure C1, the statistical groupings for lab (at p value < 0.05) are shown as the orange, purple, and green ovals encircling the lab names. Labs within an oval are statistically similar. Due to the finding of statistical differences between labs, standardization of procedures or other efforts to reduce lab-to-lab differences are recommended. An experiment designed to better understand the causes of differences between the labs may be useful.

Replicates display higher variation than pseudoreplicates. This difference may be due to actual differences between the replicate samples or due to difference imparted by sample processing and analysis. However, since a careful experimental design (filling replicates 20% at a time) was employed, it is more likely that observed differences are due to differences in the operations of individual labs. Therefore, pseudoreplicates are not recommended as a measure of the variation observed when analyzing these types of samples. Replicates should include all processing steps in analysis.

The data collected in this experiment was used to evaluate the chances of falsely rejecting a hypothesis (power) in future experiments as a function of sample replication, variability, and estimated differences between samples. Power curves were generated as a function of the

standard deviation between labs compare to the standard deviation within a lab. Based on this analysis, 5 replicates would be sufficient to distinguish results between 2 labs in most cases.

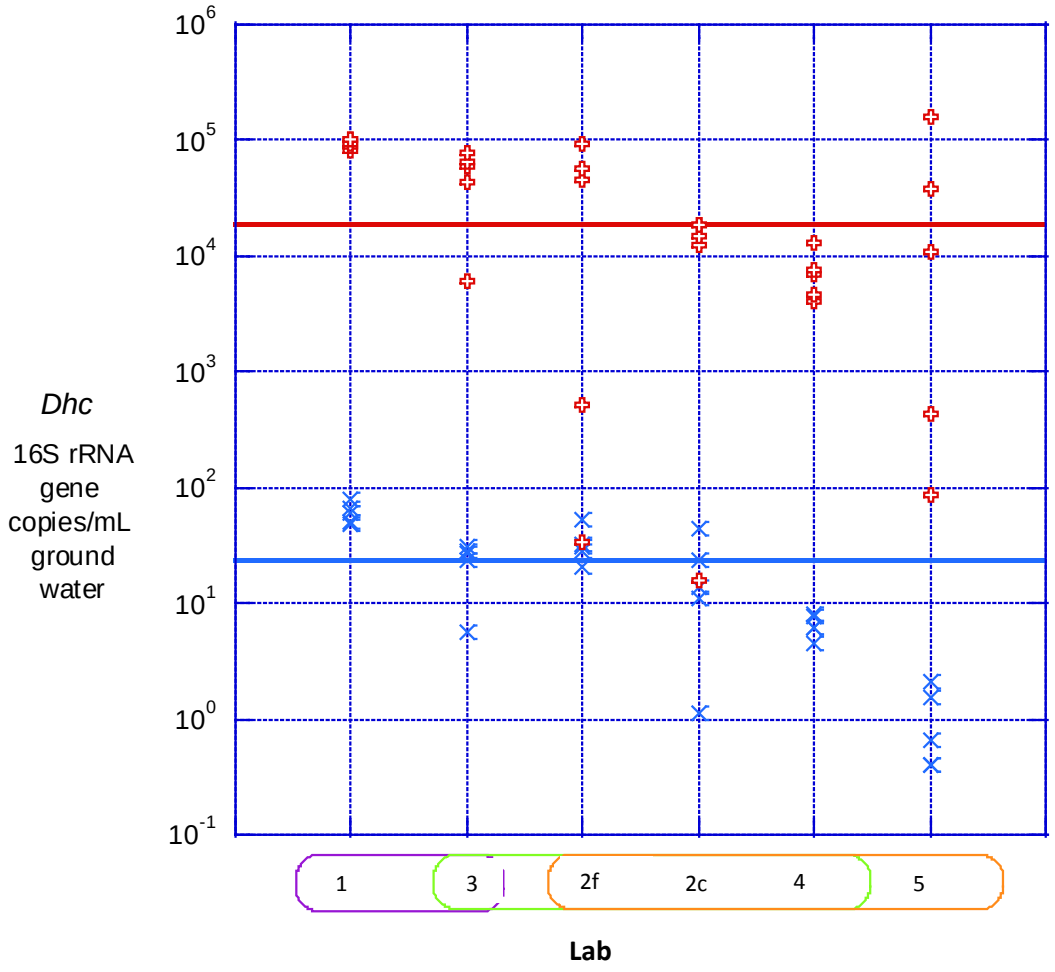


Figure C1: Data summary for whole cell round robin 2. Median data for each replicate are graphed for each lab. Red symbols represent the higher abundances, and blue represents the lower abundance samples “f “ indicates filtration and “c’ centrifugation for biomass concentration methods . The lines represent the median for all labs at each provided abundance. Labs which share a colored circle are statistically similar.

Naked DNA Round Robin (Round Robin 1)

The labs involved in the naked DNA round robin used a summary document to describe how samples were to be shipped and analyzed. There were not significant deviations from this document.

To meet assumptions of ANOVA, the data was log transformed.

Results were analyzed using a 2-way ANOVA considering lab and gene abundance. In addition to the main effects, an interaction term for lab and microbial abundance was also included. Results from the ANOVA are shown in Table 2. The data are shown in Figure C2.

Table C2: Results from ANOVA for the naked DNA round robin.

Factor	**Degrees of Freedom**	**F statistic**	**p-value**
Lab	4	41.3	<0.0001
Abundance	2	44039.8	<0.0001
Interaction	8	157.7	<0.0001

For the naked DNA round robin, abundance, lab and the interaction of lab and abundance are all statistically significant. One would expect abundances to be different. The group has also considered the possibility that the labs may be different. In this experiment, since the interaction is statistically significant, differences between labs can be evaluated by abundance level. The statistical groupings (at p-value< 0.05) are shown in Figure 2. Lab/ abundance pairs which share a letter are statistically similar. For example, in abundance 2, 4 labs share a letter D and are statistically similar. At abundance 1, Lab 1 and Lab 2 have different letters and are statistically distinct. Also, note that similar letters at one abundance do not correspond to similar letters for another abundance. For example, Lab 1 and Lab 2 are statistically different at abundance 1, but similar at abundance s 2 and 3. In this data set, the interaction occurs because the pattern of differences between labs is different at each abundance level. In addition, no lab consistently produced high or low results across the range of abundance s considered. Thus, while the interaction term is significant, there is no consistent pattern between the labs and abundances. It is possible that the use of pseudoreplicates produced low variation within labs and as a result, differences between labs are more apparent.

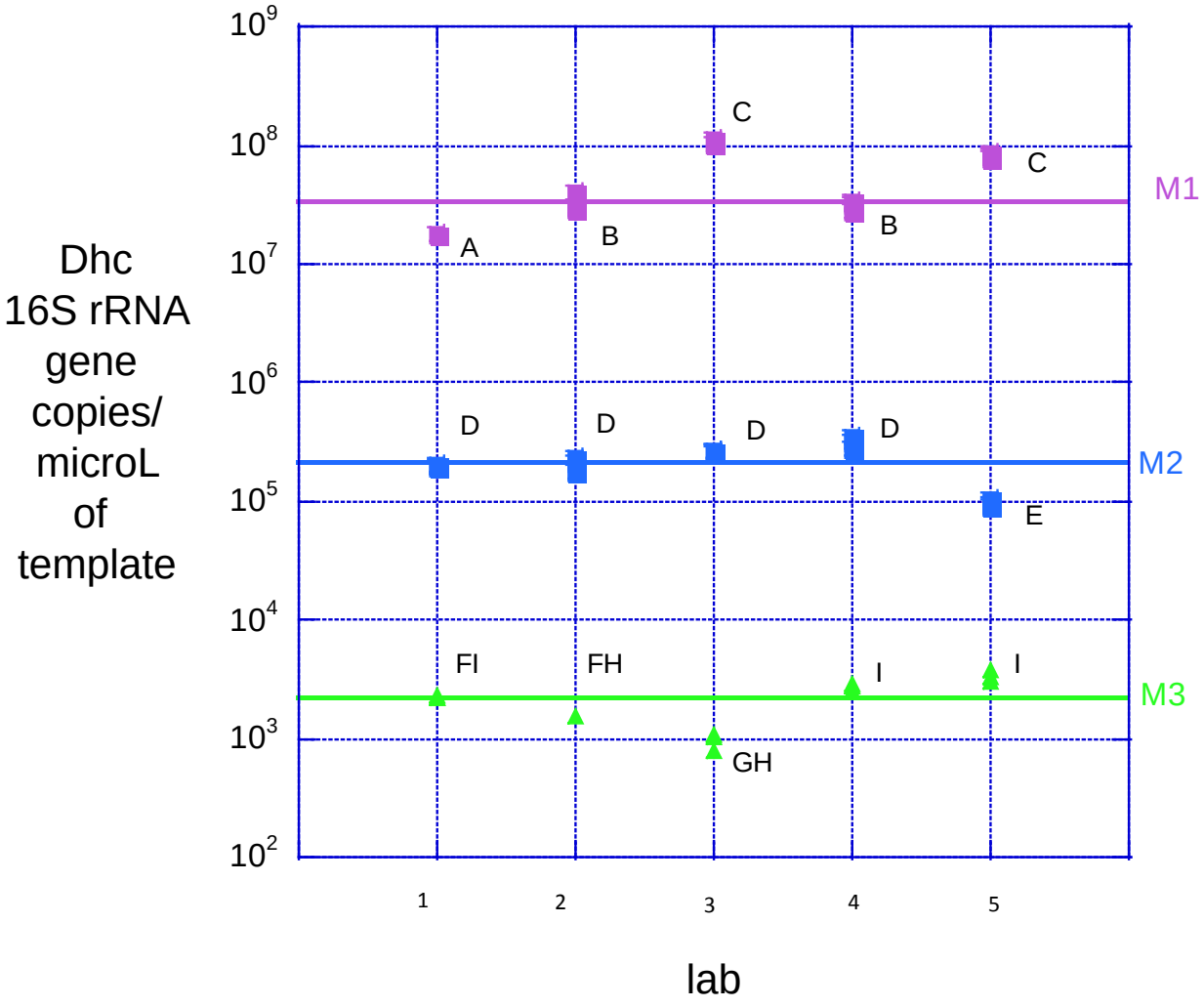


Figure C2: Summary of naked DNA round robin. Date for replicates generated at the qPCR step of analysis are graphed for each lab. The lines indicate the median for all labs at each concentration tested. The color of the points corresponds to the color of the median line. Labs with statistically similar results share a common letter.

Appendix D

Methods and Data from Growth and Testing of Natural MIACs

Growth and Testing of Naturally Occurring Microorganisms as MIAC

Materials and Methods

Groundwater from contaminated sites was screened for candidate naturally occurring MIAC. The following protocols outlines the steps necessary to grow the candidate MIAC organisms and to test MIAC DNA with suitable primers which were then used to screen groundwater for the presence of DNA of these potential MIAC.

Growth of naturally occurring MIAC *Brevundimonas diminuta* (ATCC 19146) *Micrococcus luteus* (ATCC 4698) and *Prochlorococcus marinus pastoris* CCMP2389.

Bacterial propagation and Growth ATCC Cultures

1) Propagation according to ATCC guidelines

    a. *Brevundimonas diminuta*, prepare Nutrient Agar plates for initial propagation, then grow in trypticase soy agar Temperature (mid log phase to early stationary phase) 30.0 °C

    b. *Micrococcus luteus*, prepare tryptic Soy plates for initial propagation, and grow in Trypticase soy agar Temperature: 30.0 °C broth prior to storage in glycerol (Refer to Protocol below: Preparation of Bacterial Glycerol Stocks).

2) Growth of *Prochlorococcus marinus pastoris* CCMP2389

(Strain synonyms: MED4, CCMP 1378, CCMP 1986)

Growth of CCMP 2389

Light Requirement : 80-120 uEinsteins (CCMP communication) (used OTT-LITE Model # 20EDG2R-CA Mini spiral bulb for plants, Tampa Florida)

Pro99 Medium

(Sally Chisholm, unpublished)

This medium was developed specifically for *Prochlorococcus*, but it can be used for other oceanic species tolerating high ammonia concentrations (e.g, *Bolidomonas*) and no vitamin requirement. All containers should be acid cleaned and rinsed with high quality H_2O (e.g., Milli-Q). Seawater should be collected from the oligotrophic open ocean (e.g., Sargasso Sea water), taking the usual precautions to avoid contamination. Ultrapure grade reagents should be used. This recipe was developed in Dr. Penny Chisholm's Lab (MIT), and it used smaller volumes of stock solutions. Good sterile technique is required when growing axenic strains, and a laminar flow hood is recommended.

To prepare, filter one liter of oligotrophic open ocean seawater into a Teflon-lined container, autoclave and cool before adding nutrients. Aseptically, add 1 mL each of the NaH_2PO_4, NH_4Cl and trace element solutions.

The ammonium chloride and sodium phosphate solutions should be prepared by adding the amounts indicated below, and after they are dissolved, the solution should be sterile filtered into a sterile container. The two stocks should be stored in a 4° C refrigerator.

Component	Stock Solution	Quantity	Molar Concentration in Final Medium
NaH_2PO_4	6.90 g L^{-1} dH_2O	1.0 mL	5.0 x 10^{-5} M
NH_4Cl	42.80 g L^{-1} dH_2O	1.0 mL	8.0 x 10^{-4} M
Trace Elements	(see recipe below)	1.0 mL	---

PRO99 Trace Element Solution

Primary stocks of most metals and selenium are prepared first, as indicated below. To prepare primary stocks, add the indicated amount of the component to 1 liter of high quality water. Next, the trace element solution is prepared by dissolving the EDTA in 1 liter of high quality water, by dissolving the iron, and finally by adding 1 mL of each primary stock. The final trace element solution should be sterile filtered into a clean, sterile container and stored at 4°C in a refrigerator.

Component	Stock Solution	Quantity	Molar Concentration in Final Medium
Na_2 EDTA • $2H_2O$	---	0.436 g	1.17×10^{-6} M
$FeCl_3$ • $6H_2O$	---	0.316 g	1.17×10^{-6} M
$ZnSO_4$ • $7H_2O$	2.30 g L^{-1} dH_2O	1 mL	8.00×10^{-9} M
$CoCl_2$ • $6H_2O$	1.19 g L^{-1} dH_2O	1 mL	5.00×10^{-9} M
$MnCl_2$ • $4H_2O$	17.80 g L^{-1} dH_2O	1 mL	9.00×10^{-8} M
Na_2MoO_4 • $2H_2O$	0.73 g L^{-1} dH_2O	1 mL	3.00×10^{-9} M
Na_2SeO_3	1.73 g L^{-1} dH_2O	1 mL	1.00×10^{-8} M
$NiSO_4$ • $6H_2O$	2.63 g L^{-1} dH_2O	1 mL	1.00×10^{-8} M

***B. diminuta, M. luteus*, Specific PCR assays**

Standard PCR was performed using the following primers and annealing temps, including controls *E. coli*, TB, and KB-1, ACT-3, and WBC-2 culture DNA amplification was confirmed on 1.5-2% (or higher as required) gel electrophoresis with a 100bp ladder.

PCR Reactions:

2x PCR Supermix (MBI Fermentis) 25uL

DNA free water 20 ul

Volume of primer set – 2.0 µL of primer mix (10 pmol/ µL each primer)

Volume of Template DNA – 3 µL of template (DNA diluted in TE)

Volume total = 50 µL

Thermocycler parameters:

5 min at 95°C, with 30-35 cycles of:

1 min at 94°C

1 min at 59°C

1.5 min at 72°C

5 min at 72°C

Hold at 4°C

Development of *P. marinus* MIAC qPCR method

After screening (Table L1) indicated that *P. marinus* were not found in a variety of groundwater samples. A quantitative PCR method for *Prochlorococcus (P. marinus)* was successfully implemented using *P. marinus* genomic DNA at concentrations from 5×10^7 to 5×10^1 copies per reaction to produce a standard curve using the SYBER Green based method described by (Ahlgren et al., 2006). The primers used are complementary to the 16S-23S rDNA Internal transcribed spacer (ITS) region have the sequence and produce a DNA amplicon 88 base pairs in length.

Primers used to quantify *P. marinus* were:

low BAII2f, 5´-TACCTCCACTGAATACCACCTCT-3´

low BAI2r, 5´-CGCACAAATAATAAATCTGCATCAT-3´

PCR thermocycling was performed as follows:

initial denaturation at 95°C for 1 minute, following by 40 cycles of:

Denaturation at 95°C for 45 seconds;

Annealing at 58°C for 45 seconds;

Extension at 72°C for 30 seconds.

The qPCR standard curve had an r^2 value of 0.9982 and a PCR efficiency of 90.2 %. The *P. marinus* qPCR produced melting curves and amplicon sizes similar to those described by Ahlgren et al., (2006) indicating the PCR was specific and was performing as expected.

Table D1: PCR Primers Used for Screening Samples *B. diminuta*, *M. luteus*, *Perchlorococcus (Pcc)*

Target Microbe	Primer Name	Sequence (5'-3')	Reference
B. diminuta	BD 1089	agtcctcatggcccttacag	This Study
	BD 1216	tagcgattccaacttcatgc	This Study
M. luteus	ML 570	tgcactctagtctgcccgta	This Study
	ML 455	cgagcgttatccggaattat	This Study
Prochlorococcus (Pcc)	low BAII2f,	tacctccactgaataccacctct	Algren et al., 2006
	low BAI2r	cgcacaaataataaatctgcatcat	Algren et al., 2006

Summary of Preliminary Testing for use of *Prochlorococcus* as MIAC (July 9/ 09)

Growth of *Prochlorococcus*

Prochlorococcus marinus (*P. marinus*) and Pro99 media were obtained from the Bigelow laboratory for Ocean Science, (West Boothbay Harbor, ME). *Prochlorococcus* cultures were grown under compact fluorescent lights in 500 ml flasks at a light intensity of 80-120 microeinsteins (as determined by portable light meter) on a 12 hours light, 11 hours dark schedule. Growth was monitored by visual observation of green color and spectrophotometry at a λ of 600 nanometers.

Figure D1: *P. marinus* culture as supplied and growth media

Cryopreservation

P. marinus cells in growth phase (as determined by spectrophotometry) were aliquoted (400 µL) into 500 µl screw-cap cryo-tubes, 7.5% DMSO (30 µL) was added and tube was mixed and cells were "snap frozen" in a dry ice alcohol bath. Frozen aliquots were stored at -80ºC.

Groundwater Screening for *Prochlorococcus*

One of the key priorities for a natural surrogate is it should be absent from groundwater DNA samples, otherwise the presence of DNA that "cross reacts" with the primers for the surrogate may confound spike and recovery analysis. For example, recoveries in spike and recovery could exceed 100% due to the presence of pre-existing sequences cross reacting with the surrogate targeted primers. Thus it is an essential for these sequences to be absent from groundwater DNA samples. To determine if sequences cross reacting with the *Prochlorococcus* primers were likely to be a problem a collection of 25 selected groundwater samples from varying environments were selected (see Table L2 below), these samples were previously screened with *Brevundimonas* and *Micrococcus* primers (two other potential natural surrogates) and were found to react. The DNA samples tested were derived from a wide geographic area including New Jersey, Pennsylvania, Florida, California, Alaska and Denmark and included those extracted from groundwater at number of coastal sites (*P. marinus* is an aquatic microorganism). In addition, samples from commercially bioaugmentation cultures (KB-1, WBC-2 and ACT-3) were also tested. The samples were tested using a non- quantitative PCR methods, the results of this analysis are shown in Figure 2 and indicate non-reactivity of all the DNA samples the exception of *P. marinus* DNA used as a positive control. The absence of *P. marinus* like sequences in 25 diverse samples suggests that sequences that cross react with these primers are likely to be uncommon in the environment.

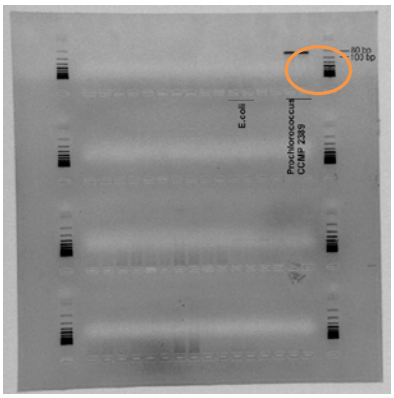


Figure D2: Agarose gel demonstrated a lack of amplification products corresponding to *P. marinus* DNA positive control (bands circled in orange) in DNA samples derived from a variety of contaminated sites. This indicated a lack of cross reactivity of primers and indicating *P. marinus* fulfills a key surrogate requirement, being absent in groundwater samples. Bands on left and right of gel are DNA size standards.

Table D1: *P. marinus* PCR Test -To determine if present indigenously in groundwater samples

Number of Samples Tested	Site Location	Criteria	Matrix	*P. marinus* like sequences
1	New Jersey	Fractured Rock site	Groundwater	Not detected
2	Pennsylvania	Fractured Rock site	Groundwater	Not detected
5	Southern California	Coastal site	Groundwater	Not detected
4	Cleveland County San Diego	Landfill Site	Groundwater	Not detected
4	Florida Coastal	Industrial Site	Groundwater	Not detected
3	Central Florida	Inland Florida	Soil	Not detected
4	Denmark	European	In situ filter/Groundwater	Not detected
2	Alaska	Arctic	Groundwater	Not detected
1	California	Industrial	Groundwater	Not detected
1	KB-1 Culture	Commercial bioaugmentation Culture	Liquid culture	Not detected
1	WBC-2 Culture	Commercial bioaugmentation Culture	Liquid culture	Not detected
1	ACT-3 Culture	Commercial bioaugmentation Culture	Liquid culture	Not detected
1	P. marinus DNA	Positive Control	Purified DNA sample	Detected

Proof of Concept use of Frozen of *P. marinus* cultures as MIAC

To confirm the viability of the use of frozen cultures of *P. marinus* as a MIAC initial spike and tests of recovery tests using -80ºC frozen aliquots of *P. marinus* were performed along with aliquots of *Dehalococcoides* BAV1 culture. The aliquots (400 µl) were spiked into phosphate buffered saline (PBS) and cell concentration (by vacuum filtration [0.2µM filter Nalgene]) and DNA extraction were performed according to standard methods (Attachment 2 [Attachment B.1]).

Table D3: Co-enumeration of P. marinus and Dehalococcoides frozen cultures in spike and recovery experiments with varying duration of bead-beating

Sample Designation	Duration of Bead Beating	*Prochlorococcus*/mL	*Dehalococcoides*/mL
6	15 Seconds	1.18E+06	1.5 E+07
10	15 Seconds	7.92E+05	8.9 E+06
3	30 Seconds	1.88E+06	1.6 +07
9	30 Seconds	1.21E+06	8.8 E+06
2	45 Seconds	1.13E+06	1.3 E+07
7	45 Seconds	1.35E+06	1.1 E+07
1	60 Seconds	9.48E+05	1.6E+07
5	60 Seconds	1.24E+06	1.5 E+07
4	120 Seconds	2.10E+06	2.2 E+07
8	120 Seconds	2.12E+06	1.8 E+07

Preliminary results (Table D3) indicated that *P. marinus* cells were present at concentrations ranging from ~8 x 10^5/mL (of original frozen culture- not of culture in PBS) to 2 x 10^6/ml depending on the length of time the bead-beating operation was performed in the DNA extraction. *Dhc* BAV1were detected from 8.8 x 10^6/mL – 2.2 x10^7/mL, depending on the duration of bead-beating. In general, it appears the longer the bead-beating the higher the recovery, with *P. marinus* possibly benefiting more than *Dhc* BAV1 from a longer bead-beating time.

The successful spike and consistent recovery of frozen *P. marinus* cells in parallel with *Dehalococcoides* is encouraging, future experiments will provide information regarding optimal extraction methods.

Table D4 Provides a summary of PCR test results using candidate MIAC specific primers, the result side9cated that *M. luteus and B. diminuta* were detected in number of groundwater samples (15/25 and 18/25) and therefore were not considered candidates for MIAC, *Prochlorococcus* was not detected in any of the samples and was considered a candidate until further testing (data not shown) suggested that *Prochlorococcus* was found in many coastal groundwater samples.

Table D4: Summary of PCR Results for *M. luteus*, *B. diminuta* and *Prochlorococcus* (*Pcc)* Primers on Groundwater DNA Samples, and Commercial Bioaugmentation Cultures

Genomic DNA Reference Number	*M. luteus* Primers	*B. diminuta* Primers	Pcc Primers	Sample Location	Site Description	Matrix
6078	-ve	+ve	-ve	New Jersey	Fractured rock site	Groundwater
0763	-ve	+ve	-ve	Pennsylvania	Fractured rock site	Groundwater
0880	+ve	+ve	-ve			
2986	+ve	+ve	-ve	California	Coastal site	Groundwater
2987	+ve	+ve	-ve			
2988	+ve	-ve	-ve			
2989	+ve	+ve	-ve			
2990	+ve	+ve	-ve			
5416	-ve	+ve	-ve	California	Landfill Sites	Groundwater
5417	-ve	-ve	-ve			
5418	-ve	+ve	-ve			
5419	-ve	-ve	-ve			
2932	+ve	-ve	-ve	Florida	Industrial Site	Groundwater
2933	-ve	-ve	-ve			
3972	-ve	+ve	-ve			
6429	-ve	-ve	-ve			
5353	+ve	+ve	-ve	Florida	Inland Florida	Soil
5354	-ve	-ve	-ve			
1902	-ve	+ve	-ve			
2477	+ve	+ve	-ve	Denmark	European site	In Situ filter/ Groundwater
2512	+ve	+ve	-ve			
3327	-ve	+ve	-ve			
3328	+ve	-ve	-ve			
5015	-ve	+ve	-ve	Alaska	Dry cleaner site	Groundwater
3688	+ve	+ve	-ve			
2463	+ve	+ve	-ve	California	Industrial Site	Groundwater
6356	-ve	-ve	-ve	-	KB-1	Bioaugmentation Culture
6368	-ve	-ve	-ve	-	ACT -3	Bioaugmentation Culture
6365	-ve	-ve	-ve	-	WBC-2	Bioaugmentation Culture
Total Positive	**15/25**	**18/25**	**0/25**	-	-	-

+ve = DNA sample produced PCR amplicon of correct size with designated primer set
-ve =DNA sample did not produce PCR amplicon of expected size with designated primer set

Reference:

Ahlgren N. A., Rocap G., and S. W. Chisholm. 2006. Measurement of *Prochlorococcus* ecotypes using real-time polymerase chain reaction reveals different abundances of genotypes with similar light physiologies *Environmental Microbiology* (2006) 8 (3), 441-454, 2006

Appendix E

Testing of Plasmid Based Internal Controls

E. coli containing a Mutated *Dhc* 16S rRNA Gene as a MIAC

Production of Modified *Dhc* 16S rRNA Gene

The IS containing target plasmid was constructed by modifying (mutating) the BAV1 16S rRNA gene (i.e., 5´-TGCA-3´ replaced by 5´-CCAT-3´) in the binding region of the TaqMan probe as described by Sen (2007). Plasmids carrying a single copy of the wild type (Wt) BAV1 16S rRNA gene (pBAV1) and the modified 16S rRNA gene IS plasmid (pIC) were used to produce standard curves in simplex qPCR assays using a single primer set (*Dhc* 1200F 5'-CTGGAGCTAATCCCCAAAGCT-3' and *Dhc* 1271R 5´-CAACTTCATGCAGGCGGG-3´) and TaqMan probes carrying different fluorophores specific for one of the two targets (*Dhc* 1240 Probe 56FAM-TCCTCAGTTCGGATTGCAGGCTGAA-BHQ3´ and IS Probe 5´VIC-TCCTCAGTTCGGATCCATGGCTGAA-BHQ3´). The TaqMan qPCR analysis was performed as described in Attachment 2 (Attachment B.5)

TaqMan MGB probes are short probes (as short as possible without being shorter than 13 nucleotides in length) that contain a short oligopeptide on their 3´ ends that possesses minor groove-binding (MGB) properties. MGB probes have more stable hybridization properties that modulate their interaction with the target sequence and typically result in increased melting temperatures (T_m) (Afonina, 1997; Kutyavin, 2000). Because of the shorter probe length compared to TaqMan probes without the MGB moiety, the greater difference in T_m for matched and mismatched probes allows for discrimination between DNA sequences with single base-pair substitutions and have been successfully used to increase probe specificity (Kutyavin, 2000).

Results

pBAV1 and the pIC plasmids were used as templates to obtain standard curves in simplex assays containing TaqMan MGB probes specific for each of the two targets (Wt MGB Probe 5´6FAM-TCGGATTGCAGGCTGA-MGBNFQ3´ and IS MGB Probe 5´VIC-TCGGATCCATGGCTGAA-MGBNFQ3´). Both assay systems used the same primer pair (*Dhc* 1200F 5´-CTGGAGCTAATCCCCAAAGCT-3´ and *Dhc* 1271R 5´CAACTTCATGCAGGCGGG-3´). The standard curves generated were linear over a range of 7 orders of magnitude for the pBAV1 plasmid and 8 orders of magnitude for the pIC plasmid (Figure 4.3A). The slopes of the linear regression lines were -3.86 and -3.44 for pBAV1 and the IS, respectively, with linear fits greater than 0.99 for each target (Figure 4.3A and Table 4.1). No cross-reactivity was observed for either of the MGB probes when used with the alternate target in simplex assays (data not shown).

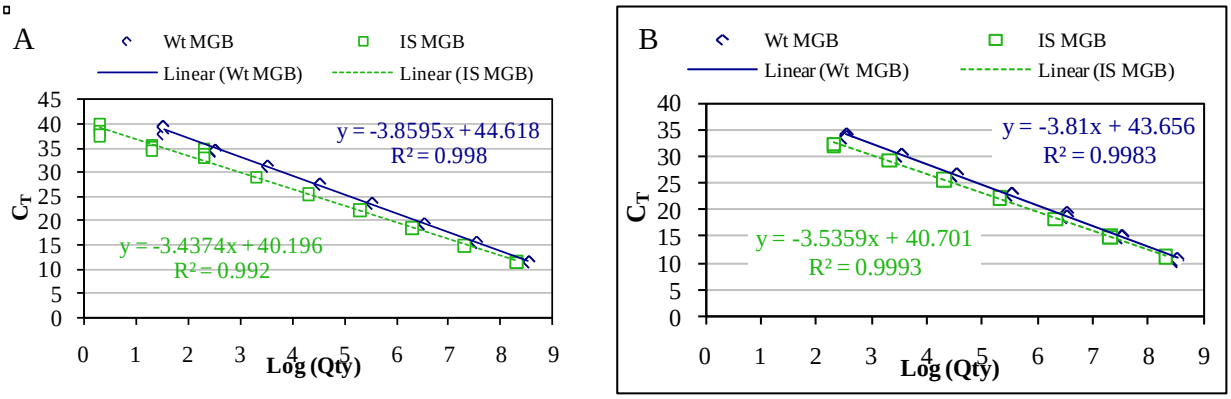


Figure E1: Simplex and Multiplex assays using MGB probes. A: Comparison of standard curves for pBAV1 and IS plasmids in a simplex qPCR assay using MGB probes. B: Comparison of standard curves for pBAV1 and IS plasmids in a multiplex qPCR assay using MGB probes. Standard curves were generated by 10-fold serial dilutions of each plasmid assayed in the ABI 7500 Fast system.

Table E1: Results of Regression Analysis of Standard Curves for qPCR Assays Under Varying Conditions

Parameters tested	Wild type Dhc MGB probe			IS MGB probe		
	Slope	y-intercept	R^2	Slope	y-intercept	R^2
Simplex assay	-3.86	44.618	0.9980	-3.44	40.196	0.9920
Multiplex assay	-5.93	65.440	0.9583	-3.76	45.447	0.9868
Multiplex assay (1/2 IS MGB probe)	**-3.81**	**43.656**	**0.9983**	**-3.54**	**40.701**	**0.9993**
50 nM IC MGB probe	-3.73	42.913	0.9978	-3.51	42.109	0.9995
High Primer Concentration (3X)	-3.99	44.542	0.9991	-3.42	36.830	0.9975
High Probe Concentration (2X)	-5.52	52.098	0.9757	-3.85	40.529	0.9885
30 second extension	-3.74	45.127	0.9976	-3.52	42.212	0.9974
62°C annealing temperature	-3.99	48.084	0.9951	-3.52	43.768	0.9905

The pBAV1 and pIC plasmids were then tested in a multiplex format to generate standard curves. The multiplex assay resulted in distorted standard curves for both the wild type target and the IS when a 1:1 ratio of wild type to IS probe was used in the assay (Table E1, lines 1 and 2). A variety of parameters were tested to improve amplification efficiency in the multiplex qPCR format. Table D1 shows the linear regression analysis of standard curves comparing a simplex qPCR assay to multiplex qPCR assays under varying conditions. The multiplex assay containing the IS MGB probe at a 125 nM concentration (Wt:IS probe ratio of 2:1) produced a standard curve (shown in bold in Table E1) comparable to that observed for the simplex assay indicating that this probe concentration was suitable for the multiplex assay.

The results shown in Figure E3 and Table E1 for each multiplex reaction included both target plasmids as templates at approximately the same concentration. Because both target sequences

are amplified simultaneously with the same primer set in the multiplex assay system, either amplification target could interfere with amplification of the other target. To determine the detection limits of the multiplex assay, a set concentration of the pIC plasmid was added to the multiplex reactions containing different concentrations of the pBAV1 plasmid. The results of a representative experiment are presented in Table E.2.

Table E2: Internal Standard Multiplex Dynamic Range Titration Experiment

Probe	10^6 pIC		10^5 pIC		10^4 pIC		10^3 pIC	
	Wt MGB	IS MGB	Wt MGB	IS MGB	Wt MGB	IS MGB	Wt MGB	IS MGB
10^8 pBav1	10.39	ND	10.57	ND	10.49	ND	9.78	ND
	10.53	ND	10.48	ND	10.52	ND	10.62	ND
10^7 pBav1		18.28	14.57	ND	14.65	ND	14.65	ND
	10.41	ND	14.6	ND	14.62	ND	14.69	ND
10^6 pBav1	18.31	18.53	18.28	26.4	18.26	ND	18.23	ND
	18.29	18.58	18.36	26.5	18.23	ND	18.32	ND
10^5 pBav1	28.62	18.37	22.96	22.44	22.82	31.62	22.57	ND
	27.85	18.35	22.97	22.31	22.67	30.75	22.56	ND
10^4 pBav1	ND	18.27	30.34	22.07	27.28	25.84	26.2	31.72
	ND	18.29	30.64	22.05	26.85	25.79	26.28	31.62
10^3 pBav1	ND	18.3	ND	22.03	34.94	25.42	30.07	29.01
	ND	18.35	ND	22.02	33.78	25.54	30.07	29.09
10^2 pBav1	ND	18.36	ND	22.16	ND	25.49	ND	33.31
	ND	18.31	ND	22.06	ND	25.48	ND	31.05
10^1 pBav1	ND	18.25	ND	22.12	ND	25.33	ND	29.53
	ND	18.23	ND	22.09	ND	25.34	ND	29.31
water	ND	18.22	ND	22.1	ND	25.36	ND	29.13
	ND	18.25	ND	22.15	ND	25.34	ND	29.01

ND = Not detected

= linear amplification

= the amplification curve is distorted

For each IC concentration tested, both target genes were accurately quantified only when the concentration of each of the templates was approximately equal (i.e., at a 1:1 ratio) (boxed results in Table 4.2). At pIC:pBAV1 ratios above 1, the multiplex assay yielded false negative results and failed to detect the wild type *Dhc* 16S rRNA gene or the amplification curve was distorted from linear. When the pIC:pBAV1 ratios were < 1 (i.e., the pBAV1 plasmid concentration was higher than the IC concentration) the IS was not detected (Table 4.2) or the amplification was distorted from linear. Similar results were observed when lower set concentrations of pIC were added to different concentrations of pBAV1 in multiplex qPCR reactions (data not shown).

To verify that false negative results were due to interference with the alternate template, multiplex assays were performed in which both templates were titrated against the other in the presence of only a single probe (Table E3). The pBAV1 wild type target was added to each reaction in 10-fold serial dilutions ranging from 108 to 100 target copies per reaction and the pIC target was added in the same reactions in an opposing gradient from 100 to 108 target copies per reaction. The results demonstrate that the lack of fluorescent signal is not due to the presence of a second probe in the multiplex assay, but rather due to a competition for primer during amplification of both templates. These data demonstrate that the more abundant template was amplified preferentially despite its sequence thus underestimating the true concentration of the low abundance template. To test if increasing the primer concentration would expand the dynamic range, 3-, 4- and 5-fold higher primer concentrations were tested in multiplex reactions. Although some improvement was observed with higher primer concentrations, the range of accurate quantification of both targets was extended only by a factor of 10 (data not shown).

Table E3: Template Interference Experiment

Wt MGB Probe		**IS MGB Probe**	
Log Qty	C_T	Log Qty	C_T
8.53	10.89	0.31	ND
8.53	10.67	0.31	ND
7.53	14.74	1.31	ND
7.53	14.76	1.31	ND
6.53	18.36	2.31	ND
6.53	18.44	2.31	ND
5.53	22.64	3.31	ND
5.53	22.6	3.31	ND
4.53	26.85	4.31	25.51
4.53	26.98	4.31	25.41
3.53	ND	5.31	21.86
3.53	ND	5.31	21.95
2.53	ND	6.31	20.45
2.53	ND	6.31	19.65
1.53	ND	7.31	15.14
1.53	ND	7.31	15.09
0.53	ND	8.31	11.36
0.53	ND	8.31	11.33

ND = Not detected

= linear amplification

Testing of an *E. coli* construct carrying the *luc* gene on plasmid (extrachromosomal element) as an internal control

The *E. coli* strain EPI300 containing a single copy pCC1 plasmid carrying the *luc* gene target (pCC1-Luc) was isolated for use as the MIAC. To explore if the *luc* gene in the transformed *E. coli* strain could be accurately quantified, the engineered strain was cultured in LB at 37ºC with shaking and 1 mL samples were taken during early-log phase (OD600 ~ 0.1), mid-log phase (OD600 ~ 1.0) and late-log phase (OD600 ~ 2.0). To mimic the treatment of environmental groundwater samples, culture samples were filtered to 0.2 µM Durapore GVWP filters and DNA from biomass collected on the filters was isolated using the MOBIO Powersoil DNA Extraction Kit. Gene copies for a single-copy *E. coli* gene (*dxs*) encoding the D-1-deoxyxylulose 5-phosphate synthase protein and the *luc* gene were determined by qPCR using SYBR Green and TaqMan chemistries, respectively, for each gene. Accurate quantification would result in a one-to-one ratio of *dxs* to *luc* gene copies.

Three independent experiments indicated that the ratio of *dxs* to *luc* ranged from 2-8 fold greater than the expected 1:1 ratio, suggesting that one or both of the assays gave inaccurate results. Differences in the detection chemistry (i.e., SYBR Green vs. TaqMan) could account for the differences observed, or, more likely, plasmid loss during the DNA extraction resulted in lower gene copies of the *luc* gene located on the plasmid compared to the *dxs* gene, which is located on the *E. coli* chromosome. Moreover, previous studies have reported that plasmid gene copy numbers show plasticity in fast-growing bacteria (i.e., the number of plasmids per cell are variable). These data indicated that the *E. coli-luc* construct carrying the *luc* gene on a plasmid does not meet the criteria for an IC.

Methods

qPCR assays were carried out as described in Attachment 2 (Appendix). For *Dhc*, the primer set *Dhc* 1200F 5'-CTGGAGCTAATCCCCAAAGCT-3' and *Dhc* 1271R 5´-CAACTTCATGCAGGCGGG-3´ with the TaqMan probe *Dhc* 1240 Probe 5'6FAM-TCCTCAGTTCGGATTGCAGGCTGAA-BHQ3' were used for qPCR analysis. For the firefly luciferase gene target, the primer set LucF 5'-TACAACACCCCAACATCTTCGA-3' and LucR 5'-GGAAGTTCACCGGCGTCAT-3' was used with the Luc Probe 5'VIC-CGGGCGTGGCAGGTCTTCCC-BHQ3' for qPCR analysis. Plasmid templates (pBAV1 and pGEM-luc) for generating standard curves were diluted as previously described and used as templates for the qPCR analysis. For the simplex reactions, the standard curves generated were linear over 6 and 7 orders of magnitude for *Dhc* 16S rRNA gene and luciferase gene targets, respectively (Figure E2).

Results

No cross-reactivity was observed for the *Dhc* probe with the luciferase-containing IC plasmid. A low level of cross-reactivity was observed for the luciferase probe in assays that contained the highest concentration (i.e., ~ 10^7 copies per reaction) of the pBAV1 plasmid. The measured fluorescence was near the detection limit of ~ 4 copies per reaction of the luciferase gene target, hence the low level of cross-reactivity is unlikely to impact the luciferase IS approach. Moreover, because *Dhc* cell titers above 10^6 per reaction are rarely found in environmental samples, cross reactivity can be avoided by adding a high titer of the luciferase gene to any experimental sample. The multiplex assay system generated results similar to those observed in the simplex reactions (Figure E2).

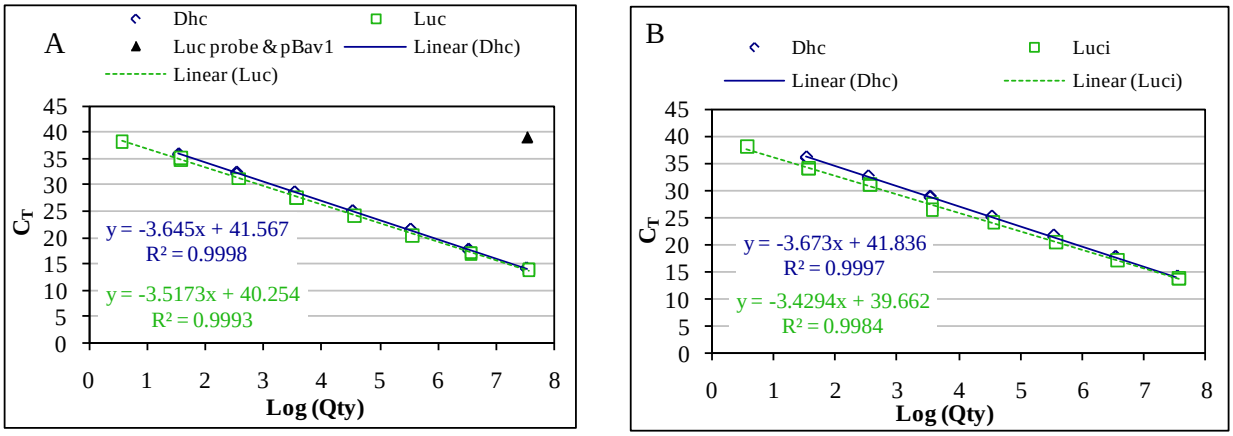


Figure E2: Simplex assays of *Dhc* and luciferase (Luc) and a Multiplex assay of both *Dhc* and luciferase. A: Comparison of standard curves for pBAV1 and pGEMLuc plasmids in a simplex qPCR assay. B: Comparison of standard curves for pBAV1 and Luc plasmids in a multiplex qPCR assay. Standard curves were generated by 10-fold serial dilutions of each plasmid assayed in the ABI 7500 Fast system.

To determine the dynamic range of the multiplex assay for both *Dhc* and luciferase targets the pBAV1 plasmid was titrated against a set concentration of the luciferase IS target. The results are shown in Table E4. At all concentrations of the IS plasmid tested (10^5 to 10^2 copies per reaction), no false negatives are observed for *Dhc* at all concentrations of *Dhc* tested (10^7-10^0 copies per reaction). The luciferase C_T values were slightly increased in the presence of ≥100-fold greater numbers of *Dhc* 16S rRNA gene targets in the qPCR reaction. Although false negatives of the luciferase gene were not observed, the fluorescence readings are distorted from expected IS values (compare to readings for IC plasmid added to water). To achieve accurate luciferase quantification, the IS target must be present at concentrations no more than 100-fold lower than the *Dhc* target gene copy number.

Table E4: *Dhc* and Luciferase Multiplex Dynamic Range Titration Experiment

	10^5 pGEM-luc		10^4 pGEM-luc		10^3 pGEM-luc		10^2 pGEM-luc	
	Dhc Probe	**Luc Probe**	**Dhc Probe**	**Luc Probe**	**Dhc Probe**	**Luc Probe**	**Dhc Probe**	**Luc Probe**
10^7 pBav1	14.10	21.53	14.45	26.16	14.06	32.20	14.16	37.79
	14.14	21.42	14.33	26.15	14.14	32.07	14.27	36.88
10^6 pBav1	17.65	20.88	17.93	24.86	17.66	30.00	17.63	35.53
	17.81	20.70	18.00	24.93	17.68	30.02	17.77	34.55
10^5 pBav1	21.40	20.42	21.53	24.19	21.43	28.62	21.45	33.04
	21.52	20.45	21.67	24.01	21.32	28.71	21.50	32.87
10^4 pBav1	24.97	20.50	24.96	24.04	25.05	28.03	24.54	33.71
	25.09	20.34	25.03	24.06	25.15	27.73	24.67	33.38
10^3 pBav1	28.46	20.36	28.44	24.04	28.40	27.53	28.10	31.98
	28.45	20.35	28.45	24.05	28.56	27.39	28.18	32.08
10^2 pBav1	32.28	20.29	32.30	24.11	32.14	27.44	32.07	31.53
	32.67	20.29	32.25	24.10	32.16	27.32	32.16	31.17
10^1 pBav1	35.45	20.38	35.72	24.08	35.67	27.40	35.39	31.12
	35.60	20.37	35.54	24.04	35.18	27.50	35.50	31.32
10^0 pBav1	38.21	20.41	ND	24.03	38.54	27.66	38.93	31.07
	ND	20.37	ND	23.99	37.48	27.56	37.80	31.40
water	ND	20.37	ND	23.99	ND	27.50	39.09	31.07
	ND	20.46	ND	23.98	ND	27.47	ND	31.12

ND = not detected

= linear amplification

= amplification is distorted

Appendix F

Production and Verification of a Chromosomally Modified *E. coli* for use as MIAC

Methods Used to Produce *E. coli-luc* MIAC

To generate an *E. coli* strain carrying the *luc* gene inserted on the chromosome, Georgia Institute of Technology (GT) took advantage of a transgene insertion vector pGRG36 (McKenzie and Craig, 2006) that utilizes the site-specific recombination machinery of the transposon Tn7. This vector facilitates a non-disruptive insertion of any gene into the site-specific Tn7 insertion site on the *E. coli* chromosome.

The pGRG36 plasmid carries a temperature sensitive origin of replication. Growth at permissive temperature (37ºC) after ligation of the transgene in the plasmid vector and transformation into *E. coli* induces the transposition machinery and transgene insertion into the chromosome. Subsequent growth at non-permissive temperature (42ºC) cures the *E. coli* cell of the delivery plasmid. The strain TOP10 attTn7::*luc* was constructed using this strategy. This strain contains the *luc* gene on the *E. coli* chromosome at the Tn7 attachment site. The chromosomal integration of the *luc* gene in the engineered strain was verified by screening for the presence of the *luc* gene using PCR and screening for the lack of the ampicillin resistance marker carried on the delivery vector. Further, PCR amplification using primers flanking the attTn7 site verified insertion of the *luc* gene at the attTn7 site (insertion of the *luc* gene results in a 3.3-kb amplicon versus a 700-bp amplicon without the insertion).

Growth of *E. coli-luc* Internal Standard

The *E. coli* strain TOP10 attTn7::*luc* containing the *luc* gene was cultured in LB medium at 37ºC with shaking. Growth was monitored by optical density measurements and the culture sampled at discrete time-points during the logarithmic and stationary growth phases for qPCR and plated on LB agar for determining colony-forming units (CFUs). The results obtained to date indicated that early stationary phase samples gave similar counts (i.e., were present at 1:1 ratios) when assayed by qPCR for both the *E. coli* marker gene *dxs* or the *luc* gene, and these numbers corresponded to those obtained by CFU counts. At early log and mid-log phases of growth, CFU counts differed from the qPCR results despite the fact that qPCR results for *dxs* and *luc* yielded comparable numbers. The *E. coli-luc* cells, the *luc* plasmid construct and associated methods have been distributed to participating labs.

Growth studies with this *E. coli* construct indicated that overnight cultures (~15 hours) incubated at 37ºC with shaking at 220 rpm yielded sufficient cells that could be quantified with independent methods. GT verified that the cell numbers determined by qPCR analysis of the chromosomally located single-copy *luc* gene and the *dxs* gene match microscopic cell counts and CFUs. When this *E. coli* construct is grown in the manner indicated, the enumeration of *E. coli* cells was reproducible and the values obtained with the different methods did not vary by no more than 3-fold. These efforts have generated understanding about the proper preparation of the MIS to ensure accurate and precise quantification before testing the IC method for estimating biomarker loss during the analysis of *Dhc* cultures.

Specificity Testing of *E. coli* (impact on *Dhc* quantification)

Tests to determine if the addition of the *E. coli-luc* construct to groundwater interferes with the quantification of *Dhc* biomarkers using a SYBR Green qPCR assay were performed by spiking varying concentrations of *E. coli-luc* genomic DNA into qPCR reactions (in triplicate) with *Dhc* genomic DNA (1.6×10^4 16S rRNA gene copies) (Table M1). The data indicated no apparent interaction of the *E. coli-luc* genomic DNA on *Dhc* biomarker gene quantification based on the observation that the *Dhc* gene copy enumeration was virtually identical irrespective of the spike concentration of *E. coli* DNA. This suggests that spiking cells of the *E. coli-luc* construct into groundwater or DNA prior to DNA extraction would not affect the quantification of *Dhc* biomarkers.

Table F1: Enumeration of *Dhc* 16S rRNA genes in the presence of *E. coli-luc* genomic DNA at varying concentrations

***E. coli-luc* genomic DNA Gene-copies spiked/reaction**	**Recovery of 1.6×10^4 gene copies/reaction spike of *Dhc* positive groundwater DNA**	**Standard deviation of *Dhc* enumeration**
1×10^6	1.7E+04	5.9E+03
1×10^5	1.4E+04	1.4E+03
1×10^4	1.8E+04	2.0E+03
1×10^3	1.6E+04	2.7E+03
0	1.6E+04	2.3E+03

Appendix G

Impact of Different Spiking Protocols for MIAC

Impact of MIAC spiking

Is the recovery of the MIAC as quantified by real-time PCR affected by the DNA extraction stage at which the MIAC is incorporated? To answer this question the recovery of the microbial internal standard (MIAC) in three different scenarios was compared:

1) The MIAC is added to the groundwater (GW) sample in the bottle/container.

2) The MIAC is preloaded on the Sterivex filter prior to filtration of the GW sample.

3) The MIAC is directly added into the DNA extraction tube together with the cut Sterivex filter. GW is thus filtered through the Sterivex filter without the MIAC.

In addition, to testing the recovery of the MIAC under the three above-mentioned scenarios, the MIAC recovery was tested for each treatment/scenario in a high or low *Dehalococcoides* (*Dhc*) background.

Materials and Methods

Artificial groundwater was prepared according to Middeldorp et al. (1998). A total of 40 L was prepared in two plastic carboys (2 carboys of 20 L each). KB-1, a mixed microbial consortium capable of complete dechlorination of TCE to non-toxic ethene was used as donor *Dehalococcoides* culture. An aliquot of KB-1 culture was used to prepare artificial GW with high *Dhc* titers. An aliquot of the later (high *Dhc* GW) was used to prepare the low *Dhc* titer artificial GW (see details below). Two controls per treatment consisting of just artificial GW were also prepared. To make the high *Dhc* titer artificial GW, 170 mL of donor KB-1 culture (approx. 10^{11} *Dhc* gene copies L^{-1}) were added to one of the carboys (1 in 100 dilution). To make the low *Dhc*, titer artificial groundwater 17mL of high *Dhc* artificial GW were spiked into the other carboy (1 in 1,000 dilution).

The MIAC (*E. coli* cells containing the luciferase gene on its chromosome) for spiking were grown in LB medium for 17 hours at 37°C and 180 rpm. At the time of harvest cell density was approx. 10^9 cells mL^{-1}. Quantification of the concentration of MIAC in the culture was achieved by plating. The average calculated concentration of the MIAC was $8.45x10^8$ cells mL^{-1} of culture. To spike approx. $1x10^6$ cells of culture and by defect of luciferase gene copies per sample, 1mL of MIAC undiluted culture was 10-fold serially diluted to 10^{-2} in PBS buffer. 118 µL of 1-in-100 diluted MIAC were used for spiking. As mentioned above, spiking of the MIAC was done in three different ways (treatments 1, 2 and 3). The nomenclature use for sample identification was as follows:

H = high *Dhc* background;

L = low *Dhc* background;

C = no *Dhc*

1 = treatment 1 (MIAC spiked into the GW);

2 = treatment 2 (pre-loaded MIAC); and

3 = treatment 3 (MIAC added to DNA tube).

For treatment 1 (MIAC spiked into the GW) the MIAC was spiked inside each bottle, mixed and the artificial GW was then filtered. For treatment 2 (pre-loaded MIAC), 118 µL of PBS-diluted MIAC were transferred into 10mL of PBS buffer and Vortexed for good homogenization. Subsequently, the 10mL of buffer containing the MIAC were filtered through Sterivex for loading of the MIAC. After this, samples were filtered through MIAC-preloaded filters. Treatment 3 bottles (MIAC added in DNA extraction tube) were first filtered through Sterivex without addition of MIAC at this stage. Here, 118 µL of PBS diluted MIAC (see above) were spiked into the DNA extraction tubes. All treatments consisted of 5 high *Dhc* background, 5 Low *Dhc* background and 2 Control (just GW, no *Dhc)* samples. Sterivex filters were recovered after removal of the filter casing using a tube cutter. DNA extraction was carried out following the manufacturer's instructions (Ultra Clean® Soil DNA isolation kit, Mo Bio Laboratories Inc.). DNA was eluted from the DNA binding column with 50 µL of UV-treated Ultrapure water (UltraPure™ Distilled Water, Invitrogen). Abundance of *Dehalococcoides* and the MIAC in samples and controls was quantified via real-time PCR targeting the 16SrRNA gene of *Dehalococcoides* and the luciferase gene, respectively, using a CFX96 real-time PCR detection system (Biorad).

Results and Discussion

Our data showed that *Dehalococcoides* abundance in the groundwater had no statistically significant effect on the recovery of the MIAC (Figure 1). Nonetheless, the recovery of the MIAC was found to depend on when the MIAC was added during sample processing. The following results were obtained:

- MIAC added to groundwater sample (treatment 1):
- Mean recovery= 28.4%+/-2.6 (high *Dhc*) and 28.4%+/-7.6 (low *Dhc*)
- MIAC added to filter prior to filtration of sample (treatment 2):
- Mean recovery = 6.1%+/-2.1 (high *Dhc*) and 10.0%+/-8.5 (low *Dhc*)
- MIAC added to DNA extraction tube (treatment 3):
- Mean recovery = 76.1%+/-20 (high *Dhc*) and 52.7%+/-12 (low *Dhc*)

Significant differences were observed between treatments. The recovery efficiency of the MIAC in descending order was: MIAC added to Extraction tube > MIAC added to GW > Preloaded MIAC.

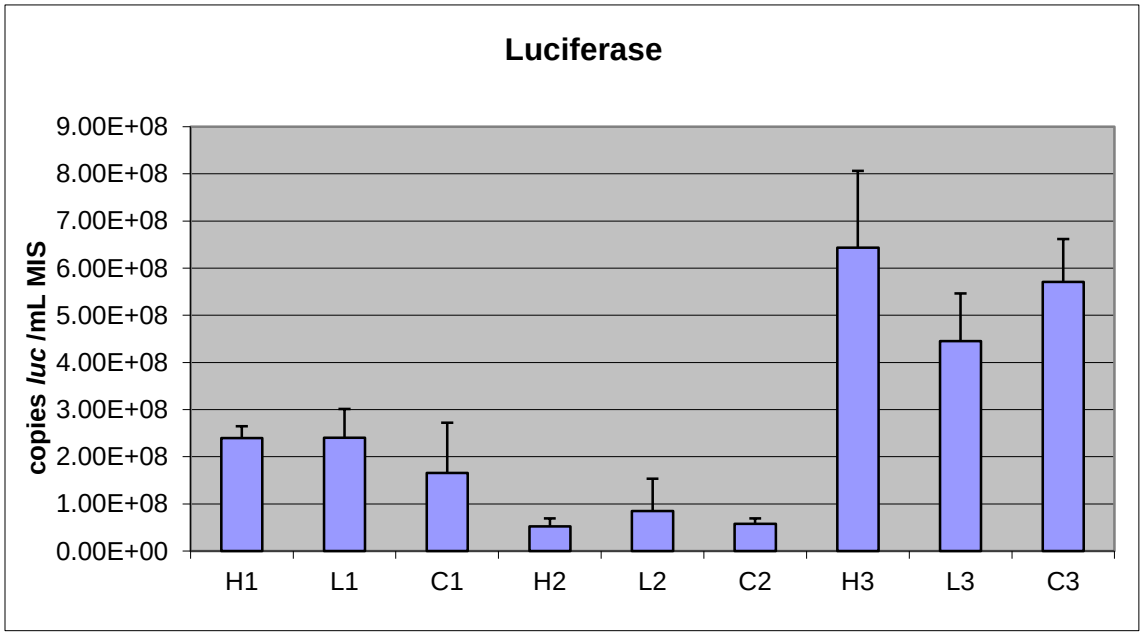


Figure G1: Mean values of luciferase gene copy numbers per mL of MIAC culture (bars) and standard deviations (errors) (n=5 for H and L samples, and n=2 for controls).

Note that errors for controls represent the range.

H = high *Dhc* background; L = low *Dhc* background; C= no *Dhc*

1 = treatment 1 (MIAC spiked into the GW); 2 = treatment 2 (pre-loaded MIAC); 3 = treatment 3 (MIAC added to DNA extraction tube)

Conclusions and implications for future research implementation

These results indicate that pre-loading the filter is not a good idea. MIAC should be added to Groundwater just prior to filtering, and if that is not possible (for example for field-filtered samples), into the DNA extraction tube.

Reference

Middeldorp et al. (1998) *Water Science Technology* Vol 37. No. 8, pp. 105-110.

Appendix H

Enumeration Data for MIAC

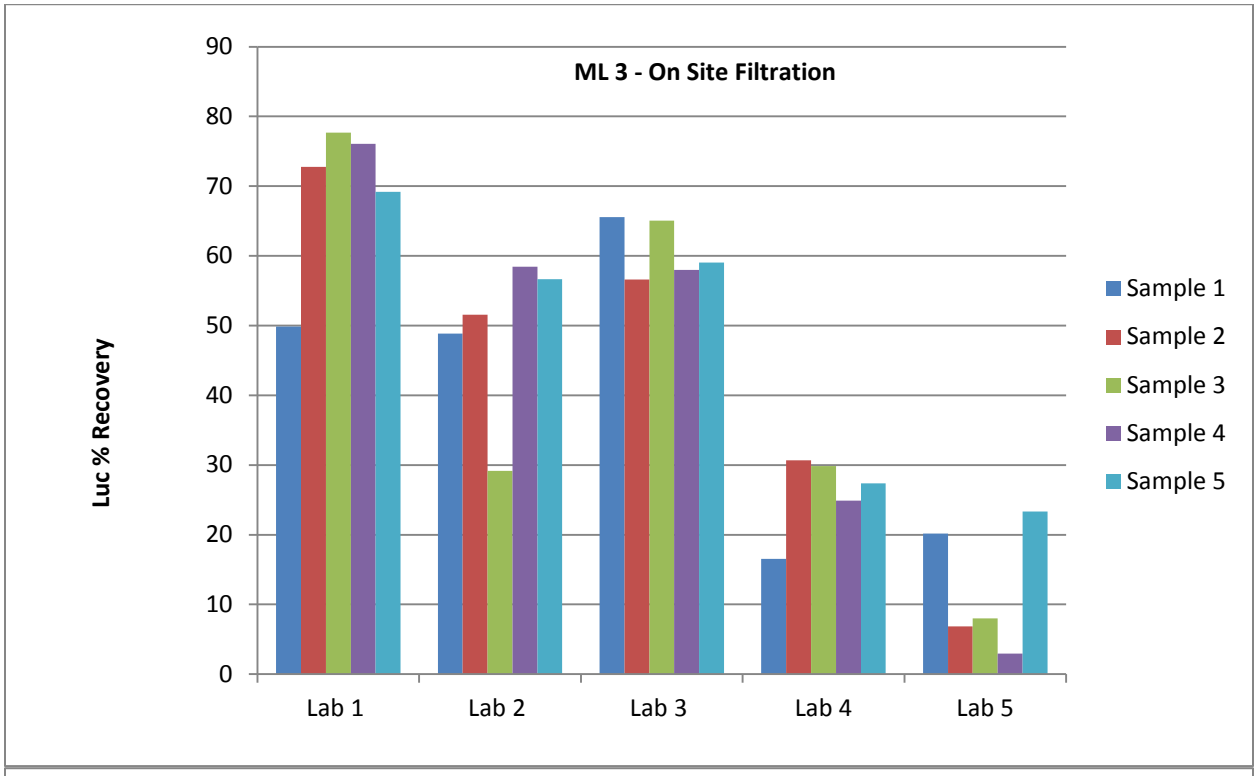
Results

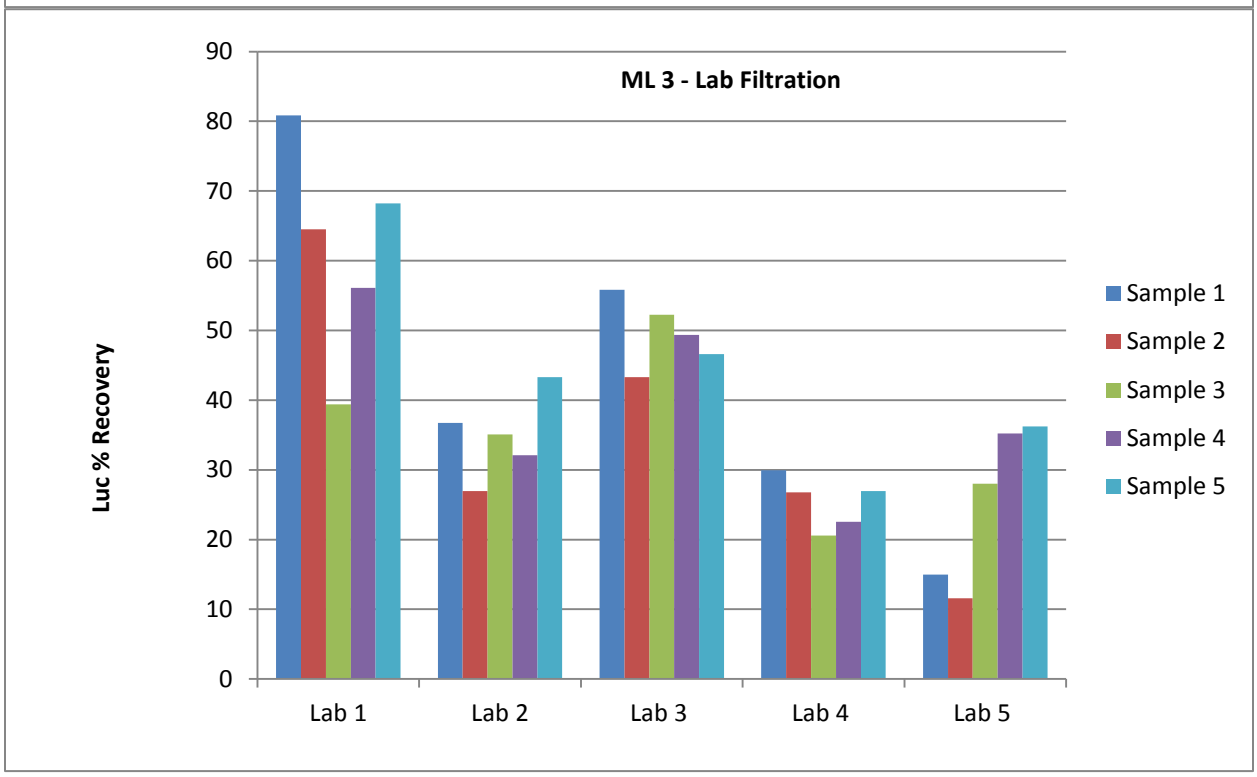
Table H1: Recovery of MIAC in Simulated Groundwater (Detailed data used in in Figure 6-2)

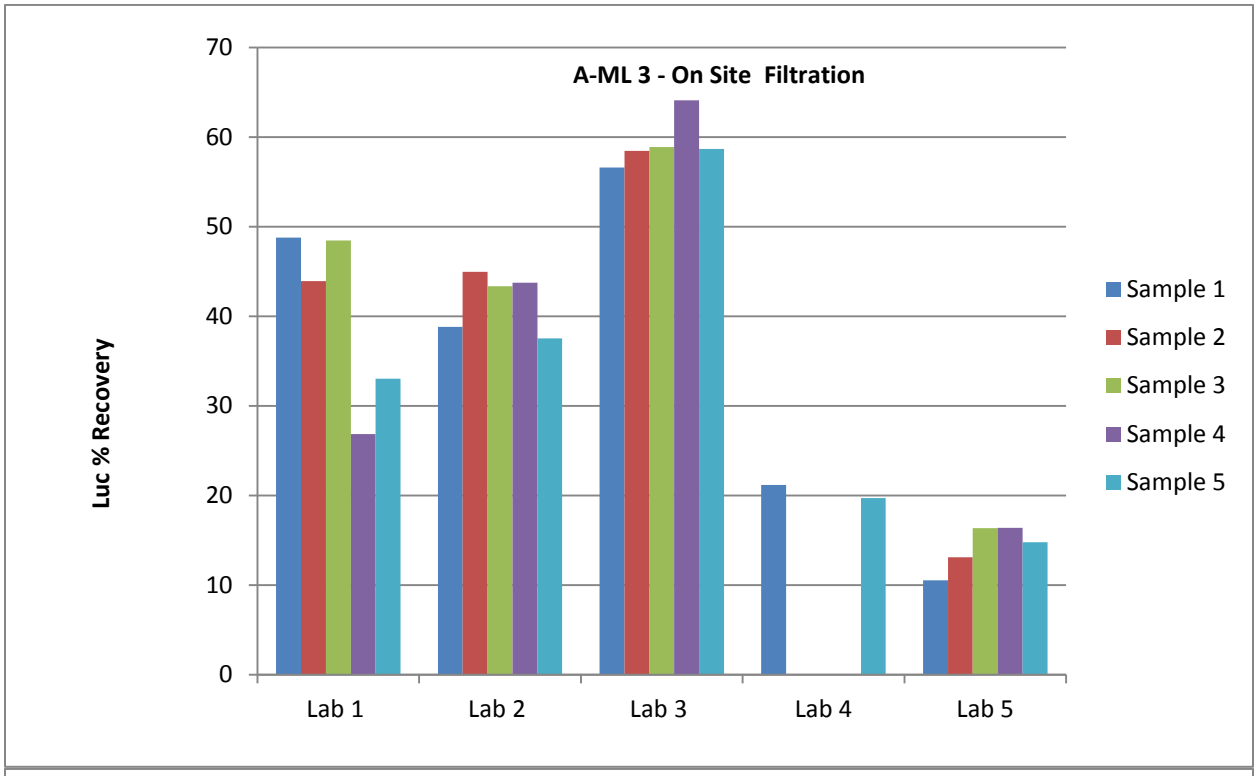
	Lab	Avg Dhc /mL Groundwater	Stdev Dhc/mL GW	luc percent recovery	Stdev luc percent recovery	Average luc/mL GW	Stdev luc/mL GW	Spike concentration per mL template
Neg (ave, n=2)	N-GT	3.8E+00		5.8%		2.6E+02		4.5E+03
	N-MI			9.1%		1.6E+02		1.8E+03
	N-SRNL-PS	3.6E+02		33.3%		1.4E+03		4.2E+03
	N-UTor	4.9E+01		3.7%		7.4E+01		2.0E+03
	N-SIREM	1.5E+01		17.0%		2.5E+02		1.5E+03
Low (ave, n=5)	L-GT	9.7E+02	3.5E+02	5.6%	0.7%	4.5E+02	1.9E+02	4.5E+03
	L-MI	1.2E+03	7.8E+01	9.5%	0.4%	1.5E+02	1.1E+01	1.6E+03
	L-SRNL-PS	2.4E+02	2.0E+02	10.7%	4.5%	4.5E+02	1.9E+02	4.2E+03
	L-UTor	3.7E+03	2.4E+03	4.4%	3.4%	8.7E+01	6.9E+01	2.0E+03
	L-SIREM	1.2E+03	3.2E+02	28.1%	7.2%	4.1E+02	1.1E+02	1.5E+03
High (ave, n=5)	H-GT	1.0E+06	1.9E+05	6.2%	2.1%	2.8E+02	9.5E+01	4.5E+03
	H-MI	1.2E+06	6.2E+04	9.3%	0.3%	1.5E+02	5.4E+00	1.6E+03
	H-SRNL-PS	4.7E+05	7.9E+04	12.4%	3.5%	5.2E+02	1.5E+02	4.2E+03
	H-UTor	2.3E+06	5.3E+05	3.1%	1.9%	6.3E+01	3.7E+01	2.0E+03
	H-SIREM	4.2E+06	7.5E+05	34.0%	7.7%	5.0E+02	1.1E+02	1.5E+03
Average Luc Recovery (n=12)	**GT**			**5.9%**	**1.4%**			
	MI			**9.4%**	**0.5%**			
	SRNL-PS			**15.2%**	**10.8%**			
	UTor			**3.7%**	**2.5%**			
	SIREM			**28.7%**	**8.9%**			

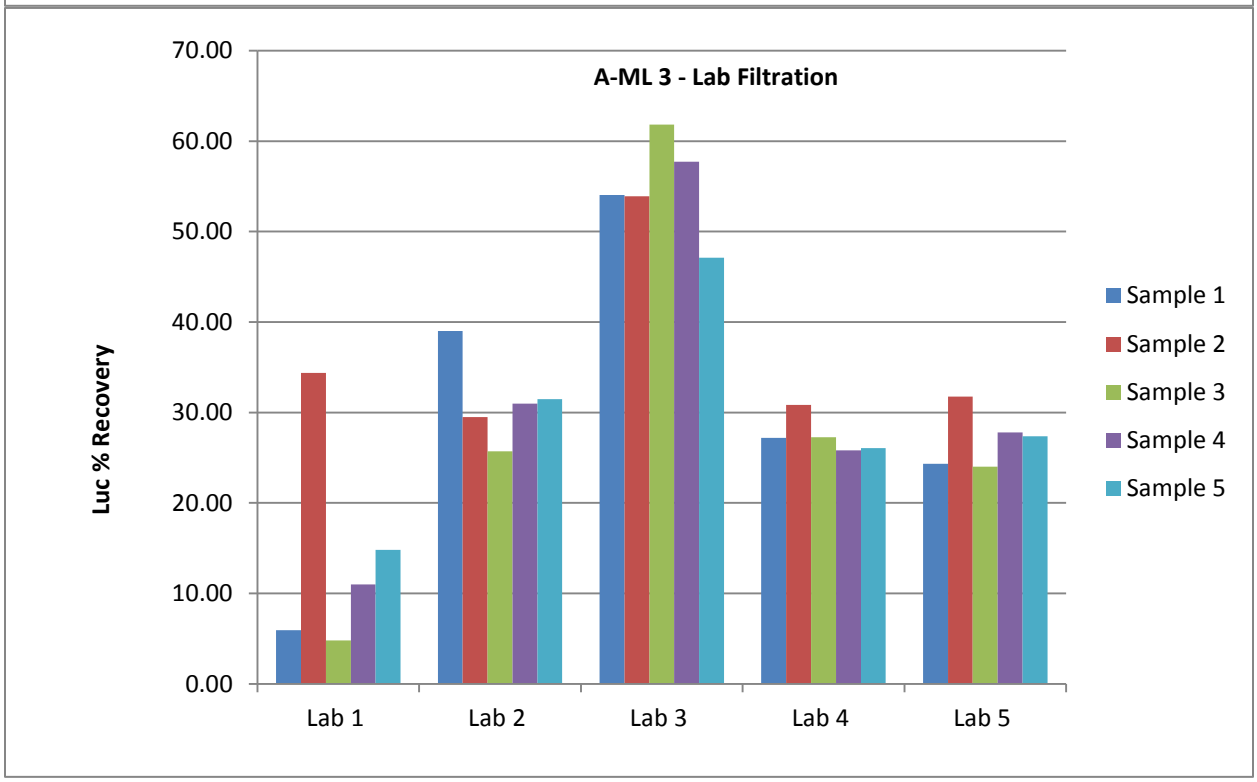
zero (not written for Log graph error)
or n/a with fewer than 3 samples

Detailed Data for Recovery of Luc in Groundwater Samples All Reps and Wells (Also see Figure 6-2 In main report for means of ML-3 data)






A-ML 3 - On Site Filtration
Luc % Recovery
70
60
50
40
30
20
10
0
Lab 1
Lab 2
Lab 3
Lab 4
Lab 5
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5


A-ML 3 - Lab Filtration
Luc % Recovery
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Lab 1
Lab 2
Lab 3
Lab 4
Lab 5
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5

Appendix I

Methods Used to Produce Frozen Aliquots and Quantify *E. coli luc* MIAC by Various Methods

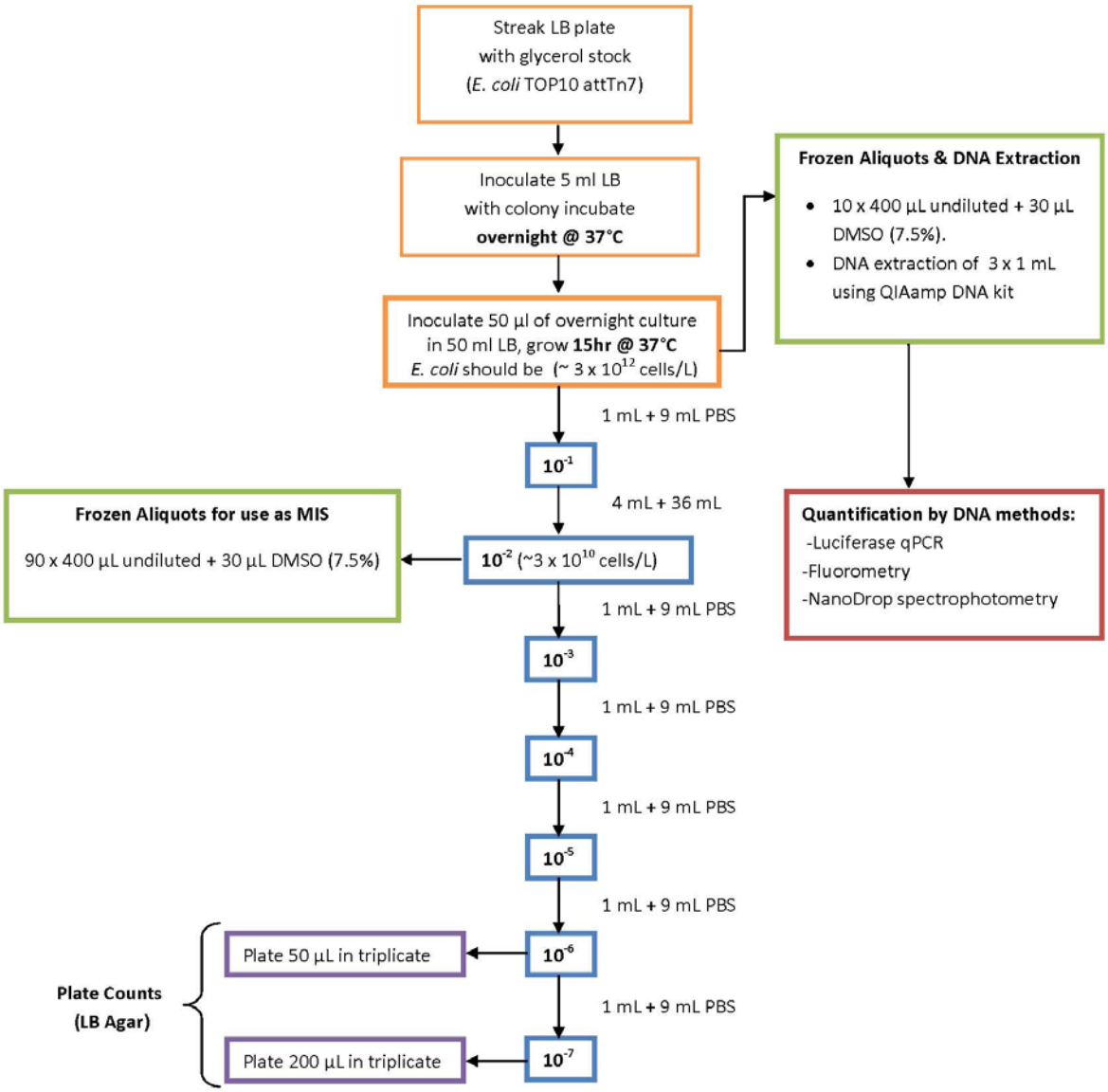


Figure I1: Overview of process for preparation of DMSO frozen stocks of the MIAC and enumeration by plate counts and DNA-based methods

Cell Growth

MIAC cells were grown according to the protocol GROWTH OF THE E. COLI TOP10 ATTTN7::LUC STRAIN TO USE AS AN INTERNAL STANDARD outlined in Attachment 3

Plate Counts

The MIAC culture was enumerated by plating 50 µL of 10^{-6} dilution and 200 µL of 10^{-7} dilution in triplicate on Lysogeny Broth (LB) plates using a sterile pipette and spreader and then

incubated at 37°C overnight. Colonies were counted manually and the colony forming units (CFU) per mL were calculated for each of the 10^{-6} plates and the 10^{-7} plates. An average was taken of the six CFU per mL values and used as the titer for the MIAC culture (i.e., cells per mL).

Genomic DNA Extraction from *E .coli* (MIAC) cultures

DNA was extracted from seven aliquots of frozen 100 fold diluted MIS culture suspension using the QiaAmp DNA Mini Kit (Qiagen). The manufacturer's protocol was followed with the following amendments: (a) approximately 400 µL of culture was extracted for each sample; (b) a 1.5 hour lysis was performed; (c) a 5 minute incubation of Elution Buffer AE (provided with the Kit) in the column instead of 1 minute; and (d) 3 elutions with 200 µL Buffer AE as opposed to 2 elutions, The final elution volume was 600 µL. The goal of this protocol was to maximize extraction efficiency.

Quantification of MIAC Genomic DNA Using Fluorometry

Fluorometry on extracted DNA was performed using the Quant-iT PicroGreen dsDNA Assay Kit (Invitrogen/Molecular Probes Eugene, OR) for duplicate genomic DNA samples, which were extracted with the QiaAmp DNA Mini Kit (Qiagen) from frozen 1/100 MIAC aliquots. Genomic DNA (10 µL) was quantified according to the manufacturer's protocol with the following amendments: (a) sample DNA was diluted in 340 µL in 1X Tris (10 mM) -EDTA (1 mM) buffer to a final volume of 350 µL; and (b) 350 µL of 1/200 diluted PicoGreen® was added to each sample for a final volume of 700 µL. Each diluted sample (200 µL) was loaded in triplicate onto a 96 well plate. Fluorescence was measured using a MyiQ Single Colour Real-Time PCR Detection System (BioRad, Mississauga, ON) and DNA concentrations were determined using the Lambda DNA stock (100 µg mL^{-1}) provided with the kit which was used to prepare 2 µg mL^{-1} and 50 ng mL^{-1} working stocks to prepare the 9-point standard curve as indicated in Table I1.

Table I1: Preparation of Lambda DNA Standard Curve for Quantifying DNA by Fluorometry

Tube #	Final λ DNA Concentration ng/ml	λ Working Stock Concentration	DNA Volume (µL)	TE Buffer Volume (µL)	PicoGreen™ Volume of Diluted Reagent (µL)	Total Volume (µL)
1	1000	2 µg/mL	700	0	700	1400
2	500	2 µg/mL	350	350	700	1400
3	200	2 µg/mL	140	560	700	1400
4	100	2 µg/mL	70	630	700	1400
5	50	2 µg/mL	35	665	700	1400
6	20	50 ng/mL	560	140	700	1400
7	10	50 ng/mL	280	420	700	1400
8	5	50 ng/mL	140	560	700	1400
9	1	50 ng/mL	28	672	700	1400
10	0	NA	0	700	700	1400

1. Sample calculation for enumerating MIAC using total genomic DNA

Total genomic DNA= 215 ng/ml

Molecular weight of E. coli genome=average 660 Daltons/base pair*4.2 million base pairs=2.7 E+09 Daltons

Moles of *E. coli* DNA = grams/molecular weight = 2.1E-07 g/2.7E+09 =7.95E-17 moles

Copies of *E. coli* genome= moles * Avogadro's number= 7.95E-17*6.023E+23= 4.8E+07 *E. coli* mL^{-1}

Quantification of the MIAC using Luciferase Gene Targeted qPCR

MIAC DNA previously quantified by fluorometry was used to generate a qPCR standard curve. Mastermix was prepared as follows: 12.5 µL SYBR Green Supermix (BioRad, Mississauga, ON) 8.7 µL DNA free water, 1.8 µL *luc* forward/reverse (f/r) primer set (Johnson et al., 2005) (10 pmol μL^{-1}) and 2.0 µL template DNA.

A 7-point standard curve was produced in duplicate using an iQ5 qPCR machine with a Multi Colour PCR Detection System (BioRad, Mississauga, ON).

qPCR thermocycling parameters were as follows: 5 minutes at 94°C; 1 minute at 94°C, 1 minute at 60°C, 2 minutes at 72°C (40 cycles); 8 minutes final extension at 72°C.

Appendix J

Beadbeating Optimization Experiment-Data

Table J1: Comparison of total DNA and *Dhc* enumeration from groundwater samples extracted using Vortex method (10 minute) or bead beater method (2 minute).

Groundwater Sample ID	*Dhc* 16S rRNA Gene copies/L		Total DNA Extracted (ng/L)		Total DNA Ratio Vortex: Bead beater	*Dhc* copies/L Ratio Vortex: Bead beater
	Vortex Method	Bead beater Method	Vortex Method	Bead beater Method		
Site A Sample 1	ND	ND	6,855	3,540	194%	NA
Site A Sample 2	ND	ND	14,925	16,628	90%	NA
Site C Sample 1	ND	ND	189	333	57%	NA
Site D Sample 1	ND	ND	438	448	98%	NA
Site D Sample 2	ND	ND	733	685	107%	NA
Site D Sample 3	ND	ND	550	343	160%	NA
Site D Sample 4	ND	ND	228	255	89%	NA
Site D Sample 5	ND	ND	253	655	39%	NA
Site D Sample 6	1.15E+05	1.11E+06	635	1,060	60%	10%
Site D Sample 7	6.33E+05	2.26E+06	470	930	51%	28%
Site E Sample 1	2.88E+07	1.06E+08	2,828	6,071	47%	27%
Site E Sample 2	5.11E+04	6.57E+04	1,193	1,260	95%	78%
Site E Sample 3	2.12E+07	1.01E+08	1,355	1,433	95%	21%
Site E Sample 4	1.62E+06	3.07E+06	3,093	4,775	65%	53%
Site E Sample 5	3.21E+05	3.07E+06	1,175	3,023	39%	10%
Site E Sample 6	9.05E+04	2.06E+05	2,770	3,085	90%	44%
Site E Sample 7	2.83E+06	8.63E+06	1,500	1,518	99%	33%
Site E Sample 8	ND	ND	1,048	1,378	76%	NA
Site E Sample 9	1.38E+05	9.04E+05	1,678	4,405	38%	15%
				Total	**82%**	**32%**

Notes:
ID - identifier
NA – not applicable
ND – non-detect

Appendix K

Protocols for TaqMan and SYBR Green Comparisons, and Multiplex TaqMan Approach for Internal Standard Analysis

Simplex Quantitative PCR for *Dhc* using TaqMan Detection Chemistry

Quantitative PCR (qPCR) was performed using a TaqMan-based qPCR chemistry with the Applied Biosystems 7500 Fast Real-Time PCR system. For quantification of Total *Dehalococcoides* the primer set *Dhc*1200F 5'-CTGGAGCTAATCCCCAAAGCT-3' and *Dhc*1271R 5'-CAACTTCATGCAGGCGGG-3' that targets the 16S rRNA gene of members of the *Dehalococcoides* group was used with the TaqMan probe *Dhc*1240 5'*FAM*-TCCTCAGTTCGGATTGCAGGCTGAA-*BHQ*-3'. Each 20 µl reaction contains the reagents as shown in Table K1. Each DNA sample (undiluted and a 1:10 dilution of the DNA) was assayed in triplicate.

Table K1: Simplex qPCR Reaction Mix

Component	Stock solution (µM)	Final concentration (nM)	µl per 20 µl Reaction Mixture
Water	-	-	6.95
Buffer [a]	2x	1x	10
Probe [b]	100	250	0.05
fwd Primer [b]	10	250	0.5
rev Primer [b]	10	250	0.5
DNA	variable	variable	2
		total volume	20

Notes:
[a] The 2x TaqMan PCR master mix was purchased from ABI (Applied Biosystems cat# 4304437). This mix includes Taq DNA polymerase, deoxynucleoside triphosphates, and $MgCl_2$.
[b] Primers and probe were ordered from ABI or IDT (www.idtdna.com).

Thermocycler parameters were as follows: 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C in the standard 7500 mode. Data collection and analysis was performed with the Applied Biosystems Sequence Detection System v1.3.1 software. For absolute quantification, a standard curve (the log of the 16S rRNA gene copy number versus the threshold fluorescence (C_T)) was obtained using serial dilutions of spectrophotometrically quantified plasmid DNA carrying a cloned 16S rRNA gene of *Dehalococcoides* sp. strain BAV1. *Dehalococcoides* gene copy numbers were calculated using the estimate of an average molecular weight of 660 for a base pair of dsDNA, one 16S rRNA gene per plasmid and a plasmid size of 5.4 x 10^3 base pairs. The equation shown below was used to calculate the number of plasmid-borne *Dehalococcoides*-derived 16S rRNA gene copies per 20µl reaction.

$$16S\ rRNA\ gene\ copies/rxn = \frac{(2\mu l/rxn) \times DNA\ (ng/\mu l) \times 6.023 \times 10^{23}}{(5.4 \times 10^3 \times 660) \times 10^9}$$

Standard curves were obtained by assaying 10-fold serial dilutions of plasmid DNA containing the target of interest at gene copies ranging from approximately 10^8gene copies to 10^0 gene copies per reaction. The SDS Software 1.3.1 calculates the *Dehalococcoides*-derived 16S rRNA gene copies of unknown samples by interpolating values from the standard curve.

Multiplex qPCR Assays using TaqMan Detection Chemistry

The reactions were essentially carried out as described above for simplex assays. Primer sets used for each reaction are described in the text. For the pIC containing multiplex reactions, the assay differed only by the addition of the pIC probe as described in the main text unless otherwise noted. Each template was added in a volume of 2 µl and the volume of water added to each reaction changed accordingly for a final reaction volume of 20µl. For the *Dhc* and luciferase multiplex reaction, the reaction mix was modified as shown below:

Table K2: Multiplex *Dhc* and Luciferase qPCR Reaction Mix

Component	Stock Solution (µM)	Final Concentration (nM)	µl per 20 µl Reaction Mixture
Water	-	-	variable
Buffer [a]	2x	1x	10
Dhc Probe [b]	100	250	0.05
*Dhc*F Primer [b]	10	250	0.5
*Dhc*R Primer [b]	10	250	0.5
Luc Probe [b]	100	250	0.05
LucF Primer [b]	10	250	0.5
LucR Primer [b]	10	250	0.5
DNA	variable	variable	variable
		Total volume	20

Notes:
[a] The 2x TaqMan PCR master mix was purchased from ABI (Applied Biosystems cat# 4304437). This mix includes Taq DNA polymerase, deoxynucleoside triphosphates, and $MgCl_2$.
[b] Primers and probe were ordered from ABI or IDT (www.idtdna.com).

Reactions were run and gene copies for each plasmid were calculated as described above.

Simplex Quantitative PCR using SYBR Green Detection Chemistry

For quantification of Total *Dehalococcoides* with SYBR Green detection chemistry in the 7500 Fast Real-Time PCR system (Applied Biosystems), the primer set *Dhc*1200F 5'-CTGGAGCTAATCCCCAAAGCT-3' and *Dhc*1271R 5'-CAACTTCATGCAGGCGGG-3' that

targets the 16S rRNA gene of members of the *Dehalococcoides* group is used. Each 20 µl reaction contains the reagents as shown in Table K3. Each DNA sample (undiluted and a 1:10 dilution of the DNA) is assayed in triplicate.

Table K3: SYBR Green qPCR Reaction Mix

Component	Stock Solution (µM)	Final Conc. (nM)	µl per 20 µl Reaction Mixture
Water	-	-	6.8
Buffer [a]	2x	1x	10
fwd Primer [b]	10	300	0.6
rev Primer [b]	10	300	0.6
DNA	variable	variable	2
		total volume	20

Notes:
[a] The 2x Power SYBR Green master mix is from Applied Biosystems (Applied Biosystems cat# 4367659). This mix includes Taq DNA polymerase, deoxynucleoside triphosphates, and $MgCl_2$.
[b] Primers were ordered from ABI or IDT (www.idtdna.com).

Thermocycler parameters are as follows: 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C and a dissociation stage of 1 cycle for 15 sec at 95°C, 1 min at 60°C and 15 sec at 95°C in the standard 7500 mode. Data collection and analysis is performed with the Applied Biosystems Sequence Detection System v1.3.1 software as described above for quantification using the TaqMan based qPCR detection chemistry.

Appendix L

Impact of PCR Primer Sets and Topological Form of DNA used for qPCR Calibration

Impact of Primer Set and Calibrator

Real-time PCR is the method of choice for the absolute quantification of gene targets in different matrixes including environmental samples. One potential source of interlab-variability for the quantification of the 16SrRNA gene of *Dhc* in groundwater is the choice of primers-a total of three different primer sets are used by the various participating groups. Differences in primer performance can be caused by various factors including: a) specificity to target, b) amplicon size and c) region of the 16Sr RNA gene targeted. Furthermore, amplification of DNA standards for calibration might be affected by the interaction standard-primer set causing additional sources of variation. Commonly, plasmid DNA containing the target gene of a reference strain is used for calibration, but other sources of DNA (e.g. PCR product or genomic DNA) might be used as well. Recent studies (Chen *et al.*, 2007 and Hou *et al.*, 2010) suggest that different topological forms of plasmid DNA (supercoiled, nicked circular and linear) can affect qPCR enumeration (under- or over-estimation) of gene targets.

To better understand how the choice of primer set might affect the quantification of *Dhc* in groundwater using real-time PCR, an experiment was conducted in which various environmental samples harboring *Dhc* were tested against the three primer sets used by the various groups; different sources of DNA harboring the 16SrRNA gene of *Dhc* for calibration were also tested. These included: i) supercoiled plasmid DNA, ii) nicked DNA (plasmid DNA treated with the restriction enzyme NtBst I), iii) linearized plasmid DNA (plasmid DNA linearized with the restriction enzyme Xba I), iv) genomic DNA (*E. coli* genomic DNA, in which the 16SrRNA gene of *Dhc* was cloned), v) PCR amplified DNA directly purified from the reaction tube and vi) PCR amplified DNA purified from an agarose gel.

Materials and Methods

The different sources of 16SrRNA gene of *Dhc* (plasmid, genomic and PCR product) were obtained and purified as follows. Plasmid DNA was isolated and purified from engineered *E. coli* cells harboring one copy of the 16S rRNA gene of *Dhc* in its plasmid using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich). Cells were grown in LB medium for 17 hours at 37°C and 180rpm. At the time of harvest cell density was approx. 10^9 cells mL^{-1}. Genomic DNA was isolated and purified from engineered *E. coli* cells harboring one copy of the 16S rRNA gene of *Dhc* in its chromosome using the Wizard® Genomic DNA Purification Kit (Promega). Cells were grown and harvested as above. PCR products harboring the 16S rRNA gene of *Dhc* were obtained by amplification via regular PCR using the primer pair T7f and M13r which target the insertion site of the plasmid/vector pCR 2.1. PCR products were either directly purified from the PCR reaction tubes using the GeneJet™ PCR Purification Kit (Fermentas), or from agarose gels using the Qiaquick Extraction Kit (Qiagen). Standard preps (plasmid, genomic and PCR) were triplicated (independently grown *E. coli* cells). Quantification of DNA concentration in standard stocks was determined using the Quan-iT™ Picogreen® technology (Biorad) and spectrophotometric analysis (Nanodrop™). In general, there was good agreement between both measurements except for the quantification of genomic DNA. Here, a difference of one order of magnitude in the quantification was observed. Data was compared to a third methodology (plate

counting). Results suggest that the spectrophotometric measurement overestimated the concentration of genomic DNA, possibly due to the high presence of RNA in the nucleic acid prep. For all calculations and comparisons, the quantification obtained with the Quan-iT™ Picogreen® technology (Biorad) was used.

Amplification and quantification of the 16SrRNA gene of *Dhc* was performed using a CFX96™ real-time PCR detection system (Biorad). All reactions (for the three primer sets) were carried out using a common protocol. The PCR cycle program was as follows: an initial denaturation at 98°C for 2min followed by 40 cycles of 5s at 98°C (denaturation) and 10s at 60°C (annealing and amplification). Data was collected at the end of each step. The reaction mixture (20 µL final volume) included 10 µL of SsoFast EvaGreen Mix (Biorad), 0.5µL of each primer (10mM each; final concentration of 0.25µM), 7µL of ultrapure distilled water (Invitrogen) and 2uL of template. All reactions were carried out in duplicate. To better compare standard curves and data from different runs, the threshold was always set at the same fluorescence intensity in such a way, that PCR amplification efficiency was maximized and the cycle threshold for each individual replicate was within the exponential amplification phase.

Results and Discussion

The interaction between primer set and standard was checked by means of amplification efficiency (slope) and intercept of the calibration curve. The slope of the standard curve and the amplification efficiency are related by the following equation:

$$eff = 10^{(-1/slope)} - 1$$

Ideally, the efficiency of the PCR should be 100%. This corresponds to a slope of 3.34.

The intercept represents the theoretical limit of detection of the reaction, although, in reality, a copy number of 10 is commonly specified as the lowest reliable copy number of target molecules. Despite not being so useful in terms of sensitivity, the intercept can be utilized for comparing different amplification systems and targets.

In terms of slope/efficiency, the primer sets GT and UT yielded relatively similar results regardless of the standard employed (Table 5.6.2.1); when nicked plasmid DNA was used, slightly lower efficiencies (87.5%; higher slope) were observed with these primer sets. Nicking the plasmid might account for additional coiling and tensions of the plasmid molecule that affect the amplification efficiency. In the case of SIR, lower efficiencies were obtained for all calibration curves compared to the GT and UT systems, with the exception of supercoiled plasmid DNA, which yielded similar results (Table 5.6.2.1). The lower efficiencies reported for the SIR system might be partly due to the length of the amplified product, which is not ideal for real-time PCR quantification. Nonetheless, other mechanisms seem to be involved as well (e.g. conformational changes in the standard) which hinder an optimal primer-standard interaction, as good efficiencies were obtained with supercoiled plasmid DNA.

Despite similar amplification efficiencies with the GT and UT systems and differences with regard to the SIR system, the intercepts of the various standard curves showed a different trend. The intercepts of GT and SIR were more alike compared to those of UT (Table 5.6.2.1), with the exception of the intercept from the standard curve of the gel-purified PCR product DNA (Table 5.6.2.1). Such differences were in general less than two units (cycles). Within the same primer system, the greatest shift in intercept was recorded for the nicked (*NtsBt I*-treated) plasmid DNA (approx. 5-6 units). Note that if the efficiency remains constant; a shift of 3 units in the intercept value translates into one-order of magnitude difference in copy numbers.

From these results a couple of important questions arise. Does the intercept pattern observed (UT yielding lower intercept than GT and SIR) also apply to environmental samples? Do such results translate into significant differences in the quantification of *Dhc*? Does the nature of the standard for calibrating matter for the quantification of *Dhc* given a specific primer set?

Table L1: Calibration curves representative of various types of standards.

	Supercoiled plasmid DNA	**Linearized plasmid DNA**	**Nicked Plasmid DNA**
GT	Y = -3.18x + 35.98	Y = -3.44x + 36.2	Y = -3.66x + 40.442
SIR	Y = -3.44x + 36.5	Y = -3.72x + 36.2	Y = -3.99x + 41.126
UT	Y = -3.27x + 34.4	Y = -3.45x + 34.3	Y = -3.65x + 38.7
	Genomic DNA	**Directly-purified PCR product DNA**	**Gel-purified PCR product DNA**
GT	Y = -3.26x + 35.7	Y = -3.50x + 34.0	Y = -3.51x + 36.7
SIR	Y = -3.69x + 35.9	Y = -3.74x + 35.1	Y = -3.80x + 43.4
UT	Y = -3.37x + 34.1	Y = -3.42x + 32.7	Y = -3.36x + 36.6

Table L1 depicts the cycle threshold (Ct) values for two environmental samples as amplified with the three different primer sets. In both cases, lower Ct values were observed for UT in a similar fashion as observed for the intercepts of the calibration curves. For sample 1, a difference of 3 units was observed, whereas for sample 2 the difference was 2 or less. Thus, there is also sample to sample variability.

Table L2: Mean values and standard deviations of cycle threshold for two environmental samples.

	GT	SIR	UT
Sample 1	27 ± 0.3	27.5 ± 0.5	24 ± 0.4
Sample 2	24.1 ± 0.2	23.7 ± 0.2	22 ± 0.2

When copy numbers for the three primer sets are compared very similar values are obtained for most calibration curves, particularly for sample 2. Two exceptions are: i) the linearized plasmid DNA results for sample 1, where a difference of an order of magnitude between UT and GT/SIR is found; and ii) results from the Gel-purified PCR product, where also a difference of an order of magnitude is observed between GT and UT. For SIR, a greater difference is even observed. These results indicate that in many cases the calibration equation buffers differences in the Ct value of the sample and similar results can be expected regardless of the primer set used.

What is the best calibrator? Plasmid DNA containing the cloned target sequence is the most common standard in quantitative PCR. Nonetheless, the calibrator most similar to environmental samples is genomic DNA, as the target gene is found in both cases within the same type of matrix. Within the same primer set, results from both supercoiled plasmid DNA and genomic DNA were very similar. Thus, any of these calibrators seems adequate. In their paper, Hou et al. (2010) warned that using supercoiled plasmid DNA results in overestimation of copy numbers and thus recommend linearizing the plasmid. Here, we did not observe any significant differences between supercoiled and linearized plasmid DNA. Caution should be taken when using purified PCR products as calibrators, since the purification method can yield substantial differences in quantification as seen for the SIR primer set. While nicking of plasmids is not a procedure for preparing plasmid preps for calibration, it shows that conformational changes can be induced which affect the way that primers interact with the calibrator. Nicking might occur naturally in plasmid preps over the course of storage or freezing and thawing cycles.

Table L3: Mean values and standard deviations of *16S rRNA* gene copies of *Dhc*.

	plasmid DNA			**Linearized plasmid DNA**			**Nicked plasmid DNA**		
	GT	SIR	UT	GT	SIR	UT	GT	SIR	UT
Sample 1	**3.9e5** ± 7.8e4	**1.9e5** ± 5.1e4	**7.9e5** ± 2.3e5	**2.5e5** ± 5.1e4	**2.1e5** ± 5.6e4	**1.6e6** ± 4.9e5	**2.4e6** ± 4.7e5	**2.3e6** ± 5.5e5	**5.4e6** ± 1.4e6
Sample 2	**2.7e6** ± 3.4e5	**2.4e6** ± 3.7e5	**3.2e6** ± 3.8e5	**1.6e6** ± 1.8e5	**2.6e6** ± 4.1e5	**6.5e6** ± 7.4e5	**1.4e7** ± 1.5e6	**2.1e7** ± 2.9e6	**1.9e7** ± 2.0e6
	Genomic DNA			**Directly-purified PCR product DNA**			**Gel-purified PCR product DNA**		
	GT	SIR	UT	GT	SIR	UT	GT	SIR	UT
Sample 1	**2.4e5** ± 5.2e4	**9.6e4** ± 2.4e4	**4.9e5** ± 1.4e5	**1.0e5** ± 2.0e4	**5.5e4** ± 1.4e4	**1.8e5** ± 3.1e4	**3.1e5** ± 6.3e4	**7.5e6** ± 1.8e6	**2.5e6** ± 7.2e5
Sample 2	**1.7e6** ± 2.1e5	**1.0e6** ± 1.5e5	**1.9e6** ± 2.2e5	**6.4e5** ± 7.1e4	**5.8e5** ± 8.4e4	**6.8e5** ± 7.8e4	**2.0e6** ± 2.2e5	**7.6e7** ± 1.1e7	**9.7e6** ± 1.1e6

Conclusions and implications for future research implementation

Primer sets can impact the amplification of a target gene as shown by differences in the Ct values of the amplified product, theoretically affecting its quantification. Nonetheless, such differences can be partly neutralized through the calibration equation. In general, differences of half an order of magnitude between primer sets in the quantification of the *16S rRNA* gene of *Dhc* can be expected, although greater differences might be observed depending on the type of calibrator used. For the most commonly employed ones, that is, supercoiled plasmid DNA or genomic DNA similar results can be expected between labs using different primer sets.

Appendix M

Comparison of DNA Quantification Methods-Detailed Data

Table M1: Detailed Data for NanoDrop Versus PicoGreen Fluorometry for Quantification of DNA samples

Sample	NanoDrop (ng/µL)	PicoGreen Fluorometry (ng/µl)	Fold Difference NanoDrop: PicoGreen	DNA Source
1	171	89	1.9	*luc* Plasmid DNA
2	158	82	1.9	*luc* Plasmid DNA
3	155	84	1.8	*luc* Plasmid DNA
4	136	94	1.4	*luc* Genomic DNA
5	124	107	1.2	*luc* Plasmid DNA
6	116	115	1.0	*luc* Plasmid DNA
7	45	10	4.6	MIAC Genomic DNA
8	45	9.3	4.8	MIAC Genomic DNA
9	44	10	4.4	MIAC Genomic DNA
10	43	49	0.9	Round Robin Sample
11	41	51	0.8	Round Robin Sample
12	37	27	1.4	*luc* Plasmid DNA
13	37	40	0.9	Round Robin Sample
14	36	38	0.9	Round Robin Sample
15	35	42	0.8	MIAC Genomic DNA
16	33	6.2	5.3	MIAC Genomic DNA
17	33	9.0	3.6	MIAC Genomic DNA
18	32	34	1.0	Round Robin Sample
19	30	13	2.3	MIAC Genomic DNA
20	27	10	2.8	MIAC Genomic DNA
21	27	6.6	4.1	MIAC Genomic DNA
22	23	20	1.1	MIAC Plasmid DNA
23	4.4	4.3	1.0	MIAC Genomic DNA
24	3.3	1.2	2.7	Round Robin Sample
25	3.0	0.9	3.2	Round Robin Sample
26	2.6	0.7	3.7	Round Robin Sample
27	1.8	0.6	3.0	Round Robin Sample
28	1.2	0.1	8.1	Round Robin Sample
29	1.1	0.9	1.3	Round Robin Sample
		Average Fold Difference NanoDrop: Fluorometry	2.5	

Appendix N

Biomass Preservation Experiments

Experiment 1

For data presented in Table N1 DNA was extracted from groundwater or from biomass collected using Sterivex™ cartridges as described (Ritalahti, 2009a; Ritalahti, 2009b).

The different storage conditions tested are shown in Table N1 below. In each case, at minimum of two replicate filters from a well were examined for each comparison, and several (2 to 5 samples per experiment) were analyzed.

Results and Discussion

Table N1: Comparison of *Dhc* Enumeration Percent Recoveries of samples types (Groundwater /Biomass on Sterivex Cartridges/as DNA) Under Different Storage Regimes

	Groundwater	Filter membrane	Sterivex Cartridge
Immediate extraction	100%	100%	100%
Overnight 4˚C	100%	80-100%*	nd
1 week 4˚C	nd	50-100%*	20-100%*
2 weeks 4˚C	50-80%	0.7-3.0%	nd
2 weeks -20˚C	nd	2 to 30%	5-50%
2 weeks -80˚C	nd	100%	100%

Notes:
nd = not done
* = typically 98-100%, some filters (depending on site) experience greater loss.

For meaningful data interpretation on the temporal scale, it is critical to utilize a consistent method for DNA extraction and quantification. Furthermore, prompt sample processing and storage is important to maintaining reliable quantification. In this experiment groundwater samples in filled glass jars stored at 4˚C for up to two weeks demonstrated 50-20% declines in *Dhc* quantification. In contrast, Sterivex filters with attached biomass may begin to experience immediate declines in abundance upon storage at 4˚C. If extended storage at 4˚C is expected, adding Bacteria Protect™ (Qiagen) reagent may extend storage at 4°C for up to 10 days (data not shown, MI). Storing filtered biomass at -20˚C for extended periods is not recommended, as enumeration of biomarkers declined rapidly after 2 weeks. This data indicates that groundwater samples should be processed as quickly as possible upon receipt in the laboratory and that biomass on filters and DNA should be stored at -80˚C until extraction and quantification respectively.

Experiment 2

Carboys with ~10^9 *Dhc* /L were prepared with KB-1 in simulated groundwater as described in Attachment 3.

Four treatment and storage regimes were tested:

1) Oxic groundwater, refrigerated [4°C] storage;

2) Anoxic groundwater, refrigerated [4°C] storage;

3) Oxic groundwater, room temperature [22 °C]storage; and

4) Anoxic groundwater, room temperature [22 °C] storage.

After the anoxic samples had been aliquoted, the carboys were purged with compressed air for a minimum of 35 minutes before distributing oxic samples. Samples were stored either at room temperature (22°C) or in a refrigerator (4°C) for five days prior to biomass collection with Sterivex™ cartridges. The membranes were cut aseptically as described (Ritalahti et al. 2012) and added to the MO BIO Ultraclean Soil DNA Extraction kit's bead beating tubes for extraction following the manufacturer's alternative protocol for higher yields. *Dhc* qPCR was conducted according to methods described in Attachment 2 [Attachment B.2]).

The results of the experiments conducted to evaluate the impact of storage temperature and aeration are presented in Figure L1. The data was inconclusive as to whether maintaining anoxic versus oxic conditions impacted *Dhc* quantification results suggesting there may be a benefit to minimizing exposure of samples to air during shipping and storage. Storage at room temperature as opposed to maintenance at 4°C reduced the *Dhc* abundance by up to 1,000 fold. This suggested that that maintaining refrigeration temperatures (4°C) is vital to accurately enumerate *Dhc*.

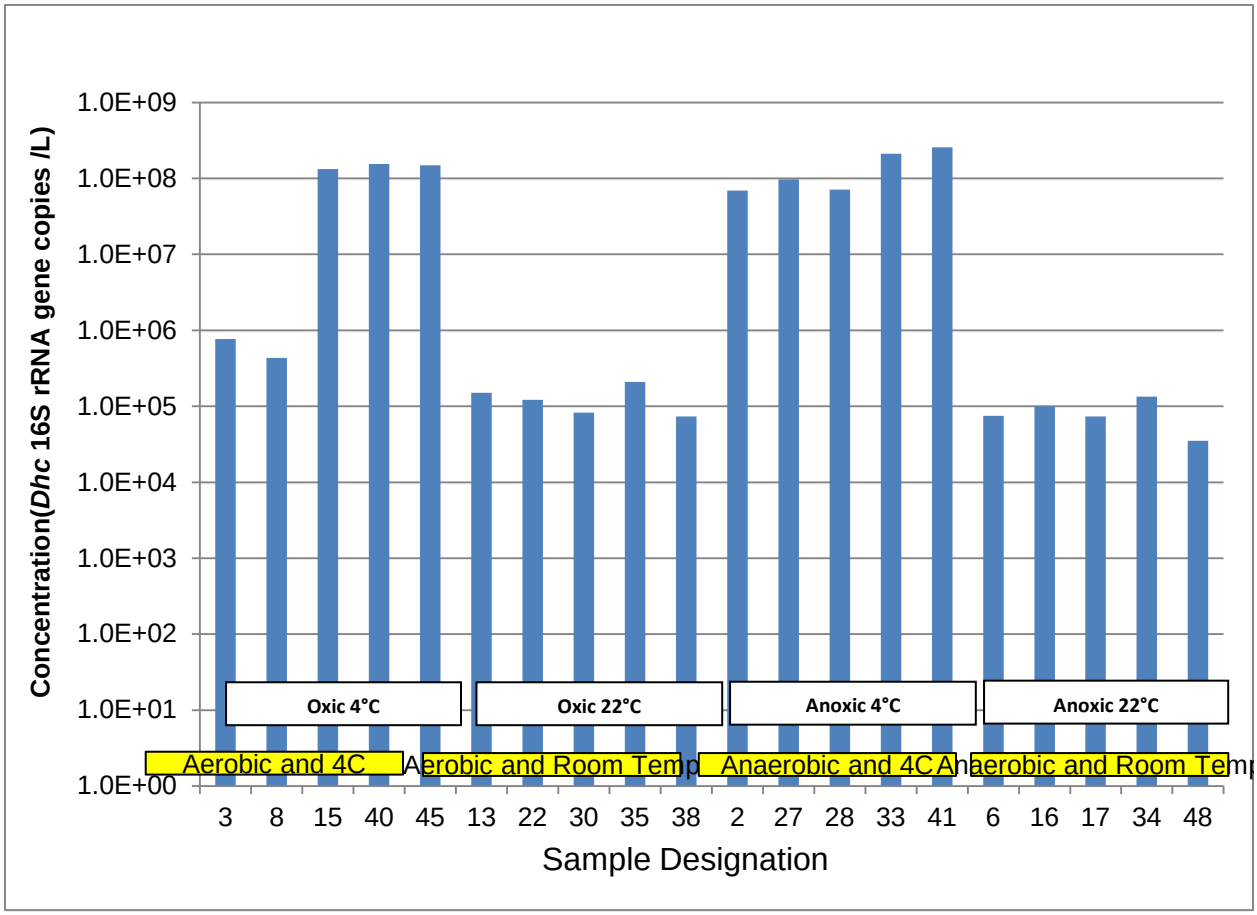


Figure N1: Impact of storage temperature of oxic versus anoxic groundwater on *Dhc* biomarker quantification. *Dhc* spiked at ~10^9 /L s were either sparged with air (oxic and 4°C), sparged with air and stored at room temperature (oxic 22°C), not sparged and stored at 4°C (anoxic 4°C), and not sparged and stored at room temperature (anoxic 22°C). The samples were stored for a total of 5 days.

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14. ABSTRACT

SERDP project ER-1561, Standardized Procedures for Use of Nucleic Acid-Based Tools for Microbial Monitoring, focused on identifying and minimizing the causes of variability during qPCR enumeration of genes of interest in groundwater, with the goal of developing a foundation for the development of standardized methods for collection, preservation, transport, storage and processing of environmental qPCR samples collected from contaminated sites.

15. SUBJECT TERMS
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