Literature Review Report

Standardized Procedures for Use Of Nucleic Acid-Based Tools

SERDP Project ER-1561

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14. ABSTRACT
A survey of peer reviewed and technical literature was conducted with a focus on the methods used in disciplines utilizing qPCR. The disciplines explored include: medical, agricultural/food, forensics, and environmental fields. The review identified that unique challenges are associated with environmental remediation samples, with a focus on groundwater, including the potential for high variability, challenges associated with representativeness, biomarker losses in sample processing and extraction and matrix interference leading to PCR inhibition. Recommendations for assessing and addressing these challenges are provided in the report and include the development of Dhc reference standards and internal microbial controls (i.e., microbial surrogates) to: a) assess current approaches to sampling, shipping, storage, biomass concentration, nucleic acid extraction, and data analysis/interpretation, and b) identify promising areas where methodological improvements may be required.

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LIST OF ABBREVIATIONS

%	percent
B. diminuta	*Brevundimonas diminuta*
cDNA	complementary DNA
CRM	certified reference material
C_t	threshold cycle
Dhc	*Dehalococcoides*
D. ethenogenes	*Dehalococcoides ethenogenes*
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EBV	Epstein Barr Virus
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EPS	extra polymeric substances
ESTCP	Environmental Security Technology Certification Program
FISH	fluorescence in situ hybridization
FLOW-FISH	flow cytometry - fluorescence in situ hybridization
GMO	genetically modified organisms
H. pylori	*Helicobacter pylori*
HIV	Human Immunodeficiency Virus
IRMM	Institute for Reference Materials and Measurements
JGI	Joint Genome Institute
L	liter
MBT	molecular biological tool
µg/L	micrograms per liter
µL	microliter
mL	milliliter
MNA	monitored natural attenuation
mRNA	messenger ribonucleic acid
MWCO	molecular weight cutoff
NA	nucleic acid
ng	nanogram
NIST	National Institute of Standards and Technology
ORP	oxidation reduction potential
PCR	polymerase chain reaction
PLFA	phospholipid fatty acid analysis
qPCR	quantitative polymerase chain reaction
QA/QC	quality assurance / quality control
RFU	relative fluorescence units
RM	reference material

LIST OF ABBREVIATIONS (CONTINUED)

RMDs	retrievable media devices
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
SERDP	Strategic Environmental Research and Development Program
USEPA	United States Environmental Protection Agency
VOC	volatile organic compound
WHO	World Health Organization

EXECUTIVE SUMMARY

Groundwater and soil samples are frequently analyzed by academic and commercial organizations using molecular biological tools (MBTs) to detect unique genetic biomarkers associated with *Dehalococcoides* (Dhc) and other environmentally relevant microorganisms. The results of these analyses are increasingly used by site owners, consultants, and regulators to design and evaluate natural degradation and enhanced bioremediation systems. Despite the widespread use and importance of MBTs, there are currently no standardized methods for collecting, preserving, transporting, storing, or processing groundwater samples for analysis. More importantly, the lack of standardized reference materials: a) is a barrier to comparison of MBT results between laboratories and over time, b) makes confident assessment of the relationship between biodegradative microorganisms, such as Dhc, and remediation success a challenge and c) obscures the impacts of sampling methodologies, detection of procedural errors, and other biases that affect the accuracy, precision and reproducibility of MBT analysis. Currently, there is little understanding of how biomarker integrity is affected throughout sample collection to quantification process.

A systematic evaluation of the factors affecting MBT data quality is required to improve the accuracy and precision of these analyses. This evaluation will lead to recommendations for standardization of sample collection and processing, and analysis/reporting procedures to establish user confidence with the goal of increasing implementation of these powerful tools to enhance site management.

The primary focus of SERDP Project number ER-1561 is the development of standardized procedures for use in nucleic acid-based MBTs. Prior to developing these procedures, a literature review of the quantitative polymerase chain reaction (qPCR) methods for the analysis of environmental samples was conducted. The purpose of this review was to confirm the project team's strategy and approach, and to identify additional promising approaches and technologies that could be incorporated as part of the research effort. Of particular interest was the evaluation of:

i. Methods that are currently available and/or emerging;

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

iii. Factors that affect sensitivity of the analysis, and the variability within/between methods;

iv. The impact of field heterogeneity on MBT results and data interpretation; and

v. Groundwater/soil sampling techniques.

Information on the above topics was obtained by surveying the peer reviewed 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 sampling and biomass concentration from groundwater samples 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 environmental remediation samples, with a focus on groundwater, including the potential for high variability, challenges associated with representativeness, biomarker losses in sample processing and extraction and matrix interference leading to PCR inhibition. Recommendations for assessing and addressing these challenges are provided and include the development of Dhc reference standards and internal microbial controls (i.e., microbial surrogates) to: a) assess current approaches to sampling, shipping, storage, biomass concentration, nucleic acid extraction, and data analysis/interpretation, and b) identify promising areas where methodological improvements may be required. The following are highlights of the key findings, additional findings and further details are provided in the specific sections devoted to these topics.

1. Development of Microbial Surrogate Standards and Reference Materials

The 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 forensics, but that the methods have not been fully applied to environmental testing.

Specifically a certified Dhc reference culture is a prerequisite for:

- Method validation and optimization;
- Assessment of inter laboratory variation;
- Assessment of laboratory personnel; and
- Assessing Dhc specific matrix effects.

Certified reference materials (CRM) are used in other disciplines for validation of qPCR analysis but are not currently used or available for environmentally relevant microorganisms. The development of a whole cell and genomic DNA-based Dhc reference material (RM) is a key need for qPCR method verification/optimization. The following key findings regarding the use and development of CRM and microbial surrogates were identified:

- The accuracy of Dhc reference materials can be verified independently using a variety of currently available methods for total biomass quantification including total DNA,

protein, phospholipid fatty acids (PLFA), and direct enumeration of cells including fluorescence in situ hybridization (FISH) and flow cytometry.

Internal controls are standards that are added directly in known quantities to the assay or sample materials and are co-monitored with the target analyte throughout the extraction and testing procedure to quantify losses and interference.

Microbial surrogates are whole cell internal process controls that are co-quantified with the test target (Dhc) and function to assess losses throughout the processing and analysis steps including:

- Incomplete biomass recovery;
- Incomplete cell lysis;
- Losses in nucleic acid extraction; and
- Losses due to PCR inhibition.

Culturable microbial surrogates are available that may be able to mimic Dhc size or cell wall characteristics including *Brevundimonas diminuta* (small), *Micrococcus sp.* (small coccoid) and *Halobacterium sp*. (Dhc-like cell wall). Another surrogate strategy is the use of genetically modified *Escherichia coli* (*E. coli*), containing a plasmid with a modified Dhc gene sequence that would serve as the PCR target.

2. Sample Collection and Preservation

Groundwater sampling and biomass concentration are likely the most highly variable steps in the qPCR sampling and analysis chain. Nonetheless, approaches for obtaining biomass from aquifers are not standardized. Managing sampling variability is contingent upon using effective approaches, and consistently applying, replicating, and monitoring field parameters that are indicators of representative sampling. After sampling, preservation of whole microorganisms and non-cell associated Deoxyribonucleic acid (DNA) without degradation is important. Currently, preservatives are not typically used with DNA samples but have potential to improve sample quality and increase hold times. Groundwater biomass sampling is performed using three basic approaches:

- Groundwater sampling, followed by laboratory filtration;
- Field filtration (filter shipped to laboratory); and
- In-well retrievable media devices (RMDs; i.e., Bio-Traps®).

Key findings related to sample collection and preservation include:

- Groundwater sampling flow rates and purge volumes affect the collection of biomass;
- A variety of filters are available but were not specifically designed for groundwater MBT analysis;
- RMDs are non-quantitative biomass concentration methods;
- A number of nucleic acid preservatives with potential to be applied directly to groundwater in the field have been identified; and
- Novel nucleic acid extraction/preservation filters have potential for groundwater samples.

3. Nucleic Acid Extraction

A multitude of nucleic acid extraction protocols and approaches exist in various disciplines that are designed to overcome specific challenges, such as lysis of cells and removal of inhibitory compounds from samples unique to their samples (e.g., blood and stool). Current methods used in environmental remediation testing rely on commercially available kits. While these methods may ultimately prove sufficient, a systematic evaluation of these methods using microbial surrogates and reference standards in the context of groundwater samples has not been performed. Methods to remove substances that inhibit PCR, such as humic compounds, tannins, phenols, metals, polysaccharides and lipids, may result in loss of PCR targets and raise minimum quantification levels. Methods that involve dilution of inhibitory compounds may prove to be a better strategy although this approach also raises detection limits

4. PCR Quantification of Nucleic Acid Targets

The key to accurate quantification of test samples is effective instrument calibration using materials themselves that are properly quantified and which reflect, to the extent possible, the properties of the test samples. The following areas were identified as key to improving current approaches to calibration in qPCR:

- Use of reference materials for ongoing method verification. Daily "check standards" are essential for improved validation and confidence in the results;
- Calibration using linear DNA may prove superior to currently used plasmid calibrators;
- Calibration using whole cells, such as the Dhc reference culture, has the potential to reduce positive bias introduced by current naked DNA calibrators; and

5. Overview of Quality Assurance / Quality Control Procedures, Data Quality and Standardization of Methods

Generating accurate results and appropriately using qPCR data requires the integration of good laboratory practice, and use of appropriate controls and replication. In addition, this information must be reviewed, compared to data quality objectives, and interpreted responsibly. The following were identified as key components of effective QA/QC protocols:

- Validation and documentation of laboratory equipment, personnel and protocols used in support of qPCR analysis;
- Consistent use of data quality samples such as trip blanks, equipment blanks, matrix spikes has the potential to improve data interpretation and quality control. The utility of specific controls should be determined so that superfluous use of controls is avoided;
- Replication at specific sampling and analysis steps combined with statistical tools such as power calculations has the potential to determine replication needs;
- Use, where possible, of non-PCR methods such as plate counts, microscopy, FISH, PLFA to validate methods and standards;
- Establishment of rigorous method detection limits (MDL), using reference materials for commercial Dhc analysis is required for data interpretation of negative results; and
- Incorporating procedures to use dilution of template DNA to evaluate whether inhibition is occurring;
- PCR efficiency is a statistical measure generated during real-time qPCR analysis has potential to be used to assess PCR inhibition.
- Normalization of numerical values to total biomass is critical for interpretation of data where biomass recovery may be inconsistent, comparison of current normalization approaches to would be informative and aid in standardization of these methods.

A survey of methods and procedures used in commercial qPCR testing and the literature has indicated that the environmental remediation field has the potential to adopt key methodological approaches derived from other disciplines in several key areas. Selected key findings viewed as having potential to improve methodologies associated with qPCR analysis have been summarized. Table 7-1 identifies numerous research activities that may address the key findings described above. Many of these activities were identified in the original proposal, but additional items have been identified and will be explored further.

This review has confirmed that the proposed focus of this project is appropriate, identified key technical issues, and has identified promising approaches and techniques that will be incorporated into the detailed laboratory work plans.

1. INTRODUCTION

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 rapidly increasing, as remedial practitioners seek to improve the design, field performance and monitoring of biologically-based remediation technologies. While academic research facilities and several commercial entities have emerged in recent years to provide these analyses, there are currently no MBT-focused standardized methods for collection, preservation, transport, storage or processing of environmental remediation samples. These factors all have the potential to significantly influence (i.e., compromise) the results of MBT analyses because biomarker recovery and integrity during the entire analytical process has not been established. A systematic evaluation of the factors affecting MBT data quality is required to improve the accuracy and precision of these analyses. This evaluation will include the development of standardized sample collection and processing procedures to establish user confidence and widespread implementation of these powerful tools for enhanced site management.

Our proposal to the Strategic Environmental Research and Development Program (SERDP) identified the fact that current MBTs for analysis of environmental remediation samples do not include surrogate standards or matrix spikes to quantify biomarker loss during shipping and storage and during each analytical step. Our technical approach involves the identification and testing of suitable internal and reference standards that will be used to conduct a systematic evaluation of the biomarker recovery of each step in the sequence of events taking place from sample collection to reporting biomarker concentrations.

The use of MBTs to quantify key microorganisms in other industries and disciplines is more common and includes more sophisticated methodologies than those typically used in the environmental remediation field. The disciplines with well developed methodologies for MBTs include medical, food, water and environmental quality, as well as criminal forensics and monitoring of industrial processes. A considerable number of peer-reviewed papers discuss the factors that affect reproducibility, sensitivity, and accuracy of MBTs. Therefore, the purpose of this review is to identify key technical issues to be resolved in the environmental remediation field and review promising methodologies for MBT application from other disciplines. We seek to improve MBT application in the bioremediation industry.

Our review focuses on the methods and materials used to develop calibrated reference standards (Section 2), sample collection and preservation (Section 3), nucleic acid extraction (Section 4), and polymerase chain reaction (PCR) quantification (Section 5). We also provide an overview of key quality assurance/quality control (QA/QC) procedures and data quality measures and approaches (Section 6). Each section highlights the current practice as it pertains to MBTs and key findings of our review. Section 7 (conclusion and recommendations) discusses our planned research activities to improve and standardize MBT application for groundwater analysis taking into account the key findings from our review.

2. DEVELOPMENT OF MICROBIAL SURROGATE STANDARDS AND REFERENCE MATERIALS

Quantified control materials used in quantitative PCR (qPCR) analysis, as well as many other analytical methods, are used for establishing standard curves (calibration) and on-going validation of the accuracy of the test procedures (validation). Two specific types of controls that have not been widely implemented in qPCR methods used in the environmental remediation field include:

1) Reference materials; and

2) Internal controls/surrogates.

2.1 Reference Materials and Certified Reference Materials

Reference materials (RM) are quantified substances, chemicals, microbes, deoxyribonucleic acid (DNA), etc. used for method calibration and accuracy validation, and could be defined as follows:

"An RM is a material or substance, one or more of whose properties are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials" (Epstein et al., 2001).

An alternate definition for RM directly applicable to qPCR analysis is:

"A material suitable for real-time PCR applications which is a specimen derived from a known biological source and whose value has been established by consensus means" (Mackay, 2007).

Certified reference materials (CRM) are a more stringently defined subcategory of RM that could be defined as follows:

"A reference material, one or more of whose property values are certified by a technically valid procedure, accompanied by or traceable to a certificate or other documentation which is issued by a certifying body, and for which each certified value is accompanied by an uncertainty at a stated level of confidence" (Epstein et al., 2001).

In chemical analytical methods, a CRM could be stock solutions of chemicals of known quantities provided by chemical vendors (for example, kerosene reference Standard-50,000 micrograms per liter [µg/L] in hexane-Supelco Inc. [Sigma-Aldrich-cat.#47517-U]).

CRMs are often produced by third parties and are typically expensive and not widely available. As such, CRMs are only typically used to validate methods and not as day-to-day calibrators for production of standard curves. A few commercial and government entities produce CRMs relevant to MBT use. The first nucleic acid international reference standard, made available

from the World Health Organization (WHO) was for Hepatitis C Virus (Saldanha et al., 1999). Since that time additional viral nucleic acid reference standards have become available including Human Immunodeficiency Virus (HIV) and Parvovirus (Muska et al., 2007). The Institute for Reference Materials and Measurements (IRMM; www.irmm.jrc.be/html/homepage.htm) produces CRM for a variety of applications including microbiological materials such as DNA stocks; however, the variability of the microbial products appears to be relatively high, for example *Campylobacter jejuni* (NCTC 11351) DNA is certified to have 71 nanograms (ng) per vial +/- 39 ng (Zika et al., 2007). Cankar et al., (2006) reported use of IRMM certified Roundup Ready Soy 5 percent (%); Roundup Ready® soybean in non-genetically modified soy for calibration of a method to detect genetically modified organisms (GMO) in soy products. Other sources of CRM include those used in the Forensic community produced by National Institute of Standards and Technology (NIST; http://ts.nist.gov/measurementservices/referencematerials/index.cfm) some of which have been available since 1992, for example, SRM 2391- PCR-Based DNA Profiling Standard for Variable Number of Tandem Repeat. SRM 2372 Human DNA Quantitation Standard for use in calibration of qPCR methods is a more recently introduced CRM, quantified using decadic attenuance, spectrophotometry (Montgomery, 2007) (Attachment A Certificate of analysis for Human DNA Quantitation Standard).

On the whole, RM and CRM have not been widely used for MBT analysis in the environmental remediation field. Nevertheless, implementation of RM/CRM for environmental remediation methods has the potential to improve calibration, validation and inter-laboratory consistency, as well for assessing differences due to laboratory personnel which has been identified as a key variable (Raggi et al., 2003).

2.1.1 Methods for Enumeration of Microorganisms and Development of a Dhc Reference Standard

The development of an RM for use in environmental remediation qPCR testing has been identified as a key need for the optimization and validation of qPCR methodologies in the original project proposal. The model organism identified in the original proposal was *Dehalococcoides* (Dhc) due to this organism's key role in the detoxification of a wide spectrum of chloroorganic contaminants, including chlorinated ethenes. Furthermore, Dhc-targeted MBTs are commercially available (primarily through Microbial Insights and SiREM) and are currently used for site assessment and monitoring. Based on the definition of a CRM as quantified by "consensus means" (Mackay, 2007) a number of quantification methods performed in parallel for pure and mixed Dhc cultures is proposed.

Enumeration of bacterial stock solutions used for calibration can in some cases be performed using classical microbiological methods such as plate counts, in cases where the subject microorganism is culturable on solid media such as agar plates (Cubero et al., 2001; Klerks et al., 2006; De Bellis et al., 2007). This is not the case for Dhc, which is typically isolated only in

liquid media (Maymó-Gatell et al., 1997; Duhamel et al., 2004; Sung et al., 2006) and has for the most part been resistant to culture on solid media. Therefore, enumeration of a Dhc CRM will require non-culture based methods, such as enumeration based on total DNA quantification of a pure Dhc culture. This information combined with the knowledge of the average size of the Dhc genome (based on 1,395,502 bases for CBDB1 (TIGR); 1,469,720 bases for strain 195 and 1,341,894 bases for BAV-1 (Joint Genome Institute (JGI)), typically1.4 x 10^6 bases is assumed, allows calculation of the titer of the culture. This is calculated by dividing the total mass DNA extracted from a certain volume of culture by a known mass of the DNA per cell to calculate the cell titer (see sample calculation below). This approach to enumeration was used to enumerate *Collimonas* genomic DNA for calibration of a qPCR method (Höppener-Ogawa et al., 2007).

-DNA extracted from 1 mL of culture = 0.1 µg (by spectrophotometry / fluorometry)

-Size of 1 Dhc genome (i.e., DNA per cell) = 1.4 x 10^6 base pairs x 650 Daltons (DA)/base pair (1 DA = 1.64 x 10^{-24} grams) =2.62 x 10^{-18}g DNA /cell = 2.62 x 10^{-12} µg DNA /cell

-Total Cells=0.1 µg DNA/1 mL culture/ 2.62 x 10^{-12} µg/cell= 3.81 x 10^{10} Dhc cells /mL

Figure 2.1: Sample Calculation Demonstrating how to Determine the Titer of Pure Dhc Culture Using Total DNA Quantification

It is important to note that the efficiency of DNA extraction is a critical parameter under this approach, as losses in DNA would lead to an underestimate of Dhc titer. A similar approach could be approached using phospholipid fatty acid analysis (PLFA) (White et al., 2005) or total protein. First, to establish the total biomass, and then, dividing by the estimated amount of the specific biomarker per Dhc cell, titer can be calculated.

Another approach used to enumerate microorganisms including Dhc is fluorescence in situ hybridization (FISH) which is a non-PCR based molecular genetic method. FISH can be performed with whole cells, which when hybridized with fluorescently labeled probes that bind to specific nucleic acid (NA) sequences within the cell, cause the cell to fluoresce, allowing the enumeration of individual cells by microscopy. For example, Yang and Zeyer (2003) developed and successfully tested a Dhc specific FISH method which was based on probes targeting consensus regions of the Dhc 16S ribosomal ribonucleic acid (rRNA) gene. The probe design was based on examination of 28 Dhc strains detected in pure culture and in environmental samples. The probes were used to enumerate the percentage of Dhc versus total bacteria; however, numeric values for Dhc were not reported. Percentages reported had associated error measurements, for example a culture containing *Dehalococcoides ethenogenes* (*D. ethenogenes*) was reported to consist of 32% *D. ethenogenes* +/-7%. As indicated by Epstein et al. (2001) the establishment of the uncertainty at a stated level of confidence is a defining feature of CRM. In another study, Aulenta et al. (2004) used FISH to enumerate Dhc as a percentage of total biomass in a dechlorinating bioreactor indicating that Dhc consisted of 41.5 % of total volatile suspended solids +/-11.2%. Heimann et al., (2006) used FISH to detect Dhc in KB-1®, a

commercially available dechlorinating culture (produced by SiREM) that is an identified source material for an RM as it is widely available and well characterized (Duhamel et al., 2006). Another study reported the use of a combination of flow cytometry and FISH (FLOW-FISH) to determine the relative concentrations of *Lactococcus* in dairy starter cultures (Friedrich et al., 2006). FLOW-FISH allows rapid enumeration of targeted microorganisms.

The comparison of multiple methods for enumeration of Dhc in pure or mixed cultures including total biomass quantification methods combined with FISH methods has the potential to provide consensus enumeration and establishing uncertainty values. Additional verification of the developed RM could be achieved by verification of the enumeration methods (such as total DNA quantification) using a third party validated CRM such as RM 2372 Human DNA Quantification Standard. This approach would provide an additional level of quality assurance for the Dhc RM by allowing its comparison to an internationally recognized standard.

2.2 Internal Standards and Microbial Surrogate Standards

Internal controls are standards which are added directly in known quantities to the assay or sample materials and are co-monitored throughout the extraction and testing procedure to quantify losses and interference. Internal controls have been used in qPCR to compensate for incomplete recovery, sample deterioration, and the presence of PCR inhibitors (Muska et al., 2007). It is essential for the use of internal controls to be able to differentiate the internal control from the assay target in the original sample. For example, in chemical analytical methods, deuterated analogs of target chemicals are sometimes used which have similar extraction and analytical characteristics but which are detected at different masses compared to the related target chemical.

Internal standards are widely used in qPCR methods for the quantification of extraction losses and interferences. Internal controls in qPCR can consist of non-cell associated “naked DNA” such as plasmids (Cubero et al., 2001; Koike et al., 2007), salmon sperm DNA (Haugland et al., 2005), synthetic oligonucleotides (personal communication with Dora Ogles, Microbial Insights) or viral Lambda DNA (Mumy et al., 2004) or M13 Phage DNA (Sum et al., 2004). Naked DNA internal controls are typically added to the DNA extraction or the qPCR reaction to quantify losses during the extraction and analysis steps. The major shortcoming of the use of naked DNA as a internal control is that it provides no information regarding losses related to whole cells, for example, cell adhesion to filters (Section 3), losses due to incomplete cell lysis (Section 4) and interactions of the original sample with bacterial cell walls (i.e., attachment of cells to solids).

The impact of cell-related factors on the accuracy of qPCR results can be best understood by the inclusion of a whole cell internal microbial control (i.e., a microbial surrogate) which is defined (for the purposes of this study) as an enumerated whole bacterial cell matrix spike. An ideal microbial surrogate has been identified in the project proposal as having a number of specific qualities including:

1. The microbial surrogate and/or the targeted nucleic acid sequences contained within the surrogate are not commonly be present in groundwater;

2. The filtration and sedimentation behavior and the cell walls of the surrogate organism should be similar to the target organism (e.g., Dhc) to prevent over or underestimation of concentration and cell lysis efficiency;

3. Unique nucleic acid sequences in the surrogate must be detectable using qPCR methods. The surrogate target sequences must be comparable to those of biomarker organism (i.e., similar length of amplicons, etc.);

4. A PCR-independent method for enumeration of the surrogate organism must be available (e.g., plate counts FISH, or microscopy methods);

5. The surrogate must be non-pathogenic; and

6. The surrogate must be a non-spore former.

Reports of the use of microbial surrogates in the peer reviewed literature are less common than the use of naked DNA internal controls. Nevertheless, the use of microbial surrogates has been reported for qPCR analysis. In one study, *Geotrichum candidum* (yeast) was added as a microbial surrogate to the DNA extraction bead tube in a procedure to quantify pathogenic *Candida* (another yeast) species (Brinkman et al., 2003). *Escherichia coli* (*E. coli*) with a plasmid containing a target DNA sequence was used as a microbial surrogate in a method for the quantification of *Salmonella* (Klerks et al., 2006). The use of *E .coli* as a surrogate for *Salmonella* is appropriate based on the fact that both of these organisms are members of the Gamma Proteobacteria, and therefore, may share certain characteristics that are relevant to extraction and quantification in qPCR. *E. coli* was also used as a microbial surrogate in an assay for *Helicobacter pylori* (*H. pylori*) in drinking water (Sen et al., 2007). The *E. coli* used in this study contained a single copy plasmid that had sequences similar to the target *H. pylori* sequence, with the exception that the surrogate sequence was mutated at four base pairs at the probe binding site. This approach has the primary advantage of providing a target sequence for PCR amplification that is identical to that of the target sequence (i.e., an "internal competitive control"), and therefore, does not introduce bias in terms of differential PCR amplification efficiencies, primer binding efficiencies, etc. (i.e., condition 3 above). Differentiation of the target sequence from the surrogate sequence occurs only at the level of the TaqMan® probe (see Section 5.1.2 for an overview of TaqMan® methods) which is labeled by a different colored fluorophore in multiplex qPCR, allowing independent co-detection of the surrogate in the reaction. The use of a modified probe surrogate also fulfills condition 1 above, in that the mutated sequence is unlikely to be present in groundwater due to the fact that it is not naturally occurring. The major disadvantage of using an internal competitive control that shares the same PCR primer sequences as the target is the potential impacts on detection limits where the target sequence is at very low concentrations and the spiked surrogate serves to out compete (and

thereby obscure) the presence of the target sequence (Muska et al., 2007). Therefore in cases where detection limits are critical the use of relatively low concentrations of surrogate must be used. For example, Sen et al. (2007) were able to optimize their assay with an *E. coli* surrogate concentration of 10 cells/liter of water for detection of 5 to 3,000 cells of *H. pylori*, a pathogenic microorganism which has significant impacts at low concentrations. The fact that Dhc is routinely detected at much higher concentrations in groundwater, up to 10^9 gene copies per liter groundwater (SiREM, unpublished data), means that competition from an internal competitive surrogate is not necessarily problematic unless extremely low detection limits are a priority.

The use of a surrogate with similar lysis, size, and other properties is essential in order for the surrogate to effectively model the target microorganism in terms of losses throughout the filtration cell lysis and NA extraction steps. Dhc is a relatively small microorganism consisting of spherical (i.e., coccoid) or disk shaped cells with a diameter of approximately 300-500 nanometers (He et al., 2005). Ideally a surrogate used in Dhc testing would be of a similar size and would have similar filtration and lysis characteristics.

The use of *E. coli* as a surrogate (Klerks et al., 2006; Sen et al., 2007) has the advantage of providing ease of introduction of cloned sequences within this microorganism because *E. coli* (the so-called white mouse of the microbial world) is the microorganism into which the vast majority of molecular cloning is performed. Protocols for the introduction of DNA (plasmids) into *E. coli* are well established, however, this is not the case for cloning into most other microorganisms. *E. coli* are gram negative rods that are typically 2-3 µM (2,000-3,000 nm) in size, significantly larger than Dhc (300-500 nm). It is unknown, and perhaps unlikely, that *E .coli* would have similar lysis properties to Dhc, however, given the advantages of *E. coli* surrogates it is worth examining their potential further.

A number of potential microorganisms have been identified that are potential Dhc surrogates based on size and cell wall characteristics. *Brevundimonas diminuta* (*B. diminuta*; ATCC-1946) is a microorganism that is used in standard protocols to validate the performance of 0.2 µM (200 nm) filters (ASTM, 2007). Thus, the size of *B. diminuta* is around 200 nm and may vary depending on the conditions under which is grown (Lee et al., 2002). This means the size of *B. diminuta* is very close to Dhc, and therefore, it may mimic the filtration characteristics of Dhc. Another potential Dhc surrogate are members of the genus *Halobacterium* including specifically *Halobacterium* sp. NRC-1 (Ng et al., 2000). *Halobacterium* are aerobic halophilic (salt loving) rod shaped members of the Archaea. Since Dhc has a Archaea-like cell wall (Maymó-Gatell et al., 1997), these microbes may share similar lysis properties. *Halobacterium* species have the additional advantages of being easily cultured and having members whose entire genome sequences are known which provides a multitude of sequences for qPCR quantification. In addition, genetic transformation and manipulation of *Halobacterium* has been demonstrated (DasSarma et al., 2006) potentially allowing genetically modified members containing Dhc genes or other modifications which could make this an ideal surrogate. Potential disadvantages include the propensity of *Halobacterium* species to lyse under non-extreme salt conditions

(Mescher et al., 1976). This property could reduce their applicability to non-saline groundwater, however modification of groundwater samples with salt prior to analysis could be examined as a possible approach to using *Halobacterium* species as a Dhc surrogate. Another potential surrogate based on size and shape is *Micrococcus* species including *Micrococcus luteus. Micrococcus luteus* (ATCC-4442) are relatively small (1,000 nm) spherical bacteria (Madigan et al., 2006).

Due to the fact that introduction and maintenance of plasmids in non-*E. coli* species is challenging, it may be necessary to use preexisting natural genetic sequences should a non-*E. coli* microorganism be selected as a microbial surrogate. This necessitates that the microorganism must be absent from groundwater samples (to meet condition 1 above) otherwise the surrogate target sequence may confound the analysis, for example leading to perceived surrogate recoveries exceeding 100%. Literature reviews may be useful for screening which microorganisms are not typically found in soil, sediment and groundwater, nevertheless, literature reviews will likely be insufficient to confidently conclude that a particular microorganism, or an identical gene sequence, are not typically found in groundwater. An alternate approach is to screen a suitable number of aquifer DNA samples with the qPCR methods proposed for the surrogate from widely distributed sites with a variety of characteristics to empirically determine whether the targeted sequence is ever detected in groundwater. The use of pre-extracted DNA archive from groundwater would provide an immediate source for this type of screening. One potential source for this archive is SiREM which has over 4,000 archived groundwater DNA samples, a portion of which would be sufficient to confirm the expected abundance of surrogate sequences in groundwater samples.

2.3 Key Findings

2.3.1 Key Findings: Reference Materials and Certified Reference Materials (CRM)

Reference Materials (RM) provide a number of advantages to the practitioner including method validation and optimization, confirmation of test results, and the ability to test for variation between laboratories and laboratory staff.

- The use of CRM is widespread in various analytical procedures and various types of commercially available products, such as standardized chemical stock solutions, are available

- The use of CRM in qPCR analysis is less common, due in part to the novelty of these methods. Nevertheless, CRM directly applicable to qPCR methods are currently available such as NIST, SRM 2372 Human DNA Quantitation Standard, and the WHO HIV and Parvovirus nucleic acid standards.

- Development of an RM requires the use of rigorous and potentially multiple independent quantification approaches in order to produce a high quality RM. Quantification and documentation of the associated variability is also critical. For the quantification of microbial cells in pure culture, methods that measure total biomass (e.g., total DNA, PLFA, protein, etc.) are potentially useful. All of the methods are contingent upon an understanding of the efficiency of biomarker extraction. Microscopy methods including FISH methods for Dhc are reported in the peer reviewed literature and have been demonstrated in various sample types despite this microbe's relatively small size. Quantitative FISH for Dhc is also commercially available.

2.3.2 Key Findings: Microbial Surrogates

There are a number of reports of microbial surrogate use in the peer reviewed literature. Microbial surrogates are not currently used in commercial methods for the quantification of Dhc in environmental samples. Several technical challenges and additional considerations need to be addressed in the context of developing an effective Dhc surrogate including:

- Surrogates should, ideally, be countable by plating (or other) methods which act as a validation/calibration approach for qPCR methods.

- *E. coli* is a commonly used microbial surrogate due to the ease of growth and genetic manipulation of this organism. Internal competitive controls cloned into *E. coli* are ideal in that they are unbiased in qPCR when compared with the target sequence, but *E. coli* may have the disadvantage of not sharing concentration and lysis properties with Dhc. In addition, internal competitive controls may lower detection limits of the target sequence.

- The use of non-*E. coli* surrogates may offer advantages in terms of size, cell wall, and lysis characteristic that more closely reflect Dhc. The major disadvantage of non-*E. coli* microorganisms is that culture methods and enumeration, genetic transformation, and preservation methods are less developed compared to *E. coli*.

- The size of the surrogate ideally should be similar to Dhc which is 300-500 nm in diameter, candidates include *Brevundimonas diminuta* (~200 nm), and *Micrococcus luteus* (1,000 nm).

- The cell wall and lysis properties of the surrogate should be similar to Dhc. Ideally, the surrogate will be amendable to plate count methods. *Halobacterium* sp. may meet these criteria, and this group has the advantage of being genetically modifiable for use as an internal competitive control (as with *E. coli* above).

3. SAMPLE COLLECTION AND PRESERVATION

The goal of sampling at bioremediation sites is to obtain analyzable samples that provide a representative "snapshot" of target microbial populations and/or activities. For MBT-based analyses, subsurface samples should be collected, stored and processed such that the amount, identity, and composition of signature biomolecules (i.e., biomarkers) can be determined and represent their true in situ abundance. An example of one major unresolved issue is the unknown distribution of target microbes between groundwater and the aquifer matrix, which is, most likely, variable (ESTCP, 2005). Despite the obvious importance of sampling procedures for MBT-based analyses, this portion of the overall procedure has received relatively little attention (Stroo et al., 2006). As stated by Chandler and Jarrell (2004) "it must be recognized that molecular microbial monitoring is an analytical process that begins with the environment, not a detector or sensor."

Beyond the requirement that MBT sampling procedures be accurate and representative, practicality and cost are critical considerations. While sediment samples may provide a representative snapshot of subsurface microbial communities attached to the aquifer matrix, the cost of obtaining subsurface cores can be prohibitive, the samples can only be taken from a location once (a severe constraint for temporal analysis), and sediments are subject to high heterogeneity. Groundwater samples are easier and less expensive to obtain and can be sampled repeatedly from the same well. The disadvantages of groundwater sampling may include the requirement of large volumes to recover sufficient biomass (Alfreider et al., 1997; Bekins et al., 1999; Vrionis et al., 2005), and the lack of standardized groundwater collection procedures. Despite these limitations, the practical advantages of groundwater sampling make it the preferred method of collecting subsurface biomass for MBT analysis. This section will focus on methods used to obtain biomass from groundwater samples, the potential for validation and improvement of these methods and the potential for use of preservatives to increase the stability of nucleic acids in sampled groundwater.

3.1 Review of Current Practice in Sample Collection

Sampling methods for extraction of nucleic acids from groundwater follow three general methodologies:

1) Groundwater sampling and laboratory filtration or centrifugation;

2) Field filtration; and

3) Retrievable media devices (RMDs; i.e., Bio-Traps®).

Each of these methods has inherent advantages and disadvantages as well as differing method development and validation needs, which are discussed in the following sections.

3.1.1 Direct Groundwater Sampling

Direct sampling methods involve the removal of water directly from a borehole using a pump or bailer and placing this material in a container, which is then shipped to the laboratory for further processing and analysis. The advantage of this method is that it is commonly used and field personnel are generally familiar with the procedure. Disadvantages include cost and inconvenience of shipping heavy and bulky groundwater samples, formation of precipitates, potential for changes in microbial composition and biomarker degradation during shipping and storage. Further, disposal of all contaminated groundwater received by the analytical laboratory increases cost and effort.

Sampling could be performed using standing well water; however, groundwater chemistry, hydrology, and the microbial community structure within the borehole are often dissimilar to that of the surrounding aquifer (Griebler et al., 2002; Stroo et al., 2006). Therefore, the use of initial volumes of well water for MBT analysis is not generally recommended and sampling typically involves well purging in order to obtain samples representative of the surrounding aquifer groundwater. Standard purge methods include:

High-flow Purge

Traditionally, the most common method of collecting groundwater samples involves using bailers or high speed pumps (>500 mL/min) to purge three to five casing volumes prior to sample collection. Although this method agitates the water column and can mobilize well sediment, the sample stream is often cleared up by the time the purge is complete (Stroo et al., 2006). Therefore, this method may decrease the turbidity of the sample, which may result in lower amounts of biomass in the sample.

Low-flow Purge

Low-flow purging (100-500 mL/min) is generally recommended to collect representative samples intended for contaminant, geochemical analysis, or MBT analysis. However, these low turbidity samples may underestimate the relevant concentration of biomass in the subsurface. A groundwater sampling protocol is being developed under ESTCP project ER-0518 (Application of Nucleic Acid-Based Tools for Monitoring Monitored Natural Attenuation [MNA], Biostimulation and Bioaugmentation at Chlorinated Solvent Sites). The low-flow techniques described in this protocol are in accordance with regulatory guidance for low flow sampling (Puls, 1996).

SiREM, a commercial provider of Dhc testing, recommends that purging be performed until concurrent oxidation reduction potential (ORP) measurements stabilize within 10%, indicating that groundwater from within the aquifer matrix is being sampled (SiREM Groundwater Sampling Protocol, Attachment B). SiREM also recommends surging (mechanical agitation of water screened section of the monitoring well) to increase the amount of aquifer material with attached biomass collected. Optional quality control blanks include trip blanks (a container filled

with distilled water at the well head) and equipment blanks (distilled water passed through non-dedicated equipment after standard decontamination procedures) in order to assess cross contamination. After sample collection, the water filled containers are placed in a cooler with ice packs and express shipped to the laboratory. Microbial Insights also makes use of direct sampling methods and like SiREM recommends a 1 liter (L) groundwater sample shipped on ice packs or ice. Although not explicitly stated in the Microbial Insights sampling protocol (Attachment C), Microbial Insights recommends purging until stable geochemical parameters have been reached.

Sample Volumes

Direct water sampling is used in other industries and sampling scenarios, for example: Haugland et al. (2005) (Table 3-1) used 1 L of beach water for detection of pathogens, McDaniels et al. (2005) and Brinkman et al. (2003) report sample volumes of 100 milliliter (mL) to 1 L to test drinking water for pathogens, Koike et al. (2007) used 100 mL to 1 L samples to test for antibiotic genes in lagoon and groundwater samples. In each of these cases, samples were shipped in coolers with wet ice or ice packs. These shipping protocols are similar to those used by SiREM and Microbial Insights for groundwater analysis. The only reported hold time (prior to filtration) was 6 hours (Haugland et al., 2005), which varies considerably from SiREM's hold times used for qPCR analysis of groundwater (2-14 days). Microbial Insights' standard procedures require filtration of groundwater samples within 24 hours after arrival.

DNA Preservation in Direct Sampling

While DNA is considered to be relatively stable (compared to ribonucleic acid [RNA]), it is still subject to degradation, which would impact the integrity of samples and the accuracy of subsequent PCR testing in the analytical laboratory. In general, degradation of DNA is enhanced under high temperature, acidic or alkaline conditions, and due to the activity of DNAses, enzymes that specifically degrade DNA. Preservatives may increase the stability of DNA in groundwater, thereby increasing the accuracy of the downstream analysis and potentially increasing possible hold times (i.e., storage of groundwater prior to biomass collection). Preservatives are not currently recommended in direct groundwater sampling methods used by the leading commercial providers of Dhc testing, including Microbial Insights and SiREM.

DNA preservatives include buffering agents, which maintain a neutral pH (Tris buffers are commonly used), dimethylsulfoxide (DMSO)/sodium chloride (Dawson et al., 1998), sodium azide (Kilpatrick, 2004), sodium dodecyl sulfate (SDS; an ionic detergent used at 0.1-1% wt/vol), and the neutral sugar trehalose (Smith, 2005). The most common DNA preservative, ethylenediaminetetraacetic acid (EDTA), works by chelating Mg^{2+}, an essential cofactor of DNAses. EDTA is typically added as Tris-EDTA (10 mM Tris, 0.1 mM EDTA, pH 8.0), which is a non-toxic, inexpensive solution that could easily be added to groundwater samples or groundwater-saturated filters prior to shipping. DMSO and sodium azide are toxic compounds, making them less suitable as general-purpose DNA preservatives and SDS can cause foaming

and enhance clogging of groundwater filtration units. If samples are small or concentrated in the field, other preservation options are available, such as storage in 70% ethanol or cryopreservation (dry ice), or several other commercial, proprietary chemical solutions (see following section).

Compared to DNA, preservation of RNA is much more problematic, as this nucleic acid is inherently less stable than DNA and the enzymes that degrade RNA (RNAses) are seemingly ubiquitous and more difficult to inactivate than DNAses. Initially, RNA preservation was achieved using cryopreservation or strong protein denaturants, such as phenol or guanidinium thiocyanate. A number of less toxic, more versatile RNA stabilization reagents have since been developed including RNAlater™ (Ambion), RNA-protect™ (Qiagen), and RNASafer™ (SuperArray Bioscience). A nucleic acid preservation method gaining prominence in the forensic and health arenas is Whatman's FTA technology. Aqueous samples are spotted onto FTA "cards" which trap and lyse bacterial cells. The filter matrix is embedded with chelators and denaturants that inactivate DNAses/RNAses, allowing released nucleic acids to be preserved at room temperature (reportedly for years). The cards are small, easy to use, designed for downstream MBT analysis, and are commercially available. Unfortunately, FTA cards can absorb only small sample volumes (< 1 mL); however, the manufacturer has recently developed the FTA concentrator, which can process up to 100 mL. Microbial Insights offers RNA based testing (see Attachment C-Bio-Flow Sampling Instruction Q-Expression [RNA]) and recommends the use of a liquid preservative that is added directly to the filtration unit to preserve RNA.

3.1.2 Concentration of Groundwater Biomass by Field Filtration Methods

Field filtration methods are commonly used for groundwater sampling for MBT analysis. In these methods the sampling well should be purged prior to groundwater sample collection, as described in 3.1.1. Groundwater samples can then be filtered on site at the location of sampling. This method has several advantages over laboratory filtration:

1. If necessary, large volumes of groundwater can be sampled in order to concentrate biomass;

2. Shipping costs and potential for sample loss are significantly reduced;

3. A greater range of biomolecule preservation methods can be immediately applied to the samples, prior to transport;

4. Iron oxide precipitates that form as the originally reduced groundwater becomes oxygenated upon sampling are avoided; and

5. Disposal of contaminated groundwater by the analytical laboratory is not required.

Current uncertainties associated with field filtration include the efficiency of biomarker recovery compared to off site (laboratory) filtration procedures. In addition, field personnel must receive proper training and gain experience in handling appropriate filtration devices, membranes, and how to deal with clogging. The commercially available filters were not developed for groundwater MBT analysis and improved designs are possible. If field filtration is more broadly applied, it is likely that manufacturers will offer new products specifically designed for this application.

Field filtration for concentrating microbial biomass has a long history in areas such as oceanography, limnology, and public health. The collection of microbial biomass from environmental waters is most commonly performed using normal flow filtration (also known as dead-end filtration) with commercially available microfiltration membranes, including those made of mixed esters of cellulose, polycarbonate, nylon, polysulfone, polyethersulfone, polyvinylidene fluoride, and cellulose acetate/nitrate. The configurations of these filters are typically flat, single layer, circular membranes ranging from 47-142 mm in diameter, but pleated or wrapped membrane filters incorporated into capsules have also been utilized. The primary advantage of using capsule filters is that the effective filtration area can be increased by 5-10 fold, which results in faster flow rates and allows for filtration of greater volumes in the presence of membrane foulants (> filter capacity). Additionally, the design of these capsules makes them ideal for shipping and storage. Examples of membrane capsule filters that have been used to concentrate microorganisms from environmental waters include: Millipore Sterivex™ filter cartridges (Humayoun et al., 2003; LaMontagne et al., 2003; Miyoshi, 2005; Vrionis et al., 2005), Envirocheck™ capsule filters (Simmons et al., 2001; Wohlsen et al., 2004), and Pall Gelman Maxi/Micro Culture filter capsules (Norrman et al., 1995; O'Connell et al., 2003). The Envirocheck capsule filter (nominal pore size of 1.0 µm) was developed specifically to recover *Cryptosporidium* oocysts and *Giardia* cysts from aqueous samples and is employed in the U.S. Environmental Protection Agency's standardized method 1623 specifically for this purpose (USEPA, 2001). Field filtration has been used significantly by Microbial Insights for the purposes of collection of biomass for subsequent DNA and/or RNA recovery. Microbial Insights recommends the use of Millipore Sterivex filter cartridges (0.2 µM Supor® membranes, hydrophilic polyethersulfone) using a peristaltic (or other) pump to filter 1-2 L of groundwater. If the filter plugs the volume passed through is recorded. If less than 1 L is filtered prior to clogging then a second filtration unit is used.

Filter Clogging

Clogging of 0.1-1.0 µm membrane filters by natural waters occurs largely due to small colloids (<100 nm in size) that adhere to pore channels, eventually causing pore blockage (Howe, 2002; Kimura et al., 2004; Laabs, 2006). For these foulants, pre-filtration based solely on size exclusion would be ineffective at preventing clogging. Materials sloughed from biofilms, planktonic cells, and dead cells (polysaccharides, lipids, peptidoglycan, etc.) are also highly effective foulants (Kimura et al., 2004; Xu, 2005; Laabs, 2006). During extended periods of

normal flow filtration (>15-20 minutes) microbial cells have even been shown to strongly attach to membrane filters via the ongoing production of extra polymeric substances (Wang, 2005; Xu, 2005). Conditions that would favor this type of clogging scenario include: a high ratio of dead to living microbes, microbial communities that produce substantial amounts of EPS or other extracellular matrices, and cell disruption due to vigorous pumping or filtration. Also of note, calcium can play a role in membrane clogging by contributing to the formation of large, humic or fulvic acid-derived aggregates that can lead to pore blockage and cake layers on membrane surfaces (Schäfer, 2000; Yuan, 2002).

Pore Size Considerations

Pore sizes for micro-filters range from approximately 0.05-10.0 µm. Bacteria are retained and can be recovered, often at high efficiency, by filters with pore sizes larger than the diameter of the target cells (Zierdt, 1979; Smith et al., 1993; Millipore, 2000). For applications such as sterilization or detection of pathogens, less than 99.9% retention of cells may be unacceptable, yet for the purpose of monitoring in situ bioremediation, it may be acceptable to have much lower levels of retention. The Dhc target cells are small (<0.5 µm), and researchers typically use membranes with pore sizes ranging from 0.1-0.45 µm. Larger-porosity-size filters (0.45–1.0 µm) could be used to capture a known percentage (e.g., >80%) of Dhc, while significantly decreasing the problems associated with membrane clogging by micro- or nano-sized foulants. Additionally, if most of the target cells naturally form aggregates or are attached to suspended particles, it will be more important that the filters be designed to capture the particulates, while the absolute cell size may be irrelevant. From these considerations, it is obvious that the choice of pore size will need to be evaluated empirically, or at least until consistent trends emerge for particular target microbes (i.e., *Dehalococcoides*, *Geobacter*, *Cryptosporidium* oocysts, etc.) or aquifer types.

Other Filtration Methods

Tangential flow filtration is well-suited for concentrating particles in very dilute samples, and has frequently been used to concentrate microbial biomass from seawater (Giovannoni et al., 1990; Venter et al., 2004) and has been the method of choice for concentrating microbes from deep, rock aquifers (Fry et al., 1997; Erwin et al., 2005). Researchers have recently begun to systematically evaluate and optimize tangential flow filtration technologies with the specific aim of recovering microorganisms and/or biomolecules from a wide variety of other sources. Hill et al. (2005) report recovery efficiencies of 70-93% for seeded bacteria in 10 L of tap water using tangential flow ultrafilters (hollow-fiber polysulfone ultrafilters with a molecular weight cutoff (MWCO) of 15,000-20,000 Da) (Hill et al., 2005). In another study, evaluating tangential flow filtration, polyacrylonitrile, hollow fiber ultrafilters (50,000 Da MWCO) were used to recover bacteria, protozoa, and viruses from surface waters that had been collected from different locations across the United States (Morales-Morales et al., 2003). Recoveries of spiked *E. coli* cells from 10 L of the various surface water samples ranged from 86-97%, including from

extremely turbid samples (up to 29 nephelometric turbidity units) from the Rio Grande River. Because the focus of tangential flow filtration research in terms of environmental applications has traditionally been on viruses, ultrafiltration has been the *de facto* method of choice. With bacteria as the primary target it may be most feasible to evaluate the application of tangential flow units incorporating microfilters, such as the experimental pleated cross-flow capsule (1,000 cm^2 filter area, 0.2 µm pore size) examined by Tanny et al. (1980) over 25 years ago. Advantages of tangential flow filtration as a method to recover biomass from groundwater include: the potential to filter large volumes of water, filters are less susceptible to clogging than with normal flow devices, the retentate is a liquid suspension, and the procedure is gentler on cells than that of pressure-driven normal flow filtration or centrifugation. The disadvantages of this approach include: the time it takes to process a water sample by recirculation can be substantially longer than for normal flow filtration, the devices are more complicated and less user friendly (especially under field conditions) and the apparatus increases cost. Tangential flow microfilters may prove to be an excellent means of concentrating bacteria from a variety of natural waters, but the method has simply not been utilized and tested for groundwater. Furthermore it is not clear there is a pressing need to process large volumes of groundwater for Dhc testing, or whether obtaining large volumes from typical wells at remediation sites is even practical.

In the early 1980's several studies demonstrated that charge-modified cellulose diatomaceous earth resins (Zeta-plus depth filters) could be used to effectively concentrate bacteria from large volumes of tap and surface water (Goyal et al., 1980; Hou et al., 1980; Mathewson et al., 1983). The Zeta-plus filters examined in these studies contained charged, microporous media with nominal pore sizes ranging from 0.2 to 2.0 µm, so retention was a function of both size exclusion and charge capture. Using *E. coli*- or *Salmonella*-seeded tap water, Goyal and Gerba (Goyal et al., 1980) processed 20 L samples in 10 min using a Zeta-plus filter with a nominal pore size of ~2.0 µm and recovered ~30% of the cells (filter retention was ~95%, thus improvements in cell elution would greatly improve this method). In another study the same filter type was used to recover 35.0% and 24.2% of spiked *Campylobacter jejuni* cells from a 100 mL sample of tap water and surface water samples, respectively (Mathewson et al., 1983).

A systematic evaluation of the performance of different filter types with groundwater, required minimum and maximum, filtration volumes, and a comparison of the results with respect to biomass recovery, allowable hold times, detection limits, etc. would be possible with surrogate calibrated reference cultures. Even so, a comparative examination of this type has not yet been performed. This is a significant shortcoming given that most of the filters used for this purpose were not designed for groundwater filtration for biomass collection. Specifically, methods that decrease filter clogging, allow maximum biomass recovery, and are compatible with downstream processing will increase the practicality and accuracy of MBT analyses.

3.1.3 Retrievable Media Devices (RMDs)

RMDs are groundwater sampling tools (e.g., biofilm coupons, in situ microcosms, groundwater dialysis chambers, porous beads) that facilitate the colonization of subsurface microorganisms onto a retrievable matrix. The nature of RMDs is they are not quantitative in the sense that it is virtually impossible to relate microbial concentrations in the groundwater or aquifer matrix to those detected on the RMD. This is due to biases imparted by the media and the fact that the groundwater to which these devices are exposed is standing well water of unknown quantity. The simplest form of RMDs are biofilm coupons, which are artificial growth surfaces which have been used to monitor biofilms from disparate environments including spent nuclear fuel pools, oil pipelines, and even the human body (Donlan, 2001; Sarró et al., 2003; Neria-Gonzalez et al., 2006). Although biofilm coupons were originally made of glass, steel, plastic film, etc., they can also be derived from materials more representative of the subsurface strata (Dodds et al., 1996; Långmark et al., 2001; Griebler et al., 2002; Peacock et al., 2004; Roberts, 2004). Diffusive polyethylene containers have been filled with various minerals and silicates and placed down boreholes to determine the attachment preferences of natural microbial populations in groundwater (Reardon et al., 2004; Roberts, 2004; Reardon, 2005). Researchers have also used more complex attachment surfaces mirroring that of the local subsurface, such as repacked cores or sediments (Hirsch et al., 1990; Nielsen et al., 1996; Griebler et al., 2002; Mailloux et al., 2003).

Microbial Insights has developed a commercially available RMD, called Bio-Trap® samplers to assess populations in the subsurface (Peacock et al., 2004). Bio-Trap® samplers contain Bio-Sep® beads (2-3 mm in diameter) as the capture matrix. Bio-Sep® beads have been used to recover biofilm biomass to assess bioremediation in tetrachloroethene, BTEX, and metal contaminated aquifers (Istok et al., 2004; Jardine, 2004; Sublette et al., 2006). These studies demonstrated that changes in aquifer microbial ecology and geochemistry were more apparent when biomass was recovered from Bio-Traps® than with groundwater samples. Compared with other RMDs incubated in wells, the Bio-Traps® more efficiently accumulated biomass in situ (Sublette et al., 2006).

Advantages of RMDs include ease of storage and transport, capture of sufficient biomass for MBT analysis, and RMD arrays may provide a more accurate temporal and spatial representation of the subsurface microbial community surrounding the site of collection than can be gleaned from groundwater samples (Peacock et al., 2004). Disadvantages of RMD applications include uncertainty as to the similarity of Bio-Trap® samplers to groundwater geochemistry and the physical conditions of the aquifer matrix, and hence, whether they truly mimic aquifer conditions from a microbe's point of view. Therefore, it is unclear if biomass captured with Bio-Trap® samplers truly represents the microbial community composition (i.e., richness and evenness) of the surrounding aquifer. These factors must be considered when interpreting Bio-Trap® sampler data, in particular when quantitative information is evaluated. Questions surrounding suitable incubation times are another issue with RMDs that have not been thoroughly addressed. Despite

the fact that RMD are potentially effective concentrators of biomass, the usefulness of such devices for site evaluation and quantitative Dhc monitoring has yet to be established.

3.2 Use of Surrogates in Sampling Shipping and Storage Procedures

The application of microbial surrogates (Section 2) during sampling shipping and storage of samples prior to extraction of nucleic acids is not widely reported. Microbial surrogates in this context could be lyophilized or dry ice frozen surrogates applied to water samples directly after sampling or passed through field filters. Surrogate application during sampling would allow the quantitative assessment of losses associated from the point of sampling including those related to shipping, use of preservatives, sample specific hold times, and to quantify losses associated with biomass recovery from field filters. Intriguingly, this kind of surrogate could also assess biomass gains, for example if growth of the surrogate occurred during prolonged storage at warm temperatures, recovery could exceed 100%. Practical considerations aside, the application of surrogates at the sampling stage is in a sense the ideal point for application as it would allow the assessment of biases throughout the entire analysis stream from shipping to final analysis. This approach would require the ability to ship stable surrogates in a form that would be stable and easy to use, for example lyophilized cells. For filtration purposes, these surrogates could be resuspended in a volume of groundwater and subsequently filtered with the native biomass. This approach would allow a quantitative assessment of filtration efficiency. The use of surrogates is most likely not relevant to RMDs due to the fundamentally non-quantitative nature of these devices and difficulties applying the surrogates to a solid matrix in any way that replicates the in situ attachment of biomass.

3.3 Key Findings

Examination of the breadth of methods used for sampling and preservation lead to the identification of several areas that have the potential to significantly impact results obtained, key finding include:

3.3.1 Key Findings, Related to Groundwater Sampling and Preservation Methods

- Effect of purging and surging pre-sampling activities
    - High-flow versus low-flow purge methods has the potential to impact sample characteristics and biomass recovery.
    - Purge volume may impact the stabilization of biomass recovery with subsequent volumes differing in composition until stabilization occurs.

- Surging is intermittently used in commercial sampling methods and may impact biomass recovery and the representativeness of the sample of the aquifer microbial community.

- The use of smaller groundwater volumes has positive implications for cost as well as the practicality of sampling from low flow environments. The key question is can data quality in terms of consistency and detection limits be maintained with smaller samples?

- Preservation of Samples

  - Preventing cell lysis prior to concentration of biomass is important, otherwise loss of NA in the concentration step is likely.

  - A number of common DNA preservatives, including Tris EDTA and trehalose, have potential to be used as nucleic acid preservatives applied directly to groundwater samples. FTA cards (Whatman) are easy to use and samples as large as 100 mL can be processed. FTA cards are potentially useful for preservation and extraction of NA from groundwater for MBT testing.

  - A variety of commercially available RNA preservatives are available which are typically applied to concentrated biomass. These include Trizol, (Invitrogen) RNAlater (Ambion), RNA-protect (Qiagen), and RNASafer (SuperArray Bioscience).

- Hold times

  - A variety of hold times are reported in peer reviewed literature and commercial MBT analyses. These times ranged from 6 hours to 14 days. The rationale for the designated hold times is often unclear and may be arbitrary.

  - Practical hold times may be impacted by storage temperature and use of preservatives. Longer hold times may decrease costs and increase the flexibility of sampling and testing is possible.

3.3.2 Key Findings, Field Filtration Methods

Findings with respect to field filtration methods include:

- Field filtration methods have inherent advantages and disadvantages compared with laboratory filtration. Advantages include: reduced shipping costs, ability to sample larger groundwater volumes, and groundwater disposal at the laboratory is not required. Disadvantages include the potential for irreversible adhesion of cells due to longer residence time on the filter, and the fact that more complex sampling procedures are

required. However, factors affecting field filtration are also relevant for laboratory filtration protocols.

- A variety of dead end filters have been used for field filtration of water samples most of which were not explicitly designed for this purpose. This raises the issue of the applicability of filter types for groundwater sampling and whether design improvements are possible.

- Filter clogging is a major issue in filtration and is dependent on the characteristics of the groundwater and of the filter. The use of larger pore size filters may in some cases reduce issues related to clogging without comprising the efficiency of capture of target microorganisms.

- Other (non-dead end) filtration methods such as tangential filtration and Zeta-plus depth filters have certain advantages with respect to their ability to filter large sample volumes. They do have the ability to recover cells more gently and are resistant to clogging. Their use in groundwater sampling, however, is currently not well developed.

3.3.3 Key Findings, Retrievable Media Devices

Key findings related to RMDs include;

- A wide variety of media types have been used for these devices ranging from various minerals and silicates to repacked cores and sediments. A commercially available version of an RMD is the Bio-Trap® offered by Microbial Insights.

- RMDs have the potential to be effective monitoring tools and offer a number of advantages over conventional sampling approaches. The advantages include the ability to concentrate biomass, the ability to reflect average groundwater conditions, and simplicity of sampling methodologies.

- RMDs are not a quantitative sampling approach but rather a biomass concentration method that is better suited to qualitative methods

3.3.4 Key Findings: Use of Microbial Surrogates in Groundwater Sampling

- The use of microbial surrogates in groundwater sampling protocols is not widely practiced/ reported but could provide benefits in terms of assessing biomass losses throughout shipping, storage, and processing.

- The use of lyophilized surrogates would be a practical method of implementing microbial surrogate use in groundwater sampling protocols.

- Microbial surrogates could be used in field filtration methods to measure biomass recovery including release of biomass from filters.

4. NUCLEIC ACID EXTRACTION

The nucleic acid (NA) extraction step is critical for subsequent detection/quantification of DNA or RNA and interpretation of DNA- or RNA-based analyses. Indeed, since the introduction of the PCR in the 1980s, one of the biggest challenges has been to produce high quality, PCR amplifiable DNA from the sample of interest (Mumford et al., 2006). A significant aspect of these challenges is due to the immense variability associated with environmental remediation samples including wide ranges in pH, solids concentration, biomass concentration, and interfering substances. The level of variation in the extraction of NA from environmental samples is undoubtedly greater than many media tested by quantitative MBTs in the medical (blood/urine/samples), agricultural (plant materials/manure), food (milk/meat/eggs/grains, etc.), and forensics (blood/semen/saliva) fields where samples tend to be more homogenous. Due to the wide range of conditions encountered in groundwater and sediment samples, the development of a "one size fits all" NA extraction protocol is highly unlikely. Instead, the use of a number of flexible, multi-phase (primary + secondary purification steps and internal controls) protocols for extraction of NA from environmental samples is required.

There are five components in a complete nucleic acid extraction protocol. These are: 1) Sample processing to concentrate or enrich biomass, 2) Cell lysis, 3) NA purification, 4) NA quantification, and 5) Storage of NA until further analysis. At each step, potential exists for introducing error, losing or degrading the NA, and co-purifying inhibitors of subsequent analyses. Current practices are reviewed below (Section 4.1) followed by a summary of key findings (Section 4.2).

4.1 Review of Current Practices for Nucleic Acid Extraction

Environmental samples collected for nucleic acid extraction generally fall into one of two categories: liquid samples containing suspended solids, such as a groundwater, surface water and microbial cultures, or solid samples, such as soil, sand, clay or mixed subsurface solids. This review will first focus on processing steps for liquid samples, as these are most commonly used in site assessment and remediation. NA acid extraction from solid samples is described subsequently (Section 4.1.5). Moreover, this review will focus on methods for DNA extraction, as DNA is, by far, the most common target for current MBTs. RNA extraction methods are discussed briefly in Section 4.1.6. It is worth noting that many of the procedures, issues and potential improvements to the QA/QC of DNA extractions from liquid samples will be equally applicable to extraction from solid samples and for extraction of RNA.

4.1.1 DNA Extraction from Liquid Samples Step 1: Concentration

For liquid samples, cell lysis and NA purification is rarely performed directly on a water sample, but rather on concentrated biomass. A concentrated sample is required for recovery of sufficient NA for detection and quantification. The two most common approaches are filtration and

centrifugation. Both of these methods also remove dissolved substances that can potentially contaminate DNA and interfere with subsequent analyses (i.e., inhibition). The efficiency of these initial processing steps is critical to the accuracy and reproducibility of all downstream procedures.

Centrifugation of groundwater samples to concentrate biomass has the advantage of removing issues surrounding recovery of the biomass from filters, but overall the effectiveness of centrifugation is rarely assessed. Centrifugation is subject to operator bias, particularly in the resuspension and recovery of pelleted material, especially in cases with near invisible pellets. These issues could be addressed with internal controls applied to the initial groundwater sample. The use of centrifugation for concentration of bacterial biomass in liquids is common. Examples include: centrifugation of environmental water samples for extraction of Dhc DNA at 9,000 x G (Hendrickson et al., 2002); centrifugation of groundwater samples at 17,700 x G to detect bacteria with tetracycline resistance genes (Koike et al., 2007); and centrifugation at 4,600 x G for extraction of *Clostridium* spores from milk (López-Enríquez et al., 2007). As can be seen by these few examples, there is a wide range of centrifugation speeds used. The capture efficiency varies as a function of cell size, density, degree of flocculation or particle association. Moreover, the centrifugation time and temperature are also important parameters that affect the efficiency of the procedure. Many research laboratories use centrifugation to minimize manipulations as compared to filtration and sample sterility is easier to maintain. However, for environmental samples, sterility is not a major concern. Centrifugation using an approach similar to that used by Hendrickson et al. (2002) was initially used by SiREM for commercial semi-quantitative Dhc assays; however, SiREM currently uses a filtration approach, as does Microbial Insights.

Concentration by filtration is the most commonly practiced approach for environmental samples. In this approach, the filtration, or capture step, is followed by either the removal of the solids from the filter prior to cell lysis, or direct lysis of cells on the filter membrane. Filtration of water samples prior to NA extraction is widely reported in the literature for filtration of drinking water (Brinkman et al., 2003; McDaniels et al., 2005), beach water (Haugland et al., 2005), wastewater and storm water (Jiang et al., 2005) and groundwater (Lehman et al., 2002; Miyoshi, 2005). As mentioned above, both SiREM and Microbial Insights use filtration prior to NA extraction with SiREM using a Nalgene cellulose nitrate membrane which is pulverized in the bead mill tube used for extraction of DNA.

All commercial membrane filters and membrane filter capsules (other than the Envirocheck™ (co) capsule and the similar Cryptest™ membrane filter cartridge [Whatman]) were developed as water purification devices, not as a biomass collection tools. As such, improvised methods have been developed to remove cells and/or biomolecules from these commercial filter matrices. Almost universally, physical disruption methods are preferred. Cells are dislodged from membranes via rinsing, sonication, vortexing, bead beating or back flushing in the presence of medium or buffer, with or without ionic or non-ionic surfactants (Smith et al., 1993; Wilson et al., 1999; Lehman et al., 2002; Weinbauer et al., 2002; Bostrom, 2004; Poretsky, 2005).

Commonly, membranes are frozen, then crushed with a mortar and pestle or cut into small pieces prior to dislodging cells by vortexing. Some methods call for cell lysis directly by bead beating. With capsule membrane filters, particularly Sterivex filters, protocols have been developed for chemically lysing the cells within the filter housing, and recovery of the solution via back flushing.

Relatively few studies have been performed to systematically evaluate cell recovery from membrane surfaces. It has been determined that the topography and chemistry of the filter matrix are important factors, and that bacterial cells can adhere, primarily through electrostatic interactions, to membrane surfaces (Zierdt, 1979; Bustnes et al., 2004). Additionally, it has been found that bacteria can attach to membrane surfaces during extended periods of filtration or storage (>30 min) via extra cellular polysaccharides and other cell surface macromolecules (Wang, 2005; Xu, 2005). Also groundwater can contain particulates made up of varying amounts of different soils and/or clay, that can also influence extraction efficiency (Zhou, 1996; Frostegard, 1999).

It has been demonstrated that the addition of surfactants, salt and sugar solutions, and other agents to wash buffers can significantly improve cell recovery from membranes. Non-ionic surfactants minimize hydrophobic interactions between organic molecules and filter surfaces; Tween 20 and Tween 80 have been found to perform well with a wide range of membrane types (Gross et al., 1995; Långmark et al., 2001; Hill et al., 2005). Another class of compounds that have proven useful in regards to removal of cells from membranes are polyphosphates (Hill et al., 2005). Depending on the target microbe(s) and the chemistry of the groundwater sample, the choice of washing agents should be determined empirically through the use of microbial surrogates and reference cultures.

Even less research has examined parameters affecting nucleic acid extraction directly from membrane surfaces. In the most complete evaluation of this type to date Burke et al. (2004) determined RNA yields and quality from eukaryotic yeast cells captured on membrane filters composed of cellulose acetate, mixed esters of cellulose, polycarbonate, polyvinylidene fluoride, and regenerated cellulose. RNA yields and quality were similar for each of the membrane types examined, with the exception of the polycarbonate filters, in which case leakage around the periphery of the membrane during filtration was suspected. The authors concluded that the choice of membrane filter should be dictated primarily by ease of filter handling, chemical compatibility, levels of filter extractables (downstream chemistry, such as PCR or cDNA synthesis, should not be inhibited by compounds released from the filter media during cell or biomolecule recovery), flow rates and clogging characteristics associated with the sample to be filtered. Yeager et al. (unpublished results) performed experiments to determine if there were gross differences in the yield, quality, and composition of RNA isolated from a mixture of the two bacterial species using several common membrane filters (polycarbonate, Supor, Duraore, nylon, and cellulose acetate) and three commercial RNA extraction methods. The data are largely congruent with those of Burke et al. in that the quantity and quality RNA extracted from

different membrane types was rather similar. The yield and integrity (23S/16S rRNA ratio) of total RNA were highest with the least disruptive RNA extraction method, which employs enzymatic/chemical lysis steps rather than bead beating. Though these last two studies were conducted with RNA (see Section 4.1.7), the filtration results are also applicable to DNA extraction and recovery..

4.1.2 DNA Extraction Steps 2&3: Cell Lysis and NA Purification

After concentration, the next critical step in NA extraction is cell lysis to liberate the nucleic acids. Cell lysis efficiency is highly variable and often difficult to measure. It depends both on the matrix and the cell type. Cell lysis can be affected using physical methods (bead-beating, sonication, freeze/thawing, or grinding under liquid N_2), chemical methods (detergents, salts and solvents) or enzymatic methods (such as lysozyme and proteinases) or a combination of these methods (Miller et al., 1999). After cell lysis, nucleic acids are purified from other cell components (proteins, lipids, etc.) and from other sample components such as minerals (for example FeS) and organic particulates or debris using extraction, precipitation and/or column filtration.

A wide variety of DNA extraction and purification methods have been developed for different sample matrices (Miller et al., 1999; Mackay, 2007). Specific approaches exist for extraction of DNA from blood, semen, plant materials, tissue samples, fecal material, and soil, and from specific organism types, including plants, bacteria, yeast, and fungi. Soils and sediments are particularly challenging matrices because PCR inhibitors such as humic acids may co-purify with DNA. Conventional DNA extraction methods based on cell lysis, followed by liquid-liquid extraction and DNA precipitation are time-consuming and susceptible to errors. Miller et al. (1999) provide a very detailed review of nine different extraction procedures and four different DNA purification methods using soil and sediment samples. The most effective method included bead-mill homogenization in phosphate-buffered SDS chloroform followed by column purification using a Sephadex G-200 column. Such extractions typically take hours to 2 days to complete.

Standardized, commercially available kits to extract nucleic acids from complex matrices in a consistent fashion have become widely available over the past decade. A variety of protocols and kits are now available for nucleic acid extraction from different matrices. In a recent review by Mackay (2007), over 19 different companies selling over 38 different kinds of NA extraction kits are listed (Table 3.1 in Mackay (2007)). The time to extract and purify DNA using these kits range from 10 minutes to approximately 2 hours. Examples of kits tailored to specific applications include the MoBio Laboratories Power Soil™ kit, the GFX Genomic Blood DNA Purification kit (GE Healthcare), and the DNeasy Tissue Kit™ (Qiagen). Despite the continued improvements in soil kits, there is still no consensus on the best method for extracting prokaryotic DNA from groundwater, soil and sediment samples. Also, no satisfactory protocol is available to assess cell lysis efficiency. This is particularly problematic because cell lysis

efficiency varies between protocols. Moreover, a tradeoff exists between using aggressive lysis steps and subsequent downstream uses (i.e., PCR amplification) of extracted nucleic acids in that aggressive lysis may impact the quality of the DNA While some methods have better lysis efficiency and higher overall DNA recovery, this DNA may be contaminated with substances that inhibit subsequent PCR amplification, or may be otherwise impaired for PCR analysis (e.g., shearing). . All of these factors need to be considered when selecting and performance monitoring of a NA extraction approach is undertaken.

In addition to diverse matrices, DNA extraction techniques, particularly for environmental samples, need to consider the diversity of cell types in the sample. Ideally, all target cells are lysed with 100% efficiency, or with a consistent (high) and quantifiable efficiency. Considering the diversity of microbial cell types in the subsurface, this requirement is daunting. In fact, there is clear data showing that for complex mixed samples, no one DNA extraction method is suitable for all the microbes in the sample. For example, DeSantis (2005) found that the length of bead beating for cell lysis greatly affected the ability to subsequently detect certain phylotypes in the sample, with some phylotypes being detected (i.e., lysed) when short bead-beating times were used, while others were only detected with longer bead beating times; however these latter conditions caused degradation of the DNA from the easily lysed cells that then were then no longer detectable. This is a disastrous situation if attempting to study or recover the whole diversity of populations within a sample. Fortunately, for the quantification of a known specific microorganism where lysis procedures can be optimized for that particular cell type (for example for the quantification of Dhc), the task is less daunting.

A number of researchers have deliberately compared various NA extraction methods for example (Carrigg, 2007; Cook et al., 2007), yet there is no consensus on which method is superior. Rather, performance of individual methods varies considerably, especially from soil and sediment samples where the matrix properties are variable. For example Cook et al. (2007) observed 3 orders of magnitude difference depending on extraction method. Tailor-made laboratory methods have generally been found to outperform commercial kits in terms of NA yield and purity (Miller et al., 1999). However, the performance margin is slim in some cases, and the availability, handling, time, and cost benefits afforded by the commercial kits make them more attractive for standardization. From our experience and an exhaustive literature survey, the MoBio Powersoil and Bio101 FastDNA™ kits are among the top candidates for NA extraction from sediments or other “dirty” samples including groundwater. The MoBio Powersoil™ kit is used by both Microbial Insights and SIREM in commercial Dhc testing protocols. For NA extraction from filtered samples, less disruptive methods (e.g., Qiagen’s Bacterial kits) may provide benefits and options if current commercial protocols are found to be insufficient.

Optimizing protocols for every type of groundwater or every treatment is not practical (Purdy, 2005; Sharma et al., 2007). In their review, Sharma et al. (2007) conclude that only a few attempts have been made to determine extraction efficiencies that allow quantitative correction for losses. Because there is currently no accurate method to measure the total bacterial NA

concentrations in sediment, soil or groundwater, extraction efficiencies have primarily been estimated using labeled cells or plasmids with a specific sequence which are spiked into the sample. Recoveries in the range of 20-90% have been documented (Mumy et al., 2004; Sharma et al., 2007), nevertheless recovery total biomass may be less variable than for spiked targets DNA Extraction Step 4: Quantification of NA Concentration

Once the DNA has been purified and concentrated into an appropriate storage buffer (see Section 4.1.4 for more information regarding storage of NA samples) the concentration of nucleic acid needs to be determined in order to assess, among other things, the success of the extraction. Nucleic acid quantification is not a trivial procedure, and significant error can inadvertently be introduced at this stage for a variety of reasons. NA quantification can be affected by the type and length of the NA, the presence of contaminants, instrument calibration, and operator accuracy in performing small volume dilutions. Moreover, not only is this determination required of a DNA sample post-extraction, it is also required to quantify DNA in standards used to generate calibration curves for quantitative real time PCR (see Section 5.2.2 for more information regarding Calibration). Thus if the measurement is erroneous, it potentially affects both the accuracy of calibration, as well as the quantification of the amount of sample DNA.

The simplest method for assessing DNA concentration is using UV absorbance at 260 nm (A260). This analysis can be performed using a spectrophotometer, and the readings converted to concentration using extinction coefficients specific to the type of NA being assayed (Mackay, 2007). These conversion factors are valid only at neutral pH and are based on a standard path length of 1 cm (Qiagen Bench Manual):

- 1 O.D. at 260 nm for double-stranded DNA = 50 µg/mL
- 1 O.D. at 260 nm for single-stranded DNA = 33 µg/mL
- 1 O.D. at 260 nm for RNA molecules = 40 µg/mL
- 1 O.D. at 260 nm for oligonucleotides = 20-30 µg/mL

These extinction coefficients are widely used, although rarely is the particular instrument in question calibrated with DNA of known concentration to calibrate and confirm reproducibility.

Spectroscopic methods can also provide qualitative information on the purity of the DNA. Most protocols suggest monitoring the A260/A280 ratio as an indicator of the purity, as proteins and phenol absorb around 280 nm. A ratio of A260/A280 greater than 1.8 is typically considered acceptable, meaning the DNA is pure enough for most downstream procedures. Spectrophotometric methods are best for samples with concentrations greater than 250 ng/mL (Mackay, 2007), and with standard spectrophotometers require enough volume of sample to fill a small cuvette. More recently, the Nanodrop™ Spectrophotometer (Nanodrop Technologies, Wilmington, DE) has been developed that has a wider dynamic range (2-3700 ng/ml) and most

importantly requires as little as 1 microliter (µL) of sample. Nanodrop spectrophotometry has become the method of choice in many labs including SiREM. While spectrophotometric methods are simple and widely used, these methods are not particularly sensitive, cannot distinguish DNA from RNA, and are sensitive not only to the type of NA, but also to the type of buffer dissolving the DNA. A260 values are reproducible in low salt alkaline buffer, but not in water, owing to changes in pH induced by absorption of carbon dioxide from air (Qiagen® Bench Guide, www.quiagen.com).

The uses of fluorescent dyes, such as Picogreen, Ribogreen, or Oligogreen (Invitrogen, Carlsbad, CA) are also very effective and widely used, particularly for high throughput analyses. These fluorometric methods can be carried out in microtiter plates using small volumes at low concentrations and have defined excitation and emission spectra upon binding to nucleic acids. Moreover, dyes have been developed that preferentially bind to certain kinds of NA, such as double stranded DNA or RNA. These methods are considerably more sensitive and have a greater dynamic range than spectrophotometric methods (Mackay, 2007). Some substances in environmental samples may interfere with fluorometric NA quantification (Zipper, 2003; Carrigg, 2007), although there are indications that some assays may perform satisfactorily (Lloyd-Jones et al., 2001). An extensive statistical comparison of spectrophotometry compared to fluorometry established that for relatively concentrated DNA samples, spectrometry outperformed fluorometry in terms of reproducibility, although the primary source of error was not the assay per say, but the error introduced in making dilutions (Haque et al., 2003).

Probably the most accurate method of quantifying NA concentrations in extracts from environmental sources is by comparison of total DNA band intensity in ethidium bromide-stained agarose gels relative to a series of lambda DNA standards (Miller et al., 1999). The disadvantages are that electrophoresis is time consuming and it can be difficult to quantify sheared NA samples. Nevertheless electrophoresis could be used in method development for assessing the quality of the extracted DNA. Electrophoresis is also the method of choice for qualitative and quantitative assessment of RNA quality and extraction efficiency. Most recently, microfluidic platforms that combine capillary electrophoresis, fluorometric analysis, and detection have been developed that generate an electropherogram for quantification of individual size fractions of a NA sample (Agilent 2100Bioanalyzer or the Biorad Experion™ systems). These platforms were developed because of the need to rapidly assess RNA quality on small volumes of samples for high throughput transcriptional analyses. While more costly per sample than other methods, these systems have the advantage of separating sample components from contaminants prior to quantification and thus are more accurate and provide a clearer picture of the degree of degradation or contamination of a particular sample (Mackay, 2007).

Because of the time-savings and handling ease that fluorometric methods provide, as well as their increased sensitivity and greater linear range compared to UV based spectrophotometric methods, a comparison of NA quantification using spectrophotometry and commercially available fluorometric assays with a range of subsurface samples may be warranted.

4.1.3 DNA Extraction Step 5: Detection and Removal of PCR Inhibitors

PCR inhibition is a commonly observed phenomenon potentially leading to false negatives or underestimates of the actual biomarker target concentration. Inhibition is caused by compounds present in the original sample or in some cases in the DNA extraction reagents, or plastic ware that are carried through the DNA extraction process into the final NA sample and affect PCR amplification. Compounds reported to inhibit PCR reactions include lipids, proteins, and high concentrations of 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). Kontanis et al. (2006) report that tannic acid at 1.4 ng/25 µl reaction is completely inhibitory to TaqMan® PCR with more moderate inhibition observed at lower concentrations. In addition, contaminated sites have the potential to harbor a wide variety of chemical contaminants and amendments (e.g., electron donors, oxidizing/reducing agents) which have the potential to impact DNA stability, DNA extraction procedures, and PCR amplification. Based on a survey of groundwater samples submitted for commercial analysis for Dhc, complete inhibition was observed for approximately 3 % of groundwater samples (SiREM, unpublished data). Incomplete inhibition, where some PCR amplification is observed is not currently quantified and most likely affects a significantly higher percentage of samples, this could lead to underestimates of target microbes

PCR inhibition can be diagnosed and possibly corrected during the nucleic acid quantification process in several ways. One of the simplest approaches for assessing, and potentially overcoming inhibition, is dilution of the extracted template DNA sample. Dilution of the inhibitor may lead to positive amplification whereas the undiluted sample yields a negative result. For example, if a sample is diluted 10-fold thereby lowering the inhibitor concentration, specific target amplification may occur, since PCR is still effective with very low template concentrations. Xiao et al. (2006) used a modification of this approach by using varying dilutions of template ranging from 0.5 to 3 µl to assess inhibition.

Another common approach for assessing PCR inhibition is testing DNA samples with universal, 16S rRNA gene-targeted primers. If amplification with these primers does not occur this is an indication that inhibitors may be present. In the literature, the dilution approach using universal primers was the most common method for assessing inhibition (Koike et al., 2007). Furthermore this approach is used by SiREM and Microbial Insights. Further confirmation of the presence of inhibitors can be carried out by spiking samples with a calibrated amount of a known plasmid template, and using PCR primers specific to this target in order to assess the degree of inhibition. The latter is certainly a recommended practice for environmental samples. These issues are also described in more detail in Section 5.1.5.

4.1.4 DNA Extraction Step 6: Storage of NA samples prior to analysis

In many cases, extracted nucleic acids need to be stored prior to use. If the DNA or RNA is to be used in a quantitative assay, it is imperative that storage conditions maintain the integrity of the DNA in the sample. DNA is generally stored with no detectable losses at 4°C for several weeks, at -20 °C for several months (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 because DNA stored in water is subject to acid hydrolysis. Any contaminants in the DNA may accelerate degradation. Repeated freeze-thaw cycles are to be avoided as these induce precipitates, therefore samples should be stored in aliquots (Qiagen® Bench Guide, www.quiagen.com). Higher concentrations of DNA are more stable, as are closed circular plasmid forms as compared to short linear fragments. If thawed DNA is to be used in a quantitative assay, DNA concentration should be re-measured to verify that the concentration is similar to that measured prior to freezing. A higher concentration could indicate that water evaporated or sublimed from the sample (due to improper sample sealing), whereas a lower concentration may indicate degradation.

In contrast to DNA, RNA is very sensitive to degradation by RNAses, and to ensure integrity, all reagents must be RNAse-free and RNAse inhibitors must be added to the sample. RNA must be stored at -80 °C (De Paoli, 2005).

4.1.5 Considerations when Extracting Nucleic Acids from Solid Samples

Nucleic acids present in cells attached to solids can be retrieved either by an indirect method where intact cells are first separated from the solid matrix, and then concentrated prior to lysis and NA purification, or by direct lysis of the cells in the solid matrix. The latter is now the preferred method in most environmental applications because it generally yields more DNA and a less biased sample (Miller et al., 1999).

In the indirect method, the separation of intact cells from sediments is a relatively difficult task to do with high efficiency. It can be performed via some physical separation methods (sonication, blending, glass beads), chemical detergents or dispersants, isopycnic density gradient centrifugation, or a combination thereof (Ström et al., 1987; Frischer et al., 2000; Mermillod-Blondin et al., 2001; Buesing et al., 2002; Barkovskii, 2004; Caracciolo et al., 2005; Lunau et al., 2005). Recovery of intact cells is low, as many cells get lysed or remain attached to the solids. The primary benefit of this approach is that whole cells can be obtained, opening up the possibility of multiple downstream applications (e.g., FISH, PCR, cell cultivation, lipid and/or protein analysis, flow cytometry, etc.). Since this method can be tailored to be less disruptive than direct nucleic acid extraction protocols, it is an excellent means of obtaining high molecular weight DNA from soil, sediments, or other particles which can be used for large-insert cloning or whole-genomic amplification to access the community metagenome (Stein et al., 1996; Berry et al., 2003; Abulencia et al., 2006).

Disadvantages of the indirect approach are low recovery efficiencies (10-50%), and depending on the environmental matrix, groundwater chemistry, and microbial community composition, the process can preferentially recover certain cell-types (Boivin-Jahns et al., 1996; Edgcomb et al., 1999). Perhaps the primary downside of this approach is that the protocols are typically much more time-consuming and laborious than direct nucleic acid extraction methods. However, recent improvements that minimize extraction biases, cell loss/damage, and the labor associated with cell separation techniques have rekindled interest in this technology and its potential applications (Lehman et al., 2001; Caracciolo et al., 2005; Lunau et al., 2005). A particularly interesting approach is to dissolve clay-dominated sediments, then recover bacterial cells (Boenigk, 2004).

In the direct approach, cell lysis occurs directly within the soil matrix. A known mass of soil or sediment is mixed with lysis buffer and subject to any one of the physical, chemical or enzymatic cell disruption techniques reviewed in Section 4.1.2. A principle disadvantage of the direct lysis method is that it entrains many more PCR-inhibiting substances into the sample. Many DNA extraction kits have been developed that are specifically designed to remove contaminants found in soil and sediment, and additional purification methods also exist to remove inhibitors. Methods and considerations provided in Sections 4.1.2 and subsequent DNA manipulations are the same for DNA obtained from direct lysis of soil samples.

4.1.6 Considerations when Extracting RNA Instead of DNA

Because RNA is much more labile than DNA, owing to the ubiquitous and hardiness or RNAses that hydrolyze RNA, extraction of RNA needs to be carried out with supreme care to remove and inhibit RNAses. Protocols for RNA extraction are very similar to those of DNA, except all solutions and glassware must be carefully treated to ensure that they are RNAse-free. Many recipes and commercially-available solutions that inhibit RNAses are now available.

As for DNA extraction, a number of commercial kits for RNA extraction are also available, greatly increasing the probability of successful extraction of RNA (e.g., FastRNA™ extraction kit from Q-biogene or the PowerSoil™ RNA kit from MoBio). However, just as in the case of DNA, specific methods need to be optimized for the sample matrix. Purdy (2005) provides a comprehensive review of the steps required to optimize extraction for a specific sample type.

In the context of developing QA/QC methods for MBTs used in site assessment and remediation, the issues surrounding the analysis of RNA samples are very similar to those for DNA samples. Thus the focus of this project is first and foremost on standardization of DNA-based methods, since these are far more widely used with the appreciation that once this is achieved, extension to standardization of RNA-based methods will be a relatively small increment.

4.2 Key Findings for Further Investigation

From the review of current DNA extraction procedures summarized in Section 4.1 a number of key issues were noted that are critical to obtaining a reproducible and quantifiable nucleic acid sample useful for subsequent PCR analysis. These issues warrant further investigation:

- A systematic evaluation of nucleic acid quantification methods is required, including the incorporation of independently quantified reference DNA for calibration. Given the importance of NA quantification both in assessing DNA recovery and in preparation of calibration standards, combined with the diverse factors affecting DNA quantification measurements make this a priority for further investigation. It is possible that co-extracted substances that confound spectrophotometric or fluorometric DNA quantification, such as humic acids, also cause poor amplification due to inhibition of PCR (Bachoon et al., 2001). Moreover, because of the time-savings and handling ease that fluorometric methods provide, as well as their increased sensitivity and greater linear range compared to UV based methods, a systematic evaluation of NA quantification using commercially available fluorometric assays applied to a range of subsurface samples is also certainly warranted.

- It is imperative to develop a whole cell internal standard that is subject to lysis to quantify and track cell lysis and DNA extraction efficiency for any and each particular case. This internal standard will be limited though, in its ability to represent only those populations with similar lysis and extraction efficiencies. This could be somewhat addressed by using two distinct whole cell internal standards, one that lyses relatively easily and one that is more difficult to lyse.

- An independent estimate of DNA content of a sample is required to assess overall extraction efficiency. Independent approaches for determining DNA content include the use of phospholipid fatty acids or protein concentrations and direct cell counting techniques (epifluorescent microscopy) to: 1) estimate the total bacterial abundance in the environmental sample, 2) calculate the theoretical NA content in the sample based on the bacterial abundance, and 3) determine the NA recovery efficiency from the difference between the theoretical NA content in the sample and the NA yield. Such proxies for DNA content will themselves need to be evaluated on known samples.

- The relationship between DNA extraction steps and presence of inhibitors of PCR needs to be assessed to attempt to identify what protocols minimize carry over of inhibitors. The use of post extraction purification steps, such as columns or organic solvents can further decrease DNA recovery from the original sample. Losses of 10-300% have been documented for these further purification steps (Sharma et al., 2007). In samples with low levels of target sequence, additional purification steps should be balanced against

potential loss of NAs (Chandler, 1997), and in many cases, dilution may be the best way to deal with inhibitors.

5. PCR QUANTIFICATION OF NUCLEIC ACID TARGETS

Quantification of nucleic acid targets by qPCR is the final step (prior to data interpretation) in the sample testing process. A number of qPCR methods and amplicon detection chemistries are available. Each procedure is subject to a variety of interferences including contamination, inhibition, and instrument calibration and normalization requirements. This section provides an overview of the current nucleic acid quantification practices used in the commercial environmental testing field as well as practices used in other disciplines that could reasonably be integrated into bioremediation testing protocols.

5.1 Review of Current Practice in PCR Quantification of Nucleic Acids

A variety of PCR approaches, chemistries, and control and calibration methodologies are used in various disciplines. Some of these are summarized in Table 4-1. While qPCR chemistries are generally consistent between the environmental remediation field and other industries, differences are more apparent in the calibration and the control strategies used in other disciplines.

5.1.1 Quantitative PCR Chemistries

A detailed discussion of qPCR chemistry is beyond the scope of this document, for a general introduction and overview of these methods see general literature or EPA guidance on this topic (Bustin, 2004; USEPA, 2004; Mackay, 2007). Current practices in various fields with respect to qPCR chemistries are summarized in Table 4-1. While the basic composition of PCR reactions is similar for all types of qPCR (Taq polymerase, deoxynucleotide triphosphates, oligonucleotide primers), there is a fundamental division in chemistries used for fluorescent detection of amplification products.

Non-specific chemistries utilize fluorogenic dyes that bind all double stranded DNA (e.g., SYBR® green I Amplifluor™, SYBER™ Gold). Non-specific dyes such as SYBR green have the advantage of being applicable to a wide range of targets and require reduced method development (Sharma et al., 2007). A major disadvantage of non-specific dyes is the potential for detection of non-specific amplicons. To rule out the possibility of non-specific amplification products (i.e., false positives), melting curve analysis is essential (USEPA, 2004). SiREM, currently, uses SYBR green chemistry for Dhc testing, with confirmation of the specificity of amplicons performed using melting melt curve analysis, combined in certain cases by gel electrophoresis.

Specific chemistries and sequence specific oligoprobes (e.g., TaqMan®, molecular beacons, Scorpion PCR) (De Bellis et al., 2007) make use of fluorescently labeled probes ("specific oligoprobes") that contain a fluorescent dye and a quencher. The quencher masks the fluorescence prior to the binding of a probe to a particular DNA sequence. The probes fluoresce

only after they have bound to their specific target sequence (in the case of TaqMan® Probes after binding and after exonuclease activity of the polymerase hydrolyzes the probe) obviating the need for amplicon confirmation in most cases. A major advantage of specific linear hybridization probes is that they allow the performance of so-called multiplex PCR, in which the use of fluorescent probes labeled with different fluorophores that emit at different frequencies e.g., Acridine™ [462 nm], 6-FAM™ [518 nm], Cy3™ [570 nm], TAMRA [580], Texas Red [603 nm], Cy5™ [667 nm] (Phillips, 2004) this allows the simultaneous quantification of up to four different target genes in the same reaction (USEPA, 2004). Multiplex PCR, for example, allows the simultaneous quantification of a target gene and a control sequence, for example a matrix spike internal control which can be used to asses DNA recovery from extraction methods, inhibition, etc. Despite the common view that specific linear hybridization probes increase the sensitivity of qPCR, non-specific and specific fluorogenic chemistries were reported to detect amplicons with equal efficiency (Mackay, 2007), and thus the chemistry used might have little impact on the detection limit of quantitative PCR methods, although this fact is somewhat controversial.

Specific linear hybridization probe methods can be divided into the basic categories of destructive oligonucleotide systems and non-destructive oligonucleotide systems.

Destructive oligonucleotide systems make use of the 5' to 3' exonuclease activity of Taq DNA polymerase to hydrolyze the probe, thereby decoupling the fluorophore and the quencher leading to a detectable increase in fluorescence. TaqMan® methods are an example of destructive oligonucleotide systems and are the most commonly used qPCR chemistry (Bustin, 2004). For example, TaqMan® chemistry was reported for quantification of Epstein Barr Virus (EBV) in plasma (Perandin et al., 2007), *Enterococcus* DNA in surface water (Haugland et al., 2005) and for monitoring the presence of genetically modified soy (Cankar et al., 2006) among others. Microbial Insights, a commercial provider of Dhc testing (offered under the trade name Census™), uses a TaqMan® based chemistry.

Non-destructive, linear hybridization probe systems do not degrade the specific probe, and thus, the probe can in effect be “reused” to examine the nature of the amplicons. For example, by monitoring the melting temperature of the probe, differences in microbial strains could be differentiated based on sequence variations which result in differences in the observed melting temperature (Mackay, 2007). There are a number of variations of non-destructive systems including hairpin linear hybridization probes (i.e., Molecular Beacons) in which a hairpin loop position the fluorophore and the quencher in close proximity that prevents detection of fluorescence unless the probe binds a specific target separating the quencher and the fluorophore. The ability to differentiate subtle differences in base composition is one of the major advantages of the Molecular Beacon approach. Alsmadi et al. (2006) used a Molecular Beacon approach to detect the blood disorder Hereditary Hemochromatosis, through the use of probes complementary to two common mutations of the gene. The assay was able to differentiate one base pair mutations from wild type. The ability to detect hepatitis A in lake water was also

demonstrated using a Molecular Beacon approach combined with an RNA based detection technology (Abd el-Galil et al., 2005).

Other more exotic oligoprobe approaches include self-priming, fluorogenic amplicon systems, which are similar to hairpin oligoprobes except the probe, primer and quencher are all contained on the same oligonucleotide. Self priming qPCR chemistries include so called "Scorpion PCR". In Scorpion PCR, the fluorophore is irreversibly incorporated into the nascent amplicon (Mackay, 2007). In "nested Scorpion PCR", the probe, primer and quencher are all contained on the same oligonucleotide, which leads to one of the major advantages of the Scorpion approach: a one to-one relationship between the number of amplicons generated and the fluorescence produced. Scorpion PCR has been reported to be more sensitive than TaqMan or Molecular Beacons (Bustin, 2004). Scorpion primers have the disadvantage of being more complex to design and more expensive to synthesize (Mackay, 2007). De Bellis et al., (2007) described the development of a Scorpion qPCR method for the detection of *Erwinia amylovora*, which causes Fire Blight of pear and apple trees. The Scorpion methods are reported to have the advantage of requiring only one probe/primer and requiring a shorter reaction time. The detection limits reported using Scorpion PCR were 3.2×10^4 CFU/ mL and approximately 100x more sensitive 2.8×10^2 CFU/ mL for nested Scorpion PCR. This indicates the potential for lower detection limits using nested qPCR approaches.

PCR chemistries, while having practical impacts in terms of specificity, detection limits and ease of analysis, and sensitivity are most likely not a key parameter in determining the ultimate quality of environmental qPCR. Accurate quantitative results can be obtained with all of the available chemistries. Despite the common view that specific oligoprobes necessarily increase the sensitivity of qPCR versus non-specific (SYBER green methods) both approaches have the potential to detect amplicons with equal efficiency (Mackay, 2007). Nevertheless, nested Scorpion PCR (De Bellis et al., 2007) has been reported to exhibit increased sensitivity (Bustin, 2004) and may be worth examining in more detail for environmental applications should lower detection limits be a high priority. In general, the use of specific oligoprobes (e.g., TaqMan®) methods will be required for performance of multiplex qPCR. A requirement for inclusion of internal standards and will necessitate that TaqMan® or equivalent methods be used in many analytical aspects of this project.

5.1.2 RNA-Based Methods

RNA is the intermediary between genes (DNA) and a particular function (typically mediated by catalytic proteins called enzymes). The ability to quantify RNA targets is significant, as RNA is more closely associated with gene expression (e.g., a specific degradation activity) than DNA, which simply represents potential for expression of the activity. Messenger RNA (mRNA) levels may vary in response to a variety of stimuli and indicate the metabolic state of microorganisms. For example, a gene may be highly expressed (high amounts of mRNA) or turned off (no mRNA). In both cases, the DNA based assay would have yielded identical

information. Despite the inherent advantages of target RNA quantification, it is by nature a more unstable molecule than DNA, being extremely susceptible to ubiquitous RNA degrading enzymes called RNAses (Bustin et al., 2004). Due to its instability, RNA presents additional challenges for quantification. Typically, target RNA is quantified by first being converted into a more stable DNA molecule (cDNA) by an enzyme called reverse transcriptase (RT). The use of this enzyme in qPCR methods to produce cDNA is called RT-PCR. RT-PCR was first reported in 1987 consists of two steps:

1) the copying of the RNA into DNA by RT to produce what is called a complementary DNA (cDNA) molecule;

2) qPCR is performed on the cDNA in order quantify the cDNA and by extension the original concentration of target RNA (Phillips, 2004).

Nielson and Boye (2005) reported the use of RT-PCR for studying gene expression of several iron acquisition genes in the porcine lung pathogen *Actinobacillus pleuropneumoniae* under iron-limited conditions. Expression of the RDase gene *tceA* in response to the presence of TCE was in *Dehalococcoides ethenogenes* strain 195 was demonstrated using RT-qPCR (Fung et al., 2007). RT-PCR is also provided commercially by Microbial Insights under the Trade name Q-Expression™, which quantifies several Dhc genes using this method including total 16S rRNA (a measure of the overall metabolic activity of the Dhc population) as well as offering tests for activity of specific RDases including *bvcA* and *tceA*.

5.1.3 Calibration and Controls in Quantitative PCR Methods

A prerequisite for accurate target gene/transcript quantification using any qPCR testing methodology are accurate procedures to establish the concentration of calibration materials and standard curves. For standard curve preparation, a number of starting materials containing DNA targets are commonly used:

- Plasmid DNA containing cloned fragments of interest (Koike et al., 2007);
- PCR-amplified DNA fragments;
- Bacterial genomic DNA (Cremonesi et al., 2006; Klerks et al., 2006);
- Viral DNA containing target DNA fragments;
- Artificially synthesized DNA (oligonucleotides); and
- Whole bacterial cells (McDaniels et al., 2005).

Calibration standards termed “Calibrators” can be defined as:

"A synthetic or natural analytic standard whose concentration value has been established by a defined methodology, which may or may not include comparison to a reference standard" (Mackay, 2007).

In most cases, the practitioner produces and quantifies a calibrator using a non-qPCR-based method, for example plasmid DNA quantification using spectrophotometry or bacterial enumeration by plate counts. This information is used to calibrate the qPCR method. Calibration or validation using CRM or third party validated materials is also used (for more information on CRM see Section 2.1).

Supercoiled plasmids commonly used for calibration are in effect circles that are rolled up on themselves (much like a balled up elastic band). They are differentiated from the two other conformations referred to as nicked and linear. Supercoiled plasmids have the potential to limit the access of primers and probes and thus may be less than ideal for use as calibrators. Supercoiled plasmids can be made linear DNA by cutting the plasmid with a restriction enzyme that cuts it a specific location thus making it linear.

SiREM uses a six point calibration curve produced using a calibrator consisting of serial dilutions of supercoiled plasmids containing the target gene. The plasmid calibrator is quantified using NanoDrop spectrophotometry and Picogreen fluorometry. Negative controls used by SiREM include a method blank (sterile water processed as groundwater) to assess contamination throughout extraction and analysis, a negative control (sterile water used in PCR analysis instead of template) to assess contamination that occurs via reagents or reaction assembly. Positive controls consist of a high and low concentration "check standards" (additional aliquots of materials used to prepare the standard curve) which are used to validate the positive control for each set of samples analyzed. Like SiREM, Microbial Insights uses spectrophotometrically quantified supercoiled plasmid to produce a seven point standard curve, which is revalidated in each run with a single check standard. Microbial Insights uses method blanks and negative controls similar to those used by SiREM. Microbial Insights performs split samples with Dr. Frank Löeffler's Laboratory (Georgia Tech) for method validation purposes (personal communication with Greg Davis and Dora Ogles, Microbial Insights).

Calibration methods used outside the environmental remediation field often make use of similar approaches. For example Koike et al. (2007) monitored tetracycline resistance genes in lagoons and groundwater, and used supercoiled plasmid DNA, which was quantified by spectrophotometry, to produce standard curves. Perandin et al. (2007) measured EBV in plasma using calibrators derived from EBV positive cell lines, quantified by spectrophotometry. Haugland et al. (2005) used *Enterococcus faecalis* cells quantified by acridine orange epifluorescence microscopy to enumerate cells, which were then collected onto filters to extract DNA. The resulting DNA extracts were used as a qPCR calibrant. This approach has the unique advantage of integrating losses related to cell concentration and DNA extraction directly into calibration of the method. McDaniels et al. (2005) utilized a similar approach for the detection of

H. pylori in drinking water in which flow cytometry enumerated cells were collected on filter membranes and DNA was extracted in a similar fashion to drinking water samples. Klerks et al. (2006) used *Salmonella* cultures enumerated using plate counts as a source of genomic DNA.

The use of a variety of calibrators such as plasmid DNA, PCR products, genomic DNA, etc. is well established for qPCR methods. A comparison of some of the available calibrators including supercoiled plasmid, linear plasmid, and potentially PCR products may be worthwhile. There is evidence to suggest that PCR products are superior calibrators compared to supercoiled plasmid, with the disadvantage that PCR products are inherently less stable than plasmids based on experience in Dr. Edward's laboratory at the University of Toronto. Beyond standard calibrators CRMs could be used for calibration to verify current calibration methods which has significant potential to increase the accuracy of and confidence in environmental qPCR methods. In addition to simply verifying standard curves, CRMs could be used directly to calibrate qPCR methods for example by processing the CRMs in the same way as the test samples like Haugland et al. (2005) who used intact cells to calibrate qPCR methods. Similarly, this in this study, certified Dhc reference cultures of known titer could be spiked into artificial groundwater samples at various dilutions. The spiked samples should be extracted and analyzed to produce a "fully biased" standard curve. Comparison of this curve with current plasmid calibrated standard curves could provide insights into the recovery efficiency of Dhc and the accuracy of current calibration procedures using plasmids, which is the current method used by the main providers of Dhc testing in the environmental remediation field.

5.1.4 Assessing and Quantifying PCR Inhibition

It is common for environmental samples to contain substances which inhibit PCR reactions. Inhibitory substances can also come from the DNA extraction process (see Section 4.1.4) or from plastic ware (Fox et al., 2007).

The use of serial dilutions is a valid procedure for assessing and quantifying inhibition (Xiao et al., 2006). To illustrate how dilution could be used to assess inhibition take the case where a ten-fold dilution leads to only a two-fold decrease in target. This result suggests inhibition as it would be expected there would be a ten-fold decrease in target concentration with a ten-fold dilution. If a further ten-fold dilution of the same sample leads to an actual ten-fold concentration decrease, it indicates that inhibition was present but it was overcome by the initial ten-fold dilution and therefore the second dilution had no impact. In this way a practitioner can reduce and diagnose the dilution level at which inhibition is relieved.

While in many cases PCR inhibition is detected qualitatively during the extraction process through the use of universal controls it can also be quantitatively assessed only through the use of spike and recovery controls which typically come to bear during qPCR. Various approaches to assess and quantify inhibition are found in the peer reviewed literature. For example, Koike et al., (2007) reported spiking known amounts of a plasmid template into samples testing negative,

in order to confirm that amplification was possible within the extracted DNA sample, and thus confirm inhibition (plasmid did not amplify) or rule out inhibition (plasmid did amplify). A variation on this approach includes quantifying samples in parallel spiked with a known amount of template DNA. This approach has the advantage of not requiring a second analysis step to confirm or deny inhibition should a sample test negative.

Inhibition may also be detected through the use of internal controls, which can be differentiated from the matrix and parallel spike and recovery approaches described above. With an internal control, a sequence other than the analysis target (e.g., whole cells [MicrobialSurrogates], plasmids, oligonucleotides genomic DNA, etc.) is monitored using a different fluorophore facilitated by the use of multiplex PCR method. While important in clinical settings, the use of internal controls is particularly relevant to environmental samples (Muska et al., 2007) due to the increased variability and potential for inhibition of PCR. If recovery of the internal control is below a certain threshold, inhibition is suspected. Internal controls are superior to the parallel spike and recovery approaches described above because the control reaction is performed in the same tube and at the same time as the test reaction, thus removing considerable sources of variation which could confound the interpretation. In commercial testing Microbial Insights uses an internal standard consisting of artificially synthesized oligonucleotide, which is unlike any DNA sequence found naturally in groundwater (personal communication with Dora Ogles, Microbial Insights). This artificial DNA sequence is quantified with specific primers and a TaqMan® probe. The sample is spiked with this internal control prior to the DNA extraction process, and is then quantified. The recovery of the internal standard is compared to recovery obtained using non-inhibitory blanks to assess the DNA extraction efficiency and possible PCR inhibition by the sample.

Inhibition may also be detected using a statistical measure called PCR efficiency. Under non-inhibited conditions each PCR cycle produces two DNA molecules for each template molecule present, in other words the copy number is doubled with each amplification cycle. Inhibitors reduce the number of molecules produced per template molecule and more cycles are needed to reach the fluorescence threshold (Kontanis et al., 2006). The production of two molecules per template is defined as 100% efficient. This percentage changes over the course of the fluorescence-PCR cycle graph, but should be close to 100% at the fluorescence threshold. Software associated with Real-time qPCR equipment is capable of calculating efficiency if a dilution series of samples is analyzed. Cankar et al. (2006) used PCR efficiency to compare the effectiveness in terms of removal of inhibitors of 5 different DNA extraction methods. They recommended the use of pre-quantification screening dilutions using a so-called “monitor run” in which the PCR efficiency of an endogenous reference gene is calculated to determine a suitable amount of dilution required to overcome inhibition. Issues surrounding use of PCR efficiency for qPCR quality control are technically complex and beyond the scope of this review, nevertheless, further analysis of this area has the potential to illustrate how efficiency could be used for practical, and experimental verification of methods in this project.

5.1.5 Nested PCR

Nested PCR generally involves the use of two (or possibly more) primer sets to increase the sensitivity and specificity of the PCR process (USEPA, 2004). Typically the first primer set used is more general (e.g., targeting all Bacteria) followed by a secondary amplification with a more specific primer set, targeting sequences contained within the initial amplicon (e.g., targeting only Dhc). The performance of two distinct amplifications increases the sensitivity, as more PCR cycles are performed. Specificity may increase because the initial primer set selects a subpopulation of total sequences for subsequent secondary amplification. Therefore nested PCR is used when trying to detect a specific DNA sequence that is a very minor component of the total DNA. Nested PCR is not a quantitative method thus the utility of nested PCR for all but confirmatory testing of negative samples is likely to be limited. Other disadvantages of nested PCR include increased analysis effort and cost and increased potential for contamination (false positives). Löeffler et al., (2000) used a nested PCR approach to decrease the detection limits of *Desulfuromonas* 16S rRNA genes by up to 3 orders of magnitude from 1×10^3 -1×10^2 *Desulfuromonas* cells to 1-10 cells per reaction. In a background of 1.2×10^8 *E. coli*, the detection limit of normal (3×10^5 gene copies) versus nested PCR (3×10^3) gene copies was decreased 100-fold. De Bellis et al. (2007) reported detection limits using non-nested PCR limits for 3.2×10^4 CFU/ mL *Erwinia amylovora* using non-nested PCR and approximately 100x more sensitive 2.8×10^2 CFU/ mL using nested Scorpion qPCR (see 4.1.2 below). Lower detection limits are possible with nested PCR approaches and this may be extremely important for pathogen testing, but may be less significant in the environmental remediation context. Highly sensitive nested PCR approaches do not offer advantages for analysis of chlorinated solvent bioremediation sites because the concentration of Dhc cells required to observe dechlorination activity is typically higher than the detection limits of regular qPCR, therefore a nested approach is not required.

5.2 Key Findings

5.2.1 Key Findings Regarding qPCR Methods

Quantitative PCR methods vary significantly at the level of detection chemistry and the type of primers used.

- Non-specific qPCR detection chemistries are not applicable to multiplex qPCR which are integral to the use of internal standards and may offer reduced specificity when compared with specific detection chemistries such as TaqMan®.

- Differences between types of specific detection chemistries (TaqMan® vs. Molecular Beacons, etc.) are relatively minor in terms accuracy of quantification of NA targets.

- Nested qPCR has the potential to reduce detection limits compared to non-nested methods, and may be useful for detection of low copy numbers, but is not quantitative.

- Quantification of RNA in environmental samples requires reverse-transcription to cDNA prior to qPCR (RT-qPCR). RT qPCR has the potential to increase the ability to monitor subtle changes in metabolism in environmental samples, and is very useful in research. However, RT qPCR provides a measure of activity, and not necessarily abundance, and typically both measurements (quantifying DNA and RNA) would be required for data interpretation. The use of these methods for environmental analysis is not common and is more technically challenging than DNA based methods.

5.2.2 Key Findings: Method Calibration

- Calibration using a variety of starting materials from plasmids to PCR products to genomic DNA to whole cells was reported, specific advantages and disadvantages of the naked DNA calibrators was not well documented.

- Quantification of calibrators was performed using a variety of methods including spectrophotometry and fluorometry for DNA and RNA, microscopy, plate counts and flow and solid phase cytometry for whole microbial cells.

- Methods in which whole cells are used as a calibrator which is passed through the extraction and analysis procedure in the same way as test samples has the potential to create “fully biased” standard curves which could integrate losses into the calibration.

5.2.3 Key Findings: Assessing and Quantifying PCR Inhibition

- The use of serial dilutions is an effective and practical strategy for dealing with inhibition, however, detection limits are increased.

- Naked NA internal controls are commonly used at the analysis stage and include synthetic oligonucleotides, salmon sperm DNA, and plasmids.

- Parallel or retroactive (for samples testing negative) spike and recoveries with test target are simple methods for measuring inhibition that require little method development.

- Internal controls are the most sophisticated methods for assessing inhibition and once developed require less effort than parallel spikes as assembly of additional reactions is not required.

- The efficiency of qPCR reactions, a statistical measure related to how well the qPCR reaction is performing, has potential for assessing inhibition and should be examined further.

6. OVERVIEW OF QUALITY ASSURANCE/QUALITY CONTROL PROCEDURES DATA QUALITY AND STANDARDIZATION OF METHODS

The previous sections provided a summary of the current state of practice regarding qPCR testing of environmental bioremediation samples and approaches used in other disciplines at each stage of the analytical process, indicating specific areas that require further investigation. The goal of this section is to integrate these findings in the context of data quality and highlight the methodological prerequisites for high data quality, specific data interpretation needs (e.g., normalization) and potential areas requiring standardization. The following descriptions of controls discussed below for the reader's reference are potentially applicable to environmental qPCR testing:

- Field blanks – PCR-grade water samples poured into a sample bottle at the site;
- Equipment blanks – PCR-grade water samples processed with field equipment at the site and transferred into a sample bottle;
- Trip spikes – Artificial groundwater samples prepared in the lab with known quantities of target or a surrogate that are shipped with sample bottles and used to evaluate changes during transportation;
- Lab water blank – PCR-grade water samples prepared and processed as a sample in the lab;
- Filter blank – Clean filter carried through analytical process;
- Matrix spike – Environmental sample to which a known number of cells added to verify recovery;
- Filter spike – Sterile filter to which a known number of Dhc cells is added to verify recovery;
- Calibration standards – A known number of cells or a known number of Dhc target gene copies is used to develop a calibration curve for qPCR equipment;
- Check standard – A known number of cells or target gene copies is added to validate calibration curve with each batch of samples analyzed;
- Microbial surrogate – A whole cell sample (distinct from Dhc) of known abundance added as an internal control to evaluate losses related to biomass recovery, incomplete cell lysis, and inhibition of qPCR reactions;

- Naked DNA internal control – DNA added during DNA extraction to assess losses related to extraction and inhibition; and
- External Verification Control – Use of third party calibrated reference material to validate quantification.

6.1 Summary and Discussion of Current Practice (Environmental Remediation)

Current practices for insuring and measuring data quality occur at several steps throughout the sampling and analysis process. In this section, data quality procedures will be summarized based on current practices, with an emphasis on commercial environmental remediation operations.

6.1.1 Validation of Laboratory Equipment and Procedures

Data quality assessment in qPCR testing is not solely dependent simply on the use of sound methodologies. It is also contingent on the accurate use of a variety of equipment and good laboratory practice that directly impact the accuracy of the method (USEPA, 2004). While a comprehensive overview of this topic is beyond the scope of this document several specific areas are worth noting.

Key equipment /procedure verification needs include:

- Thermocyclers (proper temperature cycling monitored regularly with external thermometer);
- Real-time PCR equipment (background normalization / consistency of well to well readings, etc.);
- Spectrophotometers / fluorometers (validate with certified external standard);
- Laminar flow hoods – maintain nucleic acid- and bacteria-free conditions (filters, fans functioning properly –flow rates maintained);
- Balances (used to calibrate pipettes-calibrate with weight standards on regular basis);
- Pipettes (calibrate on regular basis to determine accuracy, also wipe test to determine free of contamination);
- Disposables (free of contamination from inhibitors, proper fit of pipette tips, etc.);
- Laboratory cleaning procedures (prevent nucleic acid, DNAse/RNAse, and bacterial contamination - use wipe tests to confirm);

- Verification of personnel training (test personnel with reference standards);
- Freezers and refrigerators (internal thermometer to confirm stable storage of reagents stocks and standards) and;
- Autoclaves (validate with biological indicator such as *Bacillus stearothermophilus* to ensure proper sterilization of disposables and equipment).

Documentation of protocols and schedules for maintenance of the above equipment is required to ensure qPCR analysis is accurate, precise and validated.

6.1.2 Data Quality and Standardization Considerations for Groundwater Sampling

In direct groundwater sampling (see Section 3.1.1), both commercial labs recommend purging wells until groundwater measurements stabilize. This approach is common and appropriate when sampling for chemical analytes. While it is possible that this approach yields samples which are representative of aquifer conditions, further experiments are needed to demonstrate that this approach is appropriate for MBT analytes. These experiments should include: purge volume and supporting data collection (i.e., ORP, volatile organic compound [VOC] concentrations, pH, conductivity measurements) as variables; and Dhc qPCR reproducibility and sensitivity as measures of success.

Field or sample replicates are only rarely prescribed, in many cases due to customer cost considerations. This data gap may compromise data utility. For example, sample variability is likely to be high relative to other analytical processing steps and critical to understanding and predicting the performance of MBT related technologies and thus replicate samples are required to quantify this variability. In the literature, sample replication ranged significantly: one per batch of 20 samples (Xiao et al., 2006); duplicate or triplicate samples (Cubero et al., 2001; McDaniels et al., 2005; Nielsen et al., 2005; Cook et al., 2007); 2 to 10 replicates in technique comparisons (Aldous et al., 2005; Carrigg, 2007; Lakay et al., 2007); and one sample per event with more than 100 sample events in the data set (Haugland et al., 2005; Koike et al., 2007). In many papers, sample collection was straightforward such as surface water sampling. Since groundwater sampling is more involved (and expensive) and limits to the sample size are common, sample replication guidelines must balance practical constraints and data quality needs. Sample replication needs are currently poorly characterized for environmental remediation and are likely to vary from site to site. This project should evaluate sample replication at several locations and provide recommendations for site assessment and remedial performance evaluation.

In the sampling step, current practices recommend, but do not require data quality samples such as field and equipment blanks or matrix and trip spikes. The inconsistent use of data quality samples creates problems in identifying erroneous data such as samples affected by cross contamination or deterioration during shipping, or PCR inhibition. The inclusion of whole cell

microbial surrogates or matrix spikes at an earlier stage in the analysis, perhaps at the point of sampling, has the potential to provide useful information regarding losses throughout the entire shipping, storage, processing and analysis stream and is essential for interpreting qPCR data and identifying erroneous data.

The development of guidelines for sampling methods, replication and use of data quality samples will ultimately facilitate more representative sampling comparisons between sites/locations on a common basis and may yield more predictive data to support decisions based on qPCR data.

6.1.3 Data Quality Measures Associated with Biomass Concentration and Nucleic Acid Extraction

Commercial nucleic acid extraction protocols currently include data quality samples, such as negative controls (PCR-grade water), and measures to assess the quantity and quality of extracted DNA, including PCR universal 16S rRNA primers and spectrophotometry, which is routinely performed by SiREM and Microbial Insights. Other data quality samples such as matrix spikes, filter spikes or internal microbial surrogate standards, or other measures that assess inhibition/losses are not commonly used in this industry. One exception is Microbial Insights which uses an oligonucleotide internal standard applied during DNA extraction. This internal control provides information regarding losses during nucleic acid extraction and analysis and PCR inhibition (personal communication with Greg Davis, Microbial Insights).

The literature has a few suggestions for data quality samples associated with nucleic acid extraction. Ideally, these samples would quantify the amount of nucleic acids extracted, measure extraction efficiency, and assist in determining if PCR inhibitors were present in the sample. The amount of DNA extracted is often determined through spectrophotometric methods (Bachoon et al., 2001; Cremonesi et al., 2006; Lakay et al., 2006; Koike et al., 2007) or fluorometric methods (Bachoon et al., 2001) or gel electrophoresis. The effect of PCR inhibitors was evaluated by adding external DNA (Haugland et al., 2005; McDaniels et al., 2005), whole cells (Brinkman et al., 2003), and by examining the effects of sample dilution (Xiao et al., 2006; Cook et al., 2007). Secondary purification to neutralize inhibition was also considered (Xiao et al., 2006) and is also used by commercial laboratories. Extraction blanks and internal controls (naked DNA spikes) using salmon testes DNA were included by Haugland et al. (2005) and McDaniels et al. (2005). These procedures are not common in the environmental remediation industry.

Integrating the following controls in the nucleic acid extraction procedure (plus potentially the sample shipment and storage procedure) has the potential to provide improved data quality and merits further consideration:

- A matrix spike (~1 sample per set) using a calibrated Dhc stock to determine sample specific losses with respect to Dhc recovery;

- A whole cell internal standard for losses associated with biomass concentration, recovery and cell lysis in the context of each sample tested; and
- A naked DNA internal control applied at the DNA extraction step to assess NA losses and inhibition.

The use of the above controls applied in tandem may facilitate the assessment of the various factors that come into play at particular analysis stages (e.g., determining if nucleic acid losses are associated with low cell lysis or inhibition).

6.1.4 Data Quality Measures Relevant to qPCR Quantification

Current commercial environmental PCR quantification includes several data quality measures. Calibration curves are currently based on six or seven points with six-fold replication at each point (SiREM) using supercoiled plasmid DNA. Negative controls for PCR templates use sterile water or Tris-EDTA buffer, while positive controls use the plasmid calibration standards. Melt curves or gel electrophoresis are used to confirm amplicon specificity. While inhibition is recognized as a common problem, procedures for assessing inhibition, such as universal bacteria primer sets, are for the most part qualitative, and thus of limited utility for quantitative data interpretation. Currently, the qPCR analysis portion of the MBT analytical path currently incorporates the greatest number of data quality measures, nevertheless, additional or improved measures could be implemented to improve interpretability of data.

The literature provides some interesting ideas to enhance data quality for qPCR. As with nucleic acid extraction, the literature does not present a consensus on minimum requirements. In the area of qPCR calibration, three techniques are of particular use: calibration by the organism of interest on filters (Haugland et al., 2005), independent measurement of calibration materials (Abd el-Galil et al., 2005; Haugland et al., 2005; McDaniels et al., 2005; Alsmadi et al., 2006; Cremonesi et al., 2006; Klerks et al., 2006; Koike et al., 2007; Perandin et al., 2007), and use of CRM (Cankar et al., 2006). In enumerating *Enterococcus*, Haugland et al. (2005) independently measured calibration standards using microscopic techniques and calibrated using filters spotted with the target organisms. By using the target organism and spotting the filters, this technique incorporates nucleic acid extraction efficiency and cell lysis efficiency into the calibration standards while avoiding species to species interpolation and uses the same primers and probes.

The positive and negative controls used in qPCR vary in the literature. Positive or reference controls are used to assure that qPCR equipment and reagents are performing within specified tolerances for a sample set. Positive controls may use target DNA (Cremonesi et al., 2006; Xiao et al., 2006; Cook et al., 2007; Koike et al., 2007), cultures of target organisms (Nielsen et al., 2005; Bellis et al., 2007; Perandin et al., 2007), matrix spikes (Cubero et al., 2001), or calibration standards with target organisms (Brinkman et al., 2003; Haugland et al., 2005; McDaniels et al., 2005). Current environmental remediation protocols generally use calibration standards as a

positive control; these controls are useful in identifying problems with the qPCR equipment or reagents. Adding matrix spikes as a data quality sample, either generated in the field or during filtration/concentration, would be a useful means of identifying problems during sample processing as well as qPCR analysis. Negative controls, samples with no expected response, may use: clean matrix or clean filters (Haugland et al., 2005; Nielsen et al., 2005; Cremonesi et al., 2006; Bellis et al., 2007); no template (Kontanis et al., 2006; Cook et al., 2007); or an alternate organism (Perandin et al., 2007). The current environmental remediation industry practice is to use sterile matrix and no template negative controls. The sterile matrix negative control follows a sample set through the analytical train from filtration/concentration to qPCR, and thus, can be used to identify contamination in those steps. This method is adequate in most cases. The no template negative control is generated when setting up an analytical sequence for the qPCR equipment. This control is used to identify qPCR reagent contamination or similar problems. The use of a negative control in analysis containing DNA not normally amplified by the procedure would be a minor methodological improvement.

The cost of MBT analysis, as well as the uncertainty in data significance, has limited routine data quality sampling in the bioremediation industry. This project will evaluate data quality measures throughout the analytical train, identify the most practical and important steps for data quality assessment, and recommend minimum data quality samples. Establishing more uniform data quality assessments will facilitate comparisons between labs and sites as well as identification of spurious data.

6.2 Data Analysis and Interpretation of Results

MBTs are relatively new techniques and as a result, sources of variability and error are for the most part poorly characterized. Establishing a more uniform data quality assessment will assist in this characterization, but other issues may also affect the ability to use MBT data for bioremediation.

6.2.1 Quantification and Detection Limits

Defining quantification and detection limits is common practice within environmental analysis. However, given the lack calibrated stock solution the detection limits of commercial Dhc assays have not been rigorously determined. This fact has impacts in understanding and interpreting of negative results in particular. Currently SiREM reports a quantitation limit of 4,300 gene copies per L (based on a 500 mL sample) for the Gene-Trac Method which translates to approximately 4-5 gene copies/mL based on the lowest plasmid calibration standard used for the standard curve. Detection limits are not currently reported by SiREM, but are estimated to be at least half the quantification limit or ~2000 gene copies/L. Detection limits for the Microbial Insights Census methods are reported to be 100 cells/sample (personal communication with Greg Davis and Dora Ogles, Microbial Insights), with an assumed processed sample size of 500 mL that translates to 200 cells per L (2 gene copies/mL). Various researchers report varying detection limits with

qPCR techniques. For example 27 *Enterococcus* cells/mL in beach water (Haugland et al., 2005); 10 *H. Pylori* cells per filter (McDaniels et al., 2005) , 100 colony forming units / mL of *Xanthomonas* (Cubero et al., 2001). While many labs calculate quantitation limits it is not clear if common techniques are used. The methods of determining detection limits and quantification limits may benefit from standardization. One possibility is the use of calibrated reference Dhc cultures added to the artificial groundwater samples in decreasing quantities to determine the lowest quantifiable and detectable concentrations. This approach and others should be evaluated.

6.2.2 Assessing and Managing Variability

As with many environmental measurement techniques, multiple samples from the same location can yield differing results. These differences are due to environmental heterogeneity, as well as variations in the sampling and measurement operations. As a relatively new technique, the most important sources of variation in MBT measurements have not been determined. An effective determination of variation inherent to specific steps in the analysis could allow resources to be targeted to where replication is most useful in characterizing sample variation. Variation can be characterized through replicate data collection at many analysis points including: sample collection, preservation and transport, sample filtration/concentration, DNA extraction, and qPCR target quantification. Currently, replication primarily occurs at the analysis step (i.e., qPCR protocol calls for samples to be run in triplicate) which ironically may represent one of the least variable steps in the process. Once replicate samples are collected and analyzed, variation at the specific steps can be estimated. In some cases, standardization may reduce variation and thus reduce the need for replication. In other cases, replicate sample analysis will be identified as an important data quality assessment tool. This data will provide insights into where variability occurs, and the degree of replication needed to provide meaningful information can be estimated using statistical tools such as power calculations. Once true variation in target microorganism concentrations can be separated from sampling and analysis variation, meaningful correlation of MBT information between sites/locations becomes possible. This correlation will support the evaluation of MBT information as predictive or performance measures at bioremediation sites.

Variability between laboratories and between personnel in the same laboratory is another concern. The use of reference materials will make comparisons of this type much more meaningful than current approaches. Currently, split samples are analyzed but should differences between laboratories be are observed there is no standard to determine which laboratory is more accurate. Periodic submission of replicate split samples of known quantities of reference materials to laboratories will allow effective assessment and comparison of the performance of laboratories and laboratory personnel. Furthermore should a laboratory or individual's analysis prove inaccurate there would be a readily available reference to use for method improvement and revalidation. These measures if effectively implemented would increase the quality of and ultimately the confidence in commercial qPCR analyses.

6.2.3 Validation using non-qPCR Methods

MBT are emerging analytical techniques, with associated data gaps and uncertainty regarding performance. Nevertheless, in some cases MBT based methods are superior to, or comparable to established methods based on accuracy, precision, speed, and analytical cost. In many disciplines MBT techniques have been compared to more conventional methods, such as plate counts in order to validate MBT methods (Cubero et al., 2001; Brinkman et al., 2003; Haugland et al., 2005; McDaniels et al., 2005; Cremonesi et al., 2006; Bellis et al., 2007; Koike et al., 2007). In addition independent measurement of calibration standards is common using a variety of techniques including: microscopy (Haugland et al., 2005); spectrophotometry (Koike et al., 2007; Perandin et al., 2007); flow cytometry (McDaniels et al., 2005); plate counts (Abd el-Galil et al., 2005; Cremonesi et al., 2006; Klerks et al., 2006). Due to the difficulty in culturing organisms such as *Dhc*, microscopic (FISH) or other non-DNA based methods such as PLFA data may also be useful. Total biomass determination by PLFA can confirm spectrophotometric quantification of microorganisms, and quantitative estimates of specific organisms may be possible if a signature phospholipid is identified.

For microbial surrogates, a key selection criteria will be that they can be enumerated using plate counts (see Section 2.2), providing a validation linkage between this classic microbiological method and qPCR quantification. The bioremediation industry has not typically correlated qPCR data enumeration to data generated using traditional microbial methods, primarily because Dhc and other organisms of interest cannot be enumerated using traditional methods such as plate counts or microscopy. Nevertheless, in validating bioremediation MBT testing, comparison to other techniques is critical. Establishing alternate, and multiple parallel methods for quantification of calibrators, surrogates and calibrated reference will provide a solid "verification foundation" for these emerging methods.

6.2.4 Data Interpretation and Analysis - Threshold Fluorescence

MBTs require a significant analytical path before one can assign a value to a sample. In this document, the primary focus has been on field and laboratory operations which may introduce variability. However, interpreting the output from qPCR equipment is not necessarily straight forward (Kontanis et al., 2006) and is critical to assigning a value to a sample. Real time qPCR quantitation is based on measured fluorescence as a function of amplification cycle. In the initial PCR cycles fluorescence is too low to be detected, but as amplification of the target(s) occurs, the fluorescence signal is detected above background. Eventually the amount of fluorescence will exceed a threshold (the threshold fluorescence). The number of PCR cycles required to obtain the threshold fluorescence is termed the threshold cycle (C_t). The more copies of template in a reaction, the fewer PCR cycles needed to exceed the threshold fluorescence, and thus, the lower the C_t. (i.e., C_t is inversely related to the template concentration). During the initial detection, template DNA is the limiting factor affecting increases in fluorescence, but at later amplification cycles, PCR reagents become limiting. The fluorescence-cycle curve is generally

sigmoidal in shape (Kontanis et al., 2006; Mackay, 2007), but its shape can be affected by inhibitory substances in a sample.

The threshold fluorescence may be determined manually (Cankar et al., 2006), using mathematical procedures such as the Liu and Saint method (Kontanis et al., 2006), or using programs such as LinRegPCR. The lab-to-lab variability may result as different personnel and laboratories use different techniques. Programs such as LinRegPCR should be useful in standardizing the selection of threshold fluorescence, but since these programs may be used without evaluating fundamental assumptions, their use can be problematic. Since threshold fluorescence is a critical parameter that fixes the C_t and thus, the sample test result, standardization of the method for determining this value between laboratories and even between individual runs within a lab is important. Currently SiREM manually sets the threshold fluorescence of each analytical run based on the value obtained from the standard curve. For example, if the threshold fluorescence generated (by the software algorithm) for the standard curve was 300 relative fluorescence units (RFU), then the value for subsequent runs would be manually set to 300 RFU prior to data analysis. In many cases, the mathematically generated threshold fluorescence is significantly different between analytical runs. Manually resetting the threshold fluorescence value can improve the check standard accuracy; however, the validity of this approach as well as the factors leading to variable threshold fluorescence values in the first place are not well understood. Since threshold fluorescence is a significant calibration parameter, standardizing calculation methods and better understanding variables affecting this parameter are critical.

6.2.5 Data Presentation and Normalization

Bioremediation qPCR data are generally presented as gene copies per unit of environmental matrix, volume of groundwater or mass of soil or sediment, or in the case of the Microbial Insights Bio-Trap® sampler "per bead" of matrix. This is referred to as absolute quantification. It is worth noting that SiREM and Microbial Insights present their absolute enumeration data in different units for groundwater samples with SiREM using a "per liter" value and Microbial Insights using a "per milliliter" value. This inconsistent use of units could be a possible source of confusion for users. Note that in microbiology research, cell counts are typically provided per mL of culture or sample. In assessment of drinking water quality, counts are typically provided per 100 mL. These differences reflect differences in expected concentrations of target organisms in the samples (drinking water samples are expected to have low counts of indicator organisms).

An alternative approach to presenting data, commonly used in gene expression studies, is to represent the target as a proportion of another molecule or so called relative quantification (Mackay, 2007). A common and incorrect assumption in data interpretation of qPCR analysis on groundwater is that changes in gene copies per liter necessarily reflect actual changes in the number of microorganisms in the subsurface. This may not always be the case, should, for example, one sampling event capture more total biomass (due to a varied sampling method, e.g.,

surging/not surging) resulting in the total number of gene copies detected per liter of groundwater as higher with surging, simply due to higher total biomass recovery. This could lead to the mistaken conclusion that the concentration of Dhc had actually increased, when in fact only the biomass collection efficiency changed. One method of reducing this type of misinterpretation is to normalize the gene copy data to some measure of total biomass. For example, Microbial Insights and Prof. Löeffler (Georgia Tech) normalize Dhc to total *Bacteria* quantified by qPCR using 16S rRNA universal primers (for *Bacteria*) whereas SiREM normalizes the qPCR results to total extractable DNA as quantified by Nanodrop spectrophotometry. The normalized measurements take into account the increased amount of total biomass and thus would not change if only biomass recovery efficiency occurred. For example, SiREM presents it Dhc results in gene copies per liter (see sample test Certificate Attachment D) and also as the estimated percentage of the microbial community that is comprised of Dhc "% Dhc" a relative measure. Should a Dhc gene copies /L value increase without a corresponding increase in % Dhc, then the increase is suspect and may be a case of biomass collection efficiency bias. Other disciplines normalize by other factors such as gene expression. Data analysis and interpretation should consider several normalization options and identify those normalization techniques which reduce variability and facilitate data interpretation.

6.3 Key Findings

6.3.1 Key Findings: Validation of Laboratory Equipment and Procedures

- Verification and documentation of a variety of laboratory equipment and procedures is integral to the development of high quality data and standard protocols for MBT methods.

6.3.2 Key Findings: Data Quality and Standardization Considerations for Groundwater Sampling

- Verification of current sampling procedures with supporting data (ORP, VOCs, pH conductivity, etc.) vs. Dhc concentration and reproducibility are critical to better understanding the impact of sampling activities on qPCR results.

- Understanding the impact of replication on MBT results through replicate sampling is an important prerequisite to understanding sampling variability and a prerequisite for developing sampling guidelines.

- The inconsistent use of data quality samples for testing of various controls such as trip blanks, equipment blanks may be a detriment to data quality. Inclusion of these controls as well as matrix spikes and field application of surrogates has the potential to improve data interpretation and quality control.

6.3.3 Key Findings: Data Quality Measures Associated with Biomass Concentration and Nucleic Acid Extraction

- Use of matrix spikes, internal naked DNA controls and microbial surrogates are not widely used in commercial qPCR testing for Dhc. The use of these types of controls widely reported in the literature was related to other disciplines.

- The use of a matrix spike, microbial surrogates and naked DNA internal controls applied in tandem at the biomass concentration/DNA extraction step is a possible strategy for resolved assessment of target losses during extraction and down stream procedures.

6.3.4 Key findings: Data Quality Measures Relevant to qPCR Quantification

- Current measures to assess inhibition in commercial Dhc testing are mostly qualitative and could be improved.

- Use of non-target DNA in negative control may be a slight improvement over “water only” negative controls in that they will control for non-specific PCR amplification.

- The use of alternate calibrators for analysis such as whole cells instead of plasmid DNA has the potential to significantly improve calibration of qPCR.

6.3.5 Key Findings: Quantification and Detection Limits

- Method detection limits of commercial Dhc analysis are not commonly known/reported.

- It is not clear that methods for defining quantification limits are rigorously developed or consistently applied.

- Dhc calibrated reference standards may be useful for establishing accurate detection and quantification limits.

6.3.6 Key Findings Assessing and Managing Variability

- Sources and degree of variability from sampling to final analysis are not well understood.

- The use of replication at specific sampling and analysis steps combined with statistical tools such as power calculations, has the potential to determine replication needs at those points.

6.3.7 Key Findings: Validation using non-qPCR Methods

- Use of qPCR for environmental remediation is an emerging technique and validation of methods and standards using non-PCR methods such as plate counts, microscopy, FISH, PLFA is critical to data quality and acceptance of results.

6.3.8 Key Findings: Data Interpretation and Analysis

- Impact of qPCR threshold variations on the qPCR output (i.e., C_t values need to be better understood so that variation in this value can be minimized and in cases where the threshold varies normalization between runs can be performed so C_t values between runs are compatible and data quality is maintained).

6.3.9 Key findings: Data Presentation and Normalization

- Reporting units in commercial testing is inconsistent, with SiREM using a per liter format and Microbial Insights using a per milliliter format.

- Normalization of absolute enumeration to measures of total biomass is critical for interpretation of data where inconsistent recovery of biomass may be an issue.

7. CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER STUDY

A survey of methods and procedures used in commercial qPCR testing and the literature has indicated that the environmental remediation field has the potential to adopt key methodological approaches derived from other disciplines in several key areas.

The previous sections highlight current practices with respect to qPCR testing procedures used in various disciplines and key findings. Selected key findings, viewed as having potential to improve methodologies associated with qPCR analysis, have been selected and are summarized in Table 7-1. Included in this table are the general topic (*Activity/Topic*), the relevant sections in this document where more details can be found (*Section*), the associated Task number in the original proposal (*Proposal Task Number*), the purpose of looking into this area (*Goal*), the specific components of the subject (*Relevant Parameters / Approaches*), whether the subject was addressed in the original proposal, the priority of the area to this project (*Project Priority*), and general comments and potential research activities (*Potential Research Approach/Activities*). The items designated in Table 7-1 will be examined further for upcoming research based on their designated priority and feasibility upon further consideration.

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