GUIDANCE DOCUMENT

Standardized Procedures for Use of Nucleic Acid-Based Tools – Recommendations for Groundwater Sampling and Analysis Using qPCR

SERDP Project ER-1561

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RECOMMENDATIONS FOR GROUNDWATER SAMPLING AND ANALYSIS USING qPCR

INTRODUCTION

Molecular biological tools (MBTs) have proven to be valuable in managing groundwater contamination. In particular, the polymerase chain reaction (PCR) and quantitative PCR (qPCR) allow one to identify and quantify the numbers of individual microbial species and strains, including the key microbial species needed for efficient and complete biodegradation of chlorinated ethenes (*Dehalococcoides mccartyi*, or *Dhc*).

However, there has been a lack of clear guidance on collecting and handling groundwater and subsurface soil samples for qPCR analysis. In response, the Strategic Environmental Research and Development Program (SERDP, 2005) identified the following needs:

1) A better understanding of the effects of the sampling process on MBT results, including sample collection, transport, storage, preservation, and processing;
2) Improved techniques for groundwater and associated saturated soil sampling that would foster the use of MBTs in environmental remediation; and
3) A better understanding of the relationship between the density of relevant biomarkers in a groundwater or saturated soil sample and the true density of microorganisms in the aquifer, and the impacts that sampling procedures may have on establishing such a relationship.

SERDP project ER-1561, *Standardized Procedures for Use of Nucleic Acid-Based Tools for Microbial Monitoring*, focused on identifying and minimizing the causes of variability during qPCR enumeration of genes of interest in groundwater, with the goal of developing of the knowledge needed to standardize methods for collecting, preserving, transporting, storing, and processing environmental samples for qPCR analysis.

This document summarizes the conclusions from Project ER-1561 and recommends procedures for using qPCR analyses that will provide data of sufficient accuracy and reproducibility to allow site management decisions regarding bioremediation or monitored natural attenuation (MNA). Further details are available in the ER-1561 Final Report (Lebrón et al., 2014), available at www.serdp-estcp.org.

TECHNICAL APPROACH

A technology review on the status of MBTs was performed at the beginning of the project, to evaluate the practices used for MBT analyses in other industries. That review focused the subsequent project activities, which included:

1) Comparing qPCR to non-PCR-based enumeration methods to validate and increase confidence in qPCR methods;
2) Comparing and assessing baseline variability within and between laboratories at the outset of the project using a multi-laboratory "round robin" approach;
3) Development and testing of a microbial internal amplification control (MIAC) for use in spike and recovery protocols, and in the extraction and qPCR process to assess biomarker extraction efficiency and matrix interference;
4) Assessment and optimization of methods including sampling, biomass collection, nucleic extraction, and qPCR protocols;
5) Assessment of inter-laboratory variability after integration of the MIAC and optimized methods; and
6) Determining the distribution of *Dhc* cells between aquifer solids and groundwater to better understand and interpret groundwater enumeration results.

PROJECT FINDINGS

SERDP Project ER-1561 focused on standardizing methods for qPCR enumeration of microbes in groundwater samples, with the goal of understanding and minimizing variability caused by analytical methods and sampling procedures. This project demonstrated that qPCR methods applied to remediation samples can be accurate and consistent. The project examined a wide range of techniques and challenges with the goal of increasing our ability to confidently quantify microorganisms, and in particular *Dhc*, in groundwater samples. The goal was to achieve high precision and accuracy using qPCR, and to better understand and identify cases where test results can be biased.

The two keys to accomplishing these goals were 1) step-wise testing of critical points in the sampling and analysis stream to better understand major sources of errors and possible biases, and 2) development and testing of a microbial internal amplification control (MIAC) to account for matrix interference and biomass losses.

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

1) Agreement of qPCR methods with non-molecular methods such as plate counts and microscopy, that demonstrate the fundamental accuracy of qPCR;
2) A better understanding of the steps in the analysis where variability is most likely to occur;

3) The development of an effective microbial internal amplification control (MIAC) with the ability to quantify biomass losses and inhibition and flag suspect samples/analyses;
4) Obtaining sample-to-sample consistency within labs and between sampling events in the same well;
5) The ability for five independent labs to return similar *Dhc* enumeration results for identical groundwater samples; and
6) A better understanding of the distribution of *Dhc* between aquifer solids and groundwater.

The following sections summarize the key conclusions and recommendations of the project including sampling, shipping, storage, DNA extraction, controls, filtration, qPCR analysis, and implications. Figure 1-1 provides a summary of sample and analysis flow with selected recommendations/findings at key steps. These recommendations should increase the precision and accuracy of environmental qPCR analyses, and the confidence in the results.

SAMPLING, SHIPPING, EXTRACTION, AND ANALYSIS

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

Sampling of Groundwater

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

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

- No significant difference was observed in terms of target gene quantification between high- and low-flow sampling methods. Due to its widespread use for VOC analysis low-flow sampling is recommended.
- Shipping of Sterivex cartridges or groundwater should occur at 4°C using an overnight carrier, and processing should occur without delay wherever possible.

  If necessary, Sterivex cartridges can be stored at -80°C. Storage of groundwater at 4°C should be minimized to the extent possible.

Shipping and Preservation of Samples

After collecting a site groundwater sample, or a filter cartridge with concentrated biomass, the sample is typically shipped to a testing laboratory, which typically requires 1-2 days, and where sample storage prior to analysis may be required. Storage of samples at temperatures above 4°C led to substantial reductions in the enumeration of *Dhc*. To the extent possible, storage time prior to analysis should be minimized and exposure to temperatures above 4°C avoided.

Instability of biomass was especially apparent with biomass concentrated on filters. Therefore hold times for biomass collected on onsite (e.g., Sterivex) filters should be shorter than the typical hold times for unfiltered groundwater, which may exhibit greater stability.

Collection of Biomass by Filtration

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

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

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

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

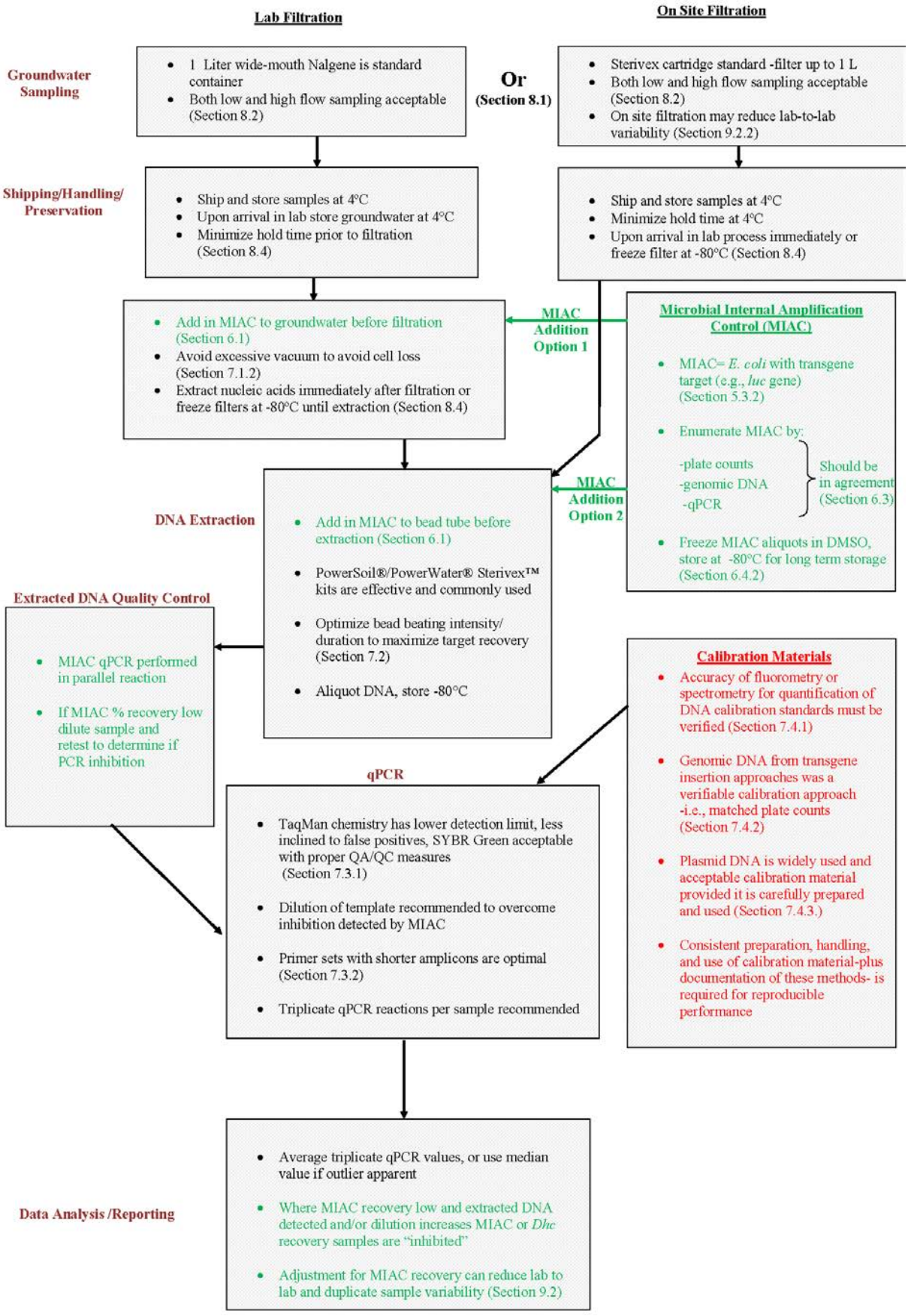

Figure 1. Key findings for improving qPCR sampling and analysis of groundwater (Section numbers are from the project Final Report)

qPCR Analysis

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

1) Both SYBR Green and TaqMan qPCR chemistries can produce accurate and comparable quantification results. Nevertheless, SYBR Green chemistry is more likely to produce false positives than TaqMan*;* therefore, greater care is required in interpreting SYBR Green assays to rule out non-specific amplification. The use of melting curve analysis and agarose gel electrophoresis confirmation target gene-specific amplification is recommended for this purpose.
2) Commonly used *Dhc* primer sets with differing amplicon lengths (from 66 bp to 514 bp) provided accurate enumeration and acceptable PCR efficiencies. Nevertheless, shorter amplicons are preferred as they are less likely to suffer from PCR inhibition and exhibit higher PCR fidelity.
3) Calibration materials used for qPCR must be suitable templates for PCR and accurate quantification of these materials is also essential for achieving accurate qPCR results. The most commonly used qPCR calibration material is plasmid DNA with a cloned target gene, which is quantified by spectrophotometry and/or fluorometry. Data from this project and the literature suggested that:
    - Spectrophotometry is likely more inclined than fluorometry to high biases due to non-specific absorbance by non-DNA molecules. For this reason, fluorometry with DNA specific dyes may offer improved accuracy for quantifying DNA.
    - Due to supercoiling, plasmids may be less efficient templates for PCR reactions than genomic DNA and have the potential to introduce high biases in qPCR enumeration. For calibration purposes, genomic DNA may offer advantages to supercoiled plasmids.

Impacts of Attached vs. Non-Attached Cells

The attachment behavior of cells (i.e., attached to aquifer solids v. non-attached) has implications for estimating the total number of target cells in an aquifer (i.e., non-attached cells plus attached cells) based on groundwater analysis. It is obviously important to understand if differences in the attached versus non-attached cell ratios occur (i) between soils with different properties, (ii) under varying geochemical

conditions, and (iii) in response to organismal characteristics of the microbe(s) of interest to better interpret groundwater MBT results.

The following conclusions can be drawn from the column studies (for details refer to Section 10 of the Final Report):

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

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

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

One of the implications of the findings is that the application of MBTs to groundwater samples may result in a significant (up to 100-fold) underestimation of the total number of *Dhc* cells in aquifers that support *Dhc* growth. Empirical data correlate the *Dhc* abundance in groundwater with reductive dechlorination activity and end points. For example, ethene formation is likely to occur when the *Dhc* cell titers exceed 10^6 cells L^{-1}. The results of ER-1561 do not change this interpretation even though the true *Dhc* abundance in the aquifer is one or two orders of magnitude higher.

At sites with intermediate (10^3-10^5 cells L^{-1}) or low (<10^3 cells L^{-1}) *Dhc* cell abundances in groundwater, the interpretation of qPCR data in terms of reductive dechlorination potential and end points is more challenging because the attached *Dhc* population size can vary and exceed the planktonic *Dhc* cell titers. Under such scenarios, additional analyses, such as determining soil organic matter content and qPCR analysis of solid materials, may be warranted.

DNA Extraction and Storage

During this process, DNA is extracted from biomass collected on filters through which groundwater is passed and extraction is performed using commercially available kits. The following procedures are recommended:

1) PowerSoil®/PowerWater® Sterivex™ kits are commonly used for effective extraction of DNA from biomass collected on filters.

2) After biomass collection, DNA extraction should occur without delay. If necessary, filters with biomass can be stored at -80ºC until extraction.

3) Manufacturer recommended DNA extraction kit bead beating intensity and duration may be insufficient for *Dhc* and protocols and should be optimized.

4) Extracted DNA should be stored at -80°C and aliquoted to minimize freeze thaw cycles when repeat analysis is required.

MICROBIAL INTERNAL AMPLIFICATION CONTROLS (MIAC)

MIAC are whole cell internal standards used in spike and recovery protocols, which can be used to detect analyte losses throughout the extraction and analysis processes. The application of a MIAC enables quality control and improves data interpretation of qPCR data. MIAC aliquots can be stored (e.g., frozen at -80ºC) to provide a consistent supply. The MIAC can be applied at different steps in the extraction process, including direct addition to groundwater, or addition at the stage of DNA extraction. Lower than expected recovery of the MIAC can indicate qPCR inhibition or problems with DNA extraction. Repeat analysis should determine if method failure was the cause of low recovery, whereas dilution of samples can be used to determine if PCR inhibition is the cause of low MIAC recovery.

The MIAC developed under the project (Hatt et al., 2013) is an *E. coli* cell with an introduced luciferase gene sequence. Use of this MIAC is recommended for qPCR sampling. The *E. coli*-luciferase MIAC has the following advantages for such uses:

1) The MIAC is quantifiable by plate count methods or other non-qPCR techniques such as direct microscopy;

2) It contains unique genetic sequences that are not found in groundwater samples; and

3) It does not interfere with quantification of the target gene(s).

CALIBRATION AND qPCR ASSAY APPROACHES

The final step in the analytical process is quantification of the genes of interest in a qPCR assay. Observations were made that may improve the accuracy of qPCR analysis including:

1) Both SYBR Green and TaqMan qPCR chemistries can produce accurate and comparable quantification results. SYBR Green chemistry is more likely to

produce false positives with environmental (e.g., groundwater) samples and greater care is required in interpreting SYBR Green assays to rule out non-specific amplification. The use of melting curve analysis and agarose gel electrophoresis confirmation of target gene-specific amplification is recommended.

2) Primer sets producing differing amplicon lengths provided accurate enumeration and acceptable PCR efficiencies. Nevertheless, shorter amplicons are preferred as they are less likely to suffer from PCR inhibition and exhibit higher PCR fidelity.

Finally, accurate quantification of qPCR calibration standards is essential for reliable qPCR results. The most commonly used qPCR calibration standard is plasmid DNA with a cloned target gene, which is quantified by spectrophotometry and/or fluorometry. The results from this project and the literature suggest that:

1) Microbial genomic DNA is an accurate qPCR calibration material and may be ideal for qPCR calibration where available/practical.
2) When using plasmids for calibration, verify plasmid DNA purity. The presence of genomic DNA or RNA could lead to an overestimation of gene copies when generating standard curves.
3) Every analytical qPCR plate should include a full suite of standards to ensure data precision and accuracy, including:
    - A DNA extraction blank, prepared with clean water processed as groundwater to rule out contamination during DNA extraction;
    - A "No Template" control, prepared by performing the qPCR reaction with sterile water to rule out contamination during reaction assembly; and
    - Positive controls, consisting of samples with target nucleic acids added at known concentrations to confirm ongoing method accuracy.

SUMMARY

The lessons learned from this project should improve the sampling and analysis of microorganisms in groundwater, especially the *Dhc* that are critical for complete biodegradation of chlorinated ethenes. In particular, use of on-site filtration, minimal holding times (or longer-term storage at -80^{o}C), relatively short amplicon lengths in primers used for analysis, appropriate MIAC (i.e., the *E. coli* strain developed for this project), and the recommended calibration procedures and standards should ensure that qPCR data are of sufficient accuracy and precision for site decision-making, and thereby increase confidence in the results, and foster the overall use of this promising technology.

Using qPCR to measure only those non-attached *Dhc* present in groundwater samples may result in a significant (up to 100-fold) underestimate of the total number of *Dhc* cells in the aquifer. Further, the ratio of attached to non-attached *Dhc* can vary greatly over time and distance. This finding should not change site management decisions if the *Dhc* levels are moderate to high ($>10^5$ cells/L), but the potential contribution of attached *Dhc* should be considered when interpreting data from sites with lower numbers.

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SERDP project ER-1561 focused on identifying and minimizing the causes of variability during qPCR enumeration of genes of interest in groundwater, with the goal of developing of the knowledge needed to standardize methods for collecting, preserving, transporting, storing, and processing environmental samples for qPCR analysis. This document summarizes the conclusions from Project ER-1561 and recommends procedures for using qPCR analyses that will provide data of sufficient accuracy and reproducibility to allow site management decisions regarding bioremediation or monitored natural attenuation (MNA). Further details are available in the ER-1561 Final Report (Lebrón et al., 2014), available at www.serdp-estcp.org.

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