EXECUTIVE SUMMARY

Development of Field Methodology to Rapidly Detect Dehalococcoides and Dehalobacter Spp. Genes On-Site

SERDP Project ER-2309

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1.0 INTRODUCTION

Microbially mediated reductive dechlorination plays a vital role in the bioremediation of the chlorinated ethenes, tetrachloroethene (PCE) and trichloroethene (TCE). Under the appropriate conditions, PCE and TCE undergo sequential reductive dechlorination via hydrogenolysis to *cis*-1,2-dichloroethene (cDCE) and vinyl chloride (VC), finally forming the non-toxic end product, ethene (ETH)[1]. When reductive dechlorination is linked to growth, it is called organohalide respiration; this metabolism is commonly associated with genera such as *Dehalococcoides* and *Dehalobacter*[2–9]. Commercially available reductive dechlorinating mixed cultures (e.g., KB-1 and SDC-9) containing such strains are frequently used for bioaugmenting contaminated groundwater aquifers[10–12]. The growth of these strains in the field and in the laboratory is commonly monitored using real-time quantitative PCR (qPCR) targeting the genes *vcrA*, *bvcA*, and *tceA*, which encode for distinct reductive dehalogenases (RDases) implicated in organohalide respiration[13]. To date, a number of qPCR protocols with DNA binding dyes or TaqMan probes to quantify *vcrA*, *bvcA*, and *tceA* genes have been developed[2, 14–16]. Although qPCR has been successful for monitoring reductive dechlorination, alternative methods would be advantageous for laboratories or practitioners without access to a real-time thermal cycler. Also, any method that is more economical and faster compared to qPCR would be beneficial.

Loop mediated isothermal amplification (LAMP) is a novel molecular method developed for the specific detection of nucleic acids[17]. LAMP, a one-step amplification reaction, amplifies a target DNA sequence using four to six primers. The *Bst* large fragment DNA polymerase has strand displacement activity and helicase-like activity, allowing it to unwind and amplify DNA strands in the 60–65ºC temperature range[17]. Because LAMP is rapid, sensitive, specific, and isothermally occurring, it has emerged as an alternative to qPCR-based methods in a wide variety of applications. Further, because LAMP does not require a real-time thermal cycler (amplification is isothermal), this method can be performed using less expensive and potentially field-deployable detection devices.

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2.0 OBJECTIVES

1. To quantify *Dehalococcoides mcccartyi* (*D. mccartyi*) 16S rRNA genes and *vcrA* genes in groundwater via LAMP without DNA extraction.

2. To use LAMP for the quantification of *D. mccartyi* RDase genes (*vcrA*, *bvcA*, and *tceA*) in two commonly used commercial bioaugmentation cultures, KB-1 (from SiREM) and SDC-9 (from APTIM), using DNA templates (rather than extracted DNA).

3. To compare quantification (i.e., gene copies/L values) between LAMP and qPCR to evaluate the effectiveness of LAMP as a tool to monitor the growth of *Dehalococcoides* spp. in KB-1 and SDC-9.

4. To validate the developed assays using DNA templates isolated from groundwater samples using LAMP with the Gene-Z (a hand-held device)[18].

5. To concentrate biomass in reaction wells of disposable Gene-Z chips for direct filter amplification (DFA) for use with LAMP assays targeting a *Dehalobacter* 16S rRNA gene and a putative 1,2-Dichloroethane reductive dehalogenase gene (*rdhA* gene)[19].

6. To compare DFA LAMP with qPCR including: i) spiking reactions with various concentrations of humic acid, ii) spiking multiple groundwater samples with *Dehalobacter* (from a commercially available bioaugmentation culture, TCA-20) following a concentration step, and iii) comparing methods for field-able target enrichment followed by direct isothermal amplification or DFA.

7. To develop visual-based SYBR green LAMP assays (requiring a bench-top centrifuge and a water bath) for quantifying *vcrA* and *tceA* genes with groundwater from contaminated sites without DNA extraction.

8. To quantify chlorinated solvent- and 1,4-dioxane-degrading microorganisms in contaminated site groundwater using shotgun sequencing and both taxonomic and functional analyses.

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3.0 TECHNICAL APPROACH

An approach for cell concentration followed by rapid direct amplification (elution-direct-amplification, EDA) and quantification without DNA extraction was developed. For amplification and quantification of the recovered cells, LAMP assays targeting the *vcrA* gene and *D. mccartyi* 16S rRNA gene were designed. Assays were tested using the Gene-Z platform, a hand-held, battery-operated, wireless and automated device that monitors fluorescence in a 64-well microfluidic card for parallel detection of multiple assays.[20]

In another study, six LAMP primers were designed for three RDase genes (*vcrA*, *bvcA*, and *tceA*) using Primer Explorer V4. The LAMP assays were compared to qPCR assays using plasmid standards and two commercially available bioaugmentation cultures, KB-1 and SDC-9 (both contain *D. mccartyi*). DNA was extracted over a growth cycle from KB-1 and SDC-9 cultures amended with TCE and VC, respectively. All three genes were quantified for KB-1 whereas only *vcrA* was quantified for SDC-9. The developed LAMP assays for *vcrA* and *tceA* genes were validated by comparing quantification on the hand-held platform, the Gene-Z, and a real-time thermal cycler using DNA isolated from eight groundwater samples obtained from a SDC-9 bioaugmented site (Tulsa, Oklahoma).

Other experiments tested DNA amplification from *Dehalobacter* with and without crude lysis and varying concentrations of humic acid. Three separate field-able methods of biomass concentration with eight aquifer samples were also tested, comparing direct LAMP with traditional DNA extraction and qPCR. A new technique was developed where filters were amplified directly within disposable Gene-Z chips (direct filter amplification, DFA).

An approach for quantifying RDase genes was developed requiring only low-cost laboratory equipment (a bench-top centrifuge and a water bath) and less time and resources compared to qPCR. This involved the concentration of biomass from groundwater, without DNA extraction, and LAMP amplification of the cell templates. The amplification products were detected by a simple visual color change (orange/green). The detection limits and overall assay performance were investigated using groundwater from contaminated sites. The approach was further adapted to incorporate deoxyuridine triphosphate (dUTP) and uracil DNA glycosylase (UNG) into the assays to reduce the probability of false positives. This optimized assay was used with the most probable number (MPN) method to quantify RDase genes (*vcrA* and *tceA*) in multiple groundwater samples.

Finally, shotgun sequencing was used for the quantification of taxonomic and functional biomarkers associated with chlorinated solvent bioremediation in twenty groundwater samples (five sites) following bioaugmentation with SDC-9. The analysis determined the abundance of 1) genera associated with chlorinated solvent degradation, 2) reductive dehalogenase (RDases) genes, 3) genes associated with 1,4-dioxane removal, 4) genes associated with aerobic chlorinated solvent degradation and 5) *D. mccartyi* genes associated with hydrogen and corrinoid metabolism.

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4.0 RESULTS AND DISCUSSION

The goal of the first study was to develop a strategy that could be used for onsite quantification of genetic biomarkers to monitor bioremediation of chlorinated solvents. Experiments were performed to: 1) evaluate the limit of detection (LOD) using the developed strategy and 2) quantitatively compare the EDA technique with conventional qPCR. A detection limit of 10^5 cells L^{-1} was obtained, corresponding to sensitivity between 10 to 100 genomic copies per reaction for assays targeting the *D. mccartyi*-specific 16S rRNA gene and *vcrA* gene, respectively. The quantity of *D. mccartyi* genomic copies from two TCE contaminated groundwater samples, measured with conventional means of quantification including filtration, DNA extraction, purification, and qPCR, was comparable to the novel LAMP-based approach. Overall, this demonstrated the method of measuring *D. mccartyi* 16S rRNA genes and *vcrA* genes in groundwater via direct amplification without intentional DNA extraction and purification.

The second study developed novel LAMP assays for *vcrA* and *tceA* using DNA extracted from groundwater samples. The values obtained were compared using qPCR on a real-time thermal cycler with LAMP on the Gene-Z. Overall, similar values were obtained for each gene on both platforms, indicating quantification with LAMP on the Gene-Z is a viable alternative to qPCR.

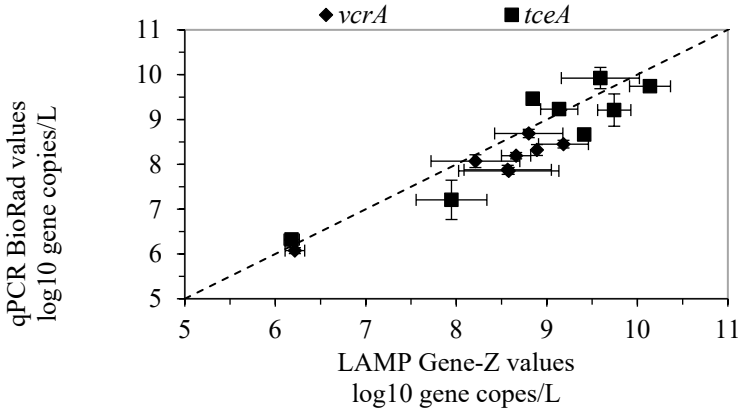

Figure 1. Comparison of *vcrA* and *tceA* Mean Gene Copies (per liter) in Triplicates or Eight Different Groundwater DNA Templates Observed Using qPCR on Real-Time Thermal Cycler and the Gene-Z.

The dashed line represents 1:1 comparison.

The LAMP assays were less susceptible to inhibition by humic acid than qPCR, amplifying with up to 100 mg/L of humic acid per LAMP reaction compared to only 1 mg/L for qPCR. Direct LAMP of *Dehalobacter* assays was less influenced by inhibition in groundwater samples (collected from remediation sites) compared to qPCR. For the DFA experiments, amplification was observed down to 10^2 *Dehalobacter* cells spiked into 100 mL. The DFA method was also tested to enrich for low target concentrations in environmental waters. While tested with different microbial targets (e.g., *Dehalococcoides* versus *Dehalobacter*), LAMP assays for both targets had similar sensitivity (10 to 100 gene copies per reaction depending on the assay tested). Placement of filters directly onto Gene-Z chips also reduced the time required for an elution step (10–15 minutes), which required an elution

buffer stored at 4°C and a means to vortex the Sterivex cartridges. Thus, DFA may provide a feasible alternative for biomass enrichment of water for genetic diagnostics.

A visual-based SYBR green LAMP-MPN approach was developed, offering a low-cost and user-friendly alternative to qPCR for quantifying RDase genes in groundwater samples. It offers three key advantages compared to existing methods: time, cost, and the potential *in situ* application. The use of centrifuged cells, instead of DNA, reduces the time and cost required for sample preparation (no DNA extraction). Also, compared to qPCR, the LAMP assay has a shorter run time and the visualization of amplification products is immediate. The assay requires only basic laboratory equipment (bench-top centrifuge and water bath), rather than an expensive real-time thermal cycler. With additional development and validation, it is possible that the method could be applied in the field. Additionally, the dUTP-UNG system reduces the probability of false positives caused by carry-over contamination and increases the overall robustness of visual detection with SYBR green LAMP. The regression equations generated for the visual-based SYBR green LAMP assay with MPN technique can be used to calibrate the assay to relate the data to traditional qPCR data.

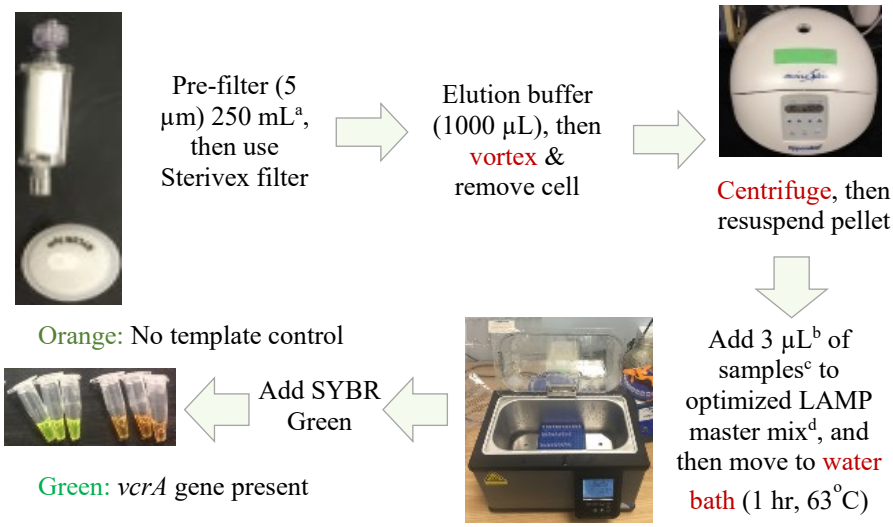

Figure 2. SYBR Green LAMP Method for Detection of RDase Genes from Groundwater Samples.

The final study examined an alternative method (shotgun sequencing) for quantifying key genes involved in contaminant biodegradation. The taxonomic analysis revealed numerous genera previously linked to chlorinated solvent degradation, including *Dehalococcoides, Desulfitobacterium* and *Dehalogenimonas*. The functional gene analysis indicated *vcrA* and *tceA* from *D. mccartyi* were the RDases with the highest relative abundance. Reads aligning with both aerobic and anaerobic biomarkers were observed across all sites. Aerobic solvent degradation genes, *etnC* or *etnE,* were detected in at least one sample from each site, as were *pmoA* and *mmoX*. The most abundant 1,4-dioxane biomarker detected was *Methylosinus trichosporium OB3b mmoX.* Reads aligning to *thmA* or *Pseudonocardia* were not found. This study illustrates the importance of shotgun sequencing to provide a more complete picture of the functional abilities of microbial communities than current methods. This method offers an advantage over current methods because it can quantify an unlimited number of functional genes.

5.0 IMPLICATIONS FOR FUTURE RESEARCH AND BENEFITS

LAMP offers two key advantages over qPCR. First, the LAMP primer sets developed in this research may be used with a variety of less expensive platforms (with diverse types of detection mechanisms), which include real-time turbidimeters, microfluidic chips (e.g., Gene-Z), and electrochemical or ultrasonic sensors[18, 21-24]. These platforms are cheaper and more accessible alternatives to qPCR thermal cyclers. Second, because these platforms use different reaction chemistries (e.g., producing significant visible fluorescence or post-reaction electrochemical changes) for the detection of amplified target sequences, they can be more economical compared to qPCR. In time-limited studies, the faster amplification during LAMP offers another potential advantage over qPCR. With the primer sets and reaction chemistries described in this research, all LAMP reactions were complete in less than one hour, significantly shorter than a typical qPCR run (>1.5 hours). In summary, the development of LAMP assays for the detection of the RDase genes *vcrA*, *bvcA*, and *tceA* will enable the use of alternative, potentially field-deployable platforms, such as the Gene-Z[18], for the rapid detection and quantification of *Dehalococcoides* spp. in groundwater from contaminated solvent sites. Further, the development of LAMP assays specific to two commonly used, commercially available cultures will facilitate specific detection of these RDase genes at sites subject to bioaugmentation.

The development of LAMP assay targeting *Dehalobacter* will also allow for their specific detection from bioaugmented sites. Minimal sample processing associated with the DFA method can potentially reduce costs and time for detection, and complements the use of field-able, real-time tools such as the Gene-Z device. The DFA method could also potentially be used in other field-able devices, in which filters are placed directly into amplification vials. Limitations of the DFA method include the inability to test multiple assays in parallel, which would require a setup for enriching and handling of numerous smaller filters. For LAMP more broadly, perhaps one of the greatest limitations is increased possibility of contamination to subsequent reactions, due to the large concentration of generated amplicons. As such, proper handling of chips and vials, ensuring they remain unopened following an amplification event, is critical.

The visual-based SYBR green LAMP approach developed is a low-cost and user-friendly alternative to qPCR for the quantitative evaluation of *D. mccartyi* RDase genes in groundwater samples. Compared to current methods, there are three key advantages to using visual detection with the LAMP and SYBR green assay: time, *in situ* application, and cost. The use of centrifuged cells, instead of DNA, reduces the time required for sample preparation. Also, compared to qPCR, the LAMP assay has a shorter run time and the visualization of amplification products is immediate. This approach has the additional potential for use in the field, as it requires equipment that could be easily transported onsite and powered by a generator. Such flexibility would enable decisions concerning remediation (e.g., to add more bioaugmentation culture) to be made immediately. A third important advantage concerns the cost of the two approaches. A 50 µL LAMP reaction with centrifuged cells is slightly cheaper (~$ 0.30) than a 20 µL qPCR reaction when consumables and reagents are considered. However, commercially available master mixes are used for qPCR, whereas reagents are mixed manually for LAMP. When commercial master mixes for LAMP become available, this will further decrease the time and cost associated with LAMP. More importantly, qPCR requires DNA extraction, which adds approximately $9.00 to each sample (almost doubling the cost). Another key difference concerns the use of low-cost laboratory equipment for LAMP (bench-top centrifuge and water bath, ~$600) compared to the high cost of

a real-time thermal cycler (~$20K) for qPCR. This makes the assay more accessible to a larger number of researchers and environmental engineers. As discussed above, if the assay is performed in triplicate in a dilution series, then the gene copies can be estimated, providing the concentration is above ~10^5 gene copies per liter. This value is less than the concentration required for effective dechlorination *in situ*.

Methods were also developed to determine the abundance of genes associated with chlorinated solvent and 1,4-dioxane biodegradation in groundwater samples (multiple samples from multiple contaminated sites). The use of shotgun sequencing enabled a larger selection of genes to be targeted compared to traditional qPCR. In fact, the number of functional genes that can be analyzed is limitless. The method also does not require primer design or primer assay verification for each target, as is the case for qPCR. The most labor-intensive part of the approach involved the collection of reference FASTA files for the DIAMOND alignment, after which all remaining steps were not time consuming. The sequencing price is perhaps the largest limitation to this method. In the current study, for 22 samples, the cost was approximately $210 per sample. However, it is likely that sequencing costs will drop as the technology evolves, making the approach more attractive. The data indicated the presence of both aerobic and anaerobic biomarkers for chlorinated solvent degradation. Not surprisingly, the taxonomic data alone was insufficient to determine the functional abilities of these communities. The relative abundance of hydrogenases and corrinoid metabolism genes suggest these may be appropriate additional biomarkers for *D. mccartyi.* The approach developed will enable researchers to investigate the abundance of any contaminant-degrading gene in any sample, greatly expanding the analytical toolbox for natural attenuation, biostimulation, or bioaugmentation.

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