

DEVELOPMENT AND APPLICATION OF A RAPID, USER-FRIENDLY AND
INEXPENSIVE METHOD TO DETECT AND QUANTIFY *DEHALOCOCCOIDES*
MCCARTYI GENES IN GROUNDWATER SAMPLES

By

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ABSTRACT

DEVELOPMENT AND APPLICATION OF A RAPID, USER-FRIENDLY AND INEXPENSIVE METHOD TO DETECT AND QUANTIFY *DEHALOCOCCOIDES MCCARTYI* GENES IN GROUNDWATER SAMPLES

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Tetrachloroethene (PCE) and trichloroethene (TCE) are the predominant contaminants at hazardous waste sites in the United States. The remediation of these chlorinated solvents at contaminated sites frequently involves bioremediation approaches such as biostimulation or bioaugmentation. Both approaches aim at increasing the population of *Dehalococcoides* cells in the subsurface. It has become common to quantify the population of these microorganisms both before and during the remediation process. TaqMan probe based quantitative polymerase chain reaction (qPCR) specific to the biomarker reductive dehalogenase (RDase) genes, such as *vcrA*, *bvcA*, and *tceA*, is now a widely accepted molecular biological tool (MBT) for this task. To date, a wide range of qPCR protocols based on *vcrA*, *bvcA*, and *tceA* genes have been successful for monitoring the reductive dechlorination driven by *Dehalococcoides* cells. However, alternate molecular methods that are faster and cheaper may make quantification significantly easier.

Loop mediated isothermal amplification (LAMP) assays were developed for the rapid and specific quantification of the RDase genes, *vcrA*, *tceA*, and *bvcA* in groundwater samples. As a first step, the developed LAMP assays were validated using DNA templates prepared from commercially available bioaugmenting cultures (SDC-9 and KB-1) and groundwater samples. To do this, the concentrations of *Dehalococcoides mccartyi* RDase genes with DNA templates obtained using LAMP were compared to concentrations obtained using qPCR on a real time thermal cycler. Additionally, the use of direct amplification was investigated. LAMP assays were then adapted for the development a field deployable kit. Here, an approach that requires only low

cost laboratory equipment (a bench top centrifuge and a water bath) and significantly less time and resources compared to qPCR is described. The method involves the concentration of biomass from groundwater (without DNA extraction) and LAMP of the cell templates. The amplification products are detected by a simple visual color change (orange/green). Finally, the most probable number technique was incorporated into the altered visual detection LAMP method for the quantitative estimation of RDase gene concentrations in groundwater samples.

Overall, quantification with LAMP on a real time thermal cycler was comparable to quantification with qPCR when DNA extracts prepared from SDC-9 and KB-1 or bioaugmented groundwater samples were used as templates for amplification. The LAMP assays to visually detect RDase genes, without DNA extraction or a thermal cycler, was successful to 1.8×10^5 gene copies per L for *vcrA* and 1.3×10^5 gene copies per L for *tceA*. Both values are below the threshold recommended for effective *in situ* dechlorination. Quantification with the MPN-LAMP assay using cell templates underestimated the concentration of RDase genes in groundwater samples by an order of magnitude compared to quantification with DNA templates and qPCR assay. Based on these results, response factors to correlate the MPN-LAMP data to estimated concentrations of RDase genes in groundwater samples are suggested. Future work should include a technology transfer of MPN-LAMP protocols to remediation practitioners.

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This thesis is dedicated to my Mom.
Thank you for always believing in me.

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KEY TO ABBREVIATIONS

PCE	Tetrachloroethene
TCE	Trichloroethene
cDCE	cis-1, 2-Dichloroethene
VC	Chloroethene
ETH	Ethene
qPCR	quantitative polymerase chain reaction
RDase	reductive dehalogenase
MBT	molecular biological tool
LAMP	loop mediated isothermal amplification
MPN	most probable number
dUTP	deoxyuridine triphosphate
UNG	Uracil-DNA glycosylase

Chapter 1: Introduction

1.1 Microbial reductive dechlorination for remediation of chlorinated ethene sites

Chlorinated solvents such as PCE and TCE have been widely used as metal degreasing agents in various industrial processes. The inappropriate disposal of hazardous waste from these processes has resulted in widespread contamination of groundwater aquifers across the US. Today, chlorinated solvents are the predominant contaminants at hazardous waste sites in the United States (1). As of 2000, EPA classifies PCE as a likely human carcinogen and TCE as a known human carcinogen by all routes of exposure. The maximum contaminant level goal (MCLG) and the maximum contaminant level (MCL) for the two contaminants have been set to 0 and 0.005 mg/L, respectively (2).

Microbially mediated reductive dechlorination plays a vital role in the degradation of the chlorinated ethenes, PCE and TCE. Under reducing conditions, PCE and TCE undergo sequential reductive dechlorination via hydrogenolysis to cDCE and VC, finally forming environmentally benign ethene (ETH). At each step, a chlorine atom is replaced by hydrogen, which is used as an electron donor (Figure 1.1). When reductive dechlorination is linked to microbial growth, it is called organohalide respiration; a metabolism commonly associated with the microorganism *Dehalococcoides mccartyi* (3-6). The biomarker RDase genes, *tceA*, *vcrA*, and *bvcA*, responsible for organohalide respiration of PCE and TCE to ETH in *Dehalococcoides mccartyi*, have been identified and annotated (Table 1.1) (4, 7-9). Today, bioremediation practitioners often use bioaugmentation and biostimulation as two key cost effective strategies for in situ attenuation of chlorinated solvent plumes. Bioaugmentation refers to the addition of commercially available reductive dechlorinating mixed cultures (e.g. KB-1 and SDC-9)

containing several *Dehalococcoides mccartyi* strains while biostimulation is defined as the nourishment of native *Dehalococcoides mccartyi* populations to metabolize PCE and TCE by the addition of various amendments to the subsurface environment. In 2009, it was estimated that bioaugmentation and biostimulation had been used at several hundred sites in the US (10, 11).

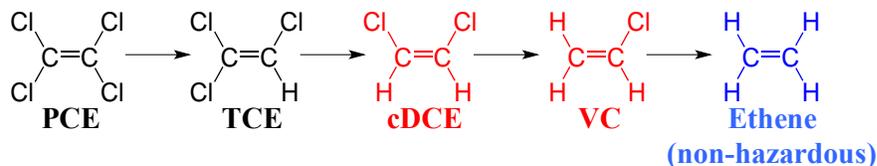


Figure 1.1 Organohalide respiration of PCE and TCE to ETH by *Dehalococcoides mccartyi*

Table 1.1 *Dehalococcoides mccartyi* RDase biomarker genes (4, 7-9)

Substrate → Product	RDase biomarker genes
TCE → cDCE	<i>tceA</i>
cDCE, VC → ETH	<i>vcrA</i>
VC → ETH	<i>bvcA</i>

1.2 Use of MBTs for quantification of *Dehalococcoides mccartyi* populations

Since bioaugmentation and biostimulation both aim to increase the concentration of *Dehalococcoides mccartyi* in the subsurface, it has become common to quantify the population of these microorganisms both before and during the remediation process. TaqMan probe based qPCR specific to the biomarker RDase genes, *tceA*, *vcrA*, and *bvcA* is now a widely accepted MBT for these tasks. To date, several qPCR protocols are available for detecting and quantifying *Dehalococcoides mccartyi* 16S rRNA and RDase genes using DNA extracts from groundwater samples as templates for amplification (12).

While alternative MBTs to qPCR have been explored, they have been scarcely used for a variety of reasons. For example, unlike qPCR, catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) is a cytogenic method, which can be used to detect active microbes. Despite being very sensitive, this MBT uses sophisticated equipment for visualization and quantification (13-15). Consequently, most remediation professionals either quantify RDase genes in-house using qPCR or use the service of a commercial laboratory with expertise in qPCR. However, there are significant costs associated with this approach. Specifically, the cost of purchasing a real time thermal cycler (~\$20K) for in-house analysis or the cost of having many samples analyzed by a commercial laboratory (typically >\$200 per sample). Given this expense and the large number of projects still addressing chlorinated solvent contamination, there is clearly a need for the development of a fast, cost-effective and user friendly approach to detect RDase genes.

1.3 Loop mediated isothermal amplification (LAMP)

LAMP is a novel molecular method recently developed for the specific detection of nucleic acids. LAMP is a one-step amplification reaction that amplifies a target DNA sequence using four to six primers. These primers are forward and backward internal primers (FIP and BIP), forward and backward outer primers (F3 and B3), and forward and backward loop primers (LF and LB). Amplification creates stem loop structures with several inverted repeats of the target and cauliflower like structures with multiple loops. The *Bst* large fragment DNA polymerase has strand displacement activity and helicase-like activity allowing it to unwind and amplify DNA strands in the 60-65 °C temperature range (16). Because LAMP is rapid, sensitive, specific and occurs isothermally, it has emerged as an alternative to PCR based methods in a wide variety of applications. For example, many LAMP assays have been developed for testing

food borne bacterial and viral contaminants. Recently, LAMP primer sets have been developed and tested for the detection of plasmids, pXO1 and pXO2, which impart infectious properties to several strains of *Bacillus anthracis*. LAMP can also be used to detect RNA viruses. A reverse transcription step is used to convert the RNA from viruses such as HIV-1 or the Ebola virus to DNA (17, 18).

In 2014, LAMP primer sets were developed for the 16S rRNA and *vcrA* genes of *Dehalococcoides mccartyi*. In that study, a field deployable approach for harvesting biomass from groundwater samples bioaugmented with SDC-9 was described. Direct amplification of templates with LAMP was performed using the hand held microfluidic platform, the Gene-Z. Detection limits below 10^7 gene copies/L were reported (this is the generally accepted threshold for acceptable *in situ* dechlorination). However, larger volumes of groundwater were required when *Dehalococcoides mccartyi* numbers were less than 10^4 gene copies/L (19).

1.4 Dissertation outline and objectives

The overall research objective of this dissertation was to develop LAMP as a rapid, sensitive, and specific method to quantify *Dehalococcoides* genes in groundwater samples without DNA extraction.

Chapter 2 – The development of specific LAMP primers for *Dehalococcoides mccartyi* RDase genes, *vcrA*, *bvcA*, and *tceA* is discussed. This chapter addresses the validation of quantification with LAMP using these primers on a real time thermal cycler. Data presented compares quantification with LAMP and qPCR using DNA templates prepared from SDC-9, KB-1, and bio augmented groundwater samples. A preliminary comparison between quantification data obtained with LAMP on the Gene-Z to qPCR for DNA templates prepared from SDC-9 bioaugmented groundwater samples is also presented.

The key objectives of this chapter were:

1. To evaluate if LAMP can be used for the quantification of *Dehalococcoides mccartyi* RDase genes (*vcrA*, *bvcA*, *tceA*) in two commonly used commercial bioaugmentation cultures, KB-1 (from SiREM) and SDC-9 (from CB&I) using DNA templates rather than direct amplification of harvested biomass.
2. To compare and contrast quantification (i.e. gene copies/L values) between LAMP and qPCR during one growth cycle in order to evaluate the effectiveness of LAMP as a tool to monitor the growth of *Dehalococcoides mccartyi* in KB-1 and SDC-9.
3. To validate quantification of the developed assays with LAMP on the Gene-Z using DNA templates isolated from eight groundwater samples.

Chapter 3 – The chapter outlines the modification of the LAMP protocol for potential field deployment. The LAMP protocol was altered to allow for the visual detection of direct cell templates without DNA extraction from SDC-9 bioaugmented groundwater samples. The new protocol can detect RDase genes, without DNA extraction or a thermal cycler, below 10^6 gene copies/L, the concentration threshold recommended for effective *in situ* dechlorination. The primary objectives of this chapter were:

1. To compare LAMP and qPCR for *tceA* and *vcrA* gene quantification using DNA extracted from numerous groundwater samples and a real time thermal cycler in order to establish that the LAMP assays were comparable to methods currently used
2. To optimize a template preparation approach for LAMP, which does not require DNA extraction.

3. To develop and test a rapid, user-friendly, and low-cost method based on LAMP to detect *vcrA* and *tceA* genes in groundwater samples from different chlorinated solvent sites.

Chapter 4 – The primary goal of this chapter was to make the visual detection LAMP assay quantitative by incorporating the MPN technique into the groundwater sample processing workflow. Moreover, visual detection with LAMP assay was optimized to include a dUTP-UNG contamination control system without significant increase in the detection limits. Two data sets are presented. The first set of data deals with the optimization of the amount of UNG required to destroy a *vcrA* gene contamination of $\sim 10^4$ gene copies per reaction while preserving amplification resulting from plasmid DNA and direct cell templates. The second set of data demonstrates the use of MPN technique coupled with visual detection LAMP to estimate the concentrations of RDase genes in groundwater samples without the extraction of DNA templates. Response factors correlating the concentrations observed with MPN visual detection LAMP and direct cell templates, to concentrations observed with qPCR and DNA templates were determined. These response factors would aid potential field deployment of the LAMP assay in the future. The key objectives of this chapter were:

1. To optimize the SYBR Green LAMP assay to incorporate dUTP and UNG based enzymatic control for prevention of false positives due to carry over contamination without a significantly increasing the detection limits of the SYBR Green LAMP assay.
2. To extend the application of MPN technique with the optimized SYBR Green LAMP assay for quantitative estimation of *Dehalococcoides mccartyi* RDase genes in groundwater samples without DNA extraction.

Chapter 5 – In this chapter, conclusions of this dissertation are outlined and a summary of the results and the future direction of the work are presented.

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Chapter 2:

Development of Loop Mediated Isothermal Amplification (LAMP) for Rapid Detection and Quantification of *Dehalococcoides mccartyi* Biomarker Genes in Commercial Reductive Dechlorinating Cultures KB-1 and SDC-9

This chapter is adapted from previously published work in Applied and Environmental Microbiology:

Kanitkar YH, Stedtfeld RD, Steffan RJ, Hashsham SA, Cupples AM. 2016. Development of loop mediated isothermal amplification (LAMP) for rapid detection and quantification of *Dehalococcoides* spp. biomarker genes in commercial reductive dechlorinating cultures KB-1 and SDC-9. Applied and Environmental Microbiology, 82:1799-1806.

2.1 Abstract

Real time quantitative polymerase chain reaction (qPCR) protocols specific to the reductive dehalogenase (RDase) genes, *vcrA*, *bvcA*, and *tceA* are commonly used to quantify *Dehalococcoides mccartyi* in groundwater from chlorinated solvent contaminated sites. In this study, LAMP was developed as an alternative approach for the quantification of these genes. LAMP does not require a real time thermal cycler (amplification is isothermal) allowing the method to be performed using less expensive and potentially field deployable detection devices. LAMP primers were designed for each of three RDase genes (*vcrA*, *bvcA*, *tceA*) using Primer Explorer V4. The LAMP assays were compared to conventional qPCR approaches using plasmid standards and two commercially available bioaugmentation cultures, KB-1 and SDC-9 (both contain *Dehalococcoides mccartyi*). DNA was extracted over a growth cycle from KB-1 and SDC-9 cultures amended with trichloroethene and vinyl chloride, respectively. All three genes

were quantified for KB-1 whereas only *vcrA* was quantified for SDC-9. A comparison of LAMP and qPCR using standard plasmids indicated quantification was similar over a large range of gene concentrations. In addition, the quantitative increase in gene concentrations over one growth cycle of KB-1 and SDC-9 using LAMP was comparable to that of qPCR. The developed LAMP assays for *vcrA* and *tceA* genes were validated by comparing quantification on the handheld 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, OK). These assays will be particularly useful at sites subject to bioaugmentation with these two commonly used *Dehalococcoides mccartyi* -containing cultures.

2.2 Introduction

Commercially available reductive dechlorinating mixed cultures (e.g. KB-1 and SDC-9) are frequently used for bioaugmenting contaminated groundwater aquifers (2, 3). 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 highly specific RDases implicated in organohalide respiration (4). To date, a number of qPCR protocols with DNA binding dyes or TaqMan probes to quantify *vcrA*, *bvcA*, and *tceA* genes have been developed (5). 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. In addition, any method that is more economical and faster compared to qPCR would be beneficial.

Loop mediated isothermal amplification (LAMP) is a novel molecular method recently developed for the specific detection of nucleic acids. It has a one-step amplification mechanism, which amplifies a target DNA sequence using four to six primers. Because LAMP is

rapid, sensitive, specific, and occurs isothermally, it has emerged as an alternative to PCR based methods in a wide variety of applications. For example, many LAMP assays have been developed for testing food borne bacterial pathogens and fungal contaminants (6, 7).

In 2014, LAMP primer sets were developed for the 16S rRNA and *vcrA* genes of *Dehalococcoides mccartyi* (8). In that study, a field deployable approach for harvesting biomass from samples of groundwater bio augmented with SDC-9 was described. Direct amplification of templates with LAMP was performed using the hand held platform, the Gene-Z. Detection limits below 10^7 gene copies/L were reported (this is the generally accepted threshold for acceptable *in situ* dechlorination). Here, the objective was to evaluate if LAMP can be used for the quantification of *Dehalococcoides mccartyi* RDase genes (*vcrA*, *bvcA*, *tceA*) in two commonly used commercial bioaugmentation cultures, KB-1 (from SiREM) and SDC-9 (from CB&I). This study involved DNA templates rather than direct amplification of harvested biomass. Quantification (i.e. gene copies/L values) was compared between LAMP and qPCR during one growth cycle to evaluate the effectiveness of LAMP as a tool to monitor the growth of *Dehalococcoides mccartyi* in KB-1 and SDC-9. Further, we used DNA templates isolated from eight groundwater samples to validate quantification of the LAMP assays on the Gene-Z. The data generated from the groundwater samples were also compared to data obtained using a real time thermal cycler.

2.3 Materials and methods

2.3.1 Cultures and growth conditions

All experiments were carried out in triplicate serum bottles (160 mL nominal volume) containing 100 mL (final volume) of culture and sealed with grey butyl rubber septa. After transferring the microcosms into an anaerobic chamber, the KB-1 or SDC-9 inoculum (10 mL)

and sterile mineral medium (90 mL) were added to the serum bottles using aseptic techniques. The bottles were capped with grey butyl rubber septa, removed from the anaerobic chamber, and sparged with 30% CO₂/70% N₂ to adjust the pH. During the growth cycle, the pH of each bottle was measured and adjusted to neutral, as needed using 1.0 M NaOH. The bottles were incubated quiescently, shielded from light, at room temperature (~22-24°C), and with the liquid in contact with the septum to minimize the loss of volatile compounds. The concentration of chlorinated ethenes was monitored by GC-FID, as previously described (9). All KB-1 serum bottles were amended with 10 µL of feed solution (1:10 dilution of neat TCE in methanol) to yield final amounts of ~23 µmol TCE and ~112 µmol of methanol in each bottle. Bottles were also amended with ethanol (~44.0 mg/L) each week if residual cDCE and VC were observed. An aliquot (1.0 mL) of culture fluid was removed on days 0, 7, 14, 21, 28, 35, 38, 41, and 44 for DNA extraction. DNA was isolated from 100 µL aliquots using Mo Bio DNA Isolation kit as per the manufacturer's protocol (Mo Bio Laboratories, Inc., Carlsbad, CA). All SDC-9 bottles were amended with ~20 µmol VC along with a 0.1 mL spike of 100 mM sodium lactate. DNA was extracted from 30 µL aliquots of 3 mL culture fluid on days 0, 6, 22, 27, 32, and 40.

2.3.2 Groundwater samples

Groundwater samples were obtained from a site in Tulsa, OK, which was recently bioaugmented with SDC-9. Eight amber glass bottles containing ~1.0 L groundwater sample representative of monitoring wells (MW) 1 - 4 (MW1-MW4) and injection wells (IW) 1-4 (IW1-IW4) were bubble wrapped and shipped overnight in a cooler packed with icepacks. Upon receipt, the bottles were stored at 4.0 °C in the absence of light for the duration of testing. Groundwater samples (100.0 mL) were filtered through 0.22 µm filter (EMD Millipore Corp., Billerica, MA) using a vacuum pump. Membranes were cut into 0.5 mm strips inside a petri dish

with a 15 blade using aseptic technique and these were added to 15.0 mL bead tubes supplied with the Mo Bio UltraClean water kit. DNA was extracted from this solution (1.5 mL) using the manufacturer's protocol. The DNA was precipitated by adding 150.0 μ L of 5 M NaCl and 3.0 mL of absolute ice-cold ethanol and incubating for 30 min at 4 °C. Following centrifugation (14000 x g, 20 min, room temperature), the DNA pellet was rinsed with 70% ethanol, air dried and suspended in 100 μ L of dH₂O. The extracted DNA was immediately used for amplification or stored at -20 °C for future use.

2.3.3 Preparation of plasmid standards

Genomic DNA was extracted from 5 mL of KB-1 (from SiREM Guelph, ON, Canada) using Mo Bio DNA isolation kit, as per the manufacturer's protocol. The *vcrA* and the *bvcA* genes were amplified using PCR with primers described previously (10). Amplified templates were cloned into *E. coli* DH5 α using pCR2.1 TOPO TA-cloning vector (Invitrogen) to generate plasmid inserts. *E. coli* cultures were grown overnight in LB medium amended with 50 mg/mL ampicillin and 7.0% glycerol at 37 °C. Plasmid standards for *tceA* were provided by Dr. Frank Löffler (University of Tennessee, Knoxville). Plasmid inserts were extracted using 5 mL *E. coli* culture and the Qiagen plasmid extraction kit. Gene copies were calculated as previously described (10). Serial dilutions of plasmid inserts from 3.16×10^8 plasmids to ~316 plasmids per μ L for *vcrA*, 2.65×10^9 plasmids to ~265 plasmids per μ L for *bvcA*, and 1.41×10^{10} to 141 plasmids per μ L for *tceA* were used as standards for the amplifications. By plotting the log of the calculated copy number against the cycle threshold (for qPCR) or threshold time (for LAMP) at which fluorescence for that sample crosses the threshold value, standard curves were obtained.

2.3.4 Design of LAMP primers

The LAMP primer sets used for this study are listed in Table 2.1. FASTA files for the functional RDase genes *vcrA* (Accession#NC_013552.1, region 1187298-1188857), *bvcA* (Accession# NC_009455.1 region 834959 to 836509), and *tceA* (Accession# AY165309.1) of *Dehalococcoides mccartyi* were downloaded and aligned with the relevant environmental sequences on the NCBI nucleotide database to identify the conserved regions. Next, LAMP primer sets were designed for those regions using Primer Explorer V4 (<https://primerexplorer.jp/e/>). For the *vcrA* gene, two new LAMP primer sets, *vcrA* set A and *vcrA* set C, targeting the 857-1072 bp region were designed and used along with *vcrA* set B, which was designed and tested (11). One primer set, *bvcA* set A, targeting the 895-1139 bp region was designed for the *bvcA* gene. Similarly, *tceA* set A was designed to target the 882-1156 bp region of the *tceA* gene. Finally, NCBI nucleotide BLAST was used to determine the fidelity of the primer sets to the target sequences in environmental submissions on the database by setting the default expect value as 1×10^{-5} .

Table 2.1 LAMP primer sets designed and used in this study

Target Gene	Primer set	Primer	Sequence (5'→3')	Target region (bp)
<i>vcrA</i>	vcrA set A	F3	GTAAGTTTTACGCGAGATGG	<i>Accesssion#</i> <i>NC_013552.</i> <i>1, region</i> <i>1187298-</i> <i>1188857</i> <i>(857-1072)</i>
		B3	GTCATCGGCTGAGCTTTC	
		FIP	ACCTCCCATTTTGGTACGCTTGTA TGGTCCGCCACAT	
		BIP	AAGACAATTTTCTAATGCTGAGGGC ATTTGGGATCTGCCAGGT	
		LF	CATCAGGTGGCGCTGAATC	
		LB	AGCTGCAAAATATTTTGGTGCTGG	
	vcrA set B (11)	F3	ACTAATATATAAGAAAGCTCAGCC	<i>Accesssion#</i> <i>NC_013552.</i> <i>1, region</i> <i>1187298-</i> <i>1188857</i> <i>(652-886)</i>
		B3	TCTTATTGAGTTCTTGTGGTTG	
		FIP	GGTCAGGAACCTTGGGATAAATTTT GATGACTCTAGGAAAAGGAACA	
		BIP	AACTTTAAGGAAGCGGATTATAGC TATGGATTCACACTTTGTTGG	
		LF	CCTGGTCCACCTAATTCAGTGA	
		LB	ACTACAATGATGCAGAGTGGGTTA	
	vcrA set C	F3	GTAAGTTTTACGCGAGATGG	<i>Accesssion#</i> <i>NC_013552.</i> <i>1, region</i> <i>1187298-</i> <i>1188857</i> <i>(857-1072)</i>
		B3	GTCATCGGCTGAGCTTTC	
		FIP	ACCTCCCATTTTGGTACGCTTGTA TGGTCCGCCACAT	
BIP		AAGACAATTTTCTAATGCTGAGGGC ATTTGGGATCTGCCAGGT		
LF		CCATCAGGTGGCGCTGAA		
LB		TGGTGCTGGTGGCGTT		
<i>bvcA</i>	bvcA Set A	F3	ACAATGCCTTTACCAGAAGA	<i>Accession#</i> <i>NC_009455.</i> <i>1 region</i> <i>834959 to</i> <i>836509</i> <i>(895-1139)</i>
		B3	ACCGTATTTGGGGCTGAT	
		FIP	TCGGCCTCCATTAAGCCATTCTC TAGGGTGGTCATGT	
		BIP	ATCAAGGACTTGGTGGCGACCTTGT TCGGAAAGACACTCA	
		LF	AGGCAATCATACTTGAAGCGTC	
		LB	TGTGGGGACCTGGTGGT	
<i>tceA</i>	tceA Set A	F3	GCCGTTTATTCCATTCATGG	<i>Accession#</i> <i>AY165309.1</i> <i>(882-1156)</i>
		B3	GCATAGACTGGATGAAGGAA	
		FIP	ACATAATTGCTGGGAGAACCCG- TCGCATAGAGAGATAAGGCC	
		BIP	GCCATTCGTGGCGGCATATAT- CAGATTATGACCCTGGTGAA	
		LF	CTTTATGGACGCTATGAAGGTTCTA	
		LB	TCTTCCCTGCGGTCGCCATA	

2.3.5 qPCR and LAMP amplification

Each 20 μ L LAMP reaction contained 1x isothermal amplification buffer (NEB, Catalog# B0537S), 1.4 mM dNTPs, 0.8 mM Betaine, 6.0 mM MgSO₄, 1.6 units of BST 2.0 Warm Start (NEB), 0.8 μ L SYTO 82 orange fluorescent dye (Life Technologies, Inc., Grand Island, NY), 0.8 μ L Pluronic (Life Technologies, Inc., Grand Island, NY), 0.8 μ L Bovine Serum Albumin, 0.25 μ M 10X Primer Mix and balance water to make up 18 μ L. Reactions were incubated at 63 °C for 60 min for amplification.

All TaqMan assays were set up as 20 μ L reactions. Each 20 μ L reaction contained 10 μ L iTaq Universal super mix supplied by Bio-Rad, 1.2 μ L TaqMan probe described previously (12-14), and balance water to make up 18 μ L. PCR amplifications were performed using cycling conditions of 95 °C for 15 s, 60 °C for 1 min, a slow ramp of 1% to 95 °C for 15 s and 60 °C for 15 s. DNA templates and plasmid standards were added to each LAMP and qPCR reaction as 2 μ L aliquots. All qPCR primers and probes used in this study are listed in Table 2.2.

All qPCR experiments were performed in the commercially available real time thermal cycler (Chromo 4 PCR thermal cycler). For KB-1 and SDC-9 templates, amplification with LAMP was carried out in the real time thermal cycler while amplification with groundwater templates was performed in both the Gene-Z (below) as well as in the real time thermal cycler. Triplicate reactions for each test, positive and no-template controls were used for all experiments.

Table 2.2 qPCR primers used in this study

Target Gene	Primer	Sequence	Reference
<i>vcrA</i>	vcrA1022F	CGGGCGGATGCACTATTTT	(10)
	vcrA1093R	GAATAGTCCGTGCCCTTCCTC	(10)
	vcrA1042Probe	FAM-CGCAGTAACTCAACCATTTCCT GGTAGTGG-TAMRA	(10)
<i>bvcA</i>	bvcA925F	AAAAGCACTTGGCTATCAAGGAC	(10)
	bvcA1017R	CCAAAAGCACCACCAGGTC	(10)
	bvcA977Probe	FAM-TGGTGGCGACGTGGCTATGTGG- TAMRA	(10)
<i>tceA</i>	tceA1270F	ATCCAGATTATGACCCTGGTGAA	(12, 14)
	tceA1336R	GCGGCATATATTAGGGCATCTT	(12, 14)

2.3.1 Gene-Z analysis of groundwater samples

Inside the Gene-Z device, an array of 64 LEDs, a bundle of optical fibers, and a single photodiode were used to measure fluorescence in real-time (8). An iPod Touch (gen 5) was used to control reaction temperature and time, start the device, stream data via Bluetooth connectivity, sort, plot, store, and transmit results. Disposable chips were made by etching channels and wells into black acrylic (1.58 mm thick) via a 40 W CO₂ laser (Full Spectrum). Etched chips were cleaned and prepared as previously described (15). Briefly, chips were cleaned with distilled water, soaked in 70% ethanol for 10 min, and dried for 10 min at 70 °C. Once dry, primers were dispensed and dried in wells at 70 °C for 5 min. Wells were enclosed with optical adhesive film (MicroAmp, Applied Biosystems) and chips were stored at -20 °C until use. Chips were cut with eight reaction wells per sample, and four samples per chip (i.e. 32 reaction wells per chip with 20 μ L reaction volume). Six chips were used to test groundwater samples and two additional chips were used to test plasmid dilution standards. *vcrA* and *tceA* primers were each dispensed into three separate reaction wells per sample lane.

2.4 Results

2.4.1 Amplification with LAMP primers and their application

As stated previously, *vcrA* set B was previously designed and tested using templates obtained from groundwater spiked with SDC-9 (11). In that study, larger volumes of groundwater samples (1 to 4 L) were required when the *vcrA* gene copies were less than 10^4 gene copies/L. In this study, we developed two new LAMP primer sets (*vcrA* set A and *vcrA* set C) that exhibited faster LAMP threshold times than *vcrA* set B with $10^{3.5}$ gene copy templates (23.9 ± 0.4 min. for *vcrA* set A, 21.2 ± 0.2 min. for *vcrA* set C, and 28.3 ± 0.3 min. for *vcrA* set B). Moreover, the new primer sets had equivalent or better detection limits compared to *vcrA* set B ($10^{3.5}$ gene copies/reaction for *vcrA* set A, $10^{2.5}$ gene copies/reaction for *vcrA* set C, and $10^{3.5}$ gene copies/reaction for *vcrA* set B). Here, the first set of experiments targeted *vcrA* in SDC-9 using both *vcrA* set A and *vcrA* set B. The second set of experiments involved *vcrA* set C, the most refined LAMP primer set for *vcrA*, with KB-1 templates.

Additionally, new primer sets were developed for *bvcA* and *tceA* genes and these were tested with KB-1 templates. One aim was to evaluate if the new LAMP primer sets could be used to track the growth of *Dehalococcoides mccartyi* in actively dechlorinating KB-1 and SDC-9 cultures over one growth cycle. As SDC-9 does not contain *bvcA*, this assay was not tested with this culture. qPCR was used as a control assay for all experiments. While in-silico specificity of the new primer sets was not evaluated, LAMP reaction requires six primers 6 different primers for amplification it is less likely to produce false positives.

2.4.2 Monitoring *Dehalococcoides mccartyi* growth in KB-1 and SDC-9 cultures

The mean mass of TCE, cDCE, VC, and ETH in triplicate KB-1 cultures and an abiotic control is shown (Figure 2.1). As expected, TCE was reduced to cDCE, VC, ETH. cDCE

accumulated and peaked at 7 days after inoculation, while VC peaked at ~35 days before being rapidly degraded to ETH. Stoichiometric amounts of ETH accumulated at the end of the growth cycle. TCE, cDCE, and VC were not detected at the end of the 48-day incubation period. At each time point, the concentration of two *Dehalococcoides mccartyi* strains, VS and BAV1, were investigated using qPCR and LAMP targeting the *vcrA* and *bvcA* genes in DNA extracted from the KB-1 cultures. Figure 2.2 illustrates the gene copies of *vcrA* (A), *bvcA* (B), and *tceA* (C) per L in triplicate cultures of KB-1 while growing on TCE. We observed a comparable steady increase in the number of *vcrA* gene copies from $\sim 5.8 \times 10^6$ gene copies/L on day 7 to $\sim 6.4 \times 10^9$ gene copies/L on day 38 using both LAMP and qPCR. This was followed by a more rapid increase between days 38 and 44 from $\sim 6.4 \times 10^9$ gene copies/L to $\sim 1.1 \times 10^{11}$ gene copies/L coupled to significant reduction of VC to ETH. A similar trend was observed with the *bvcA* gene. Gene copy numbers steadily increased from 3.2×10^6 gene copies/L at day 7 to 5.7×10^8 gene copies/L, followed by a more rapid increase to 5.49×10^9 gene copies/L. Gene copies of *tceA* increased to $\sim 1.4 \times 10^8$ gene copies/L from day 0 to day 14, coupled to the reduction of TCE to cDCE and VC, which was then followed by a slight increase to $\sim 3.8 \times 10^8$ gene copies/L on day 44.

Similarly, the growth of *Dehalococcoides mccartyi* in SDC-9 culture was investigated using the *vcrA* gene. The mean mass of VC and ETH in triplicate SDC-9 cultures and an abiotic control is shown in Figure 2.3. The bars represent standard deviation from the mean values. Rapid reductive dechlorination of $\sim 24 \mu\text{mol}$ VC from day 20 to day 40 was coupled to the stoichiometric accumulation of ETH.

Figure 2.4 illustrates *vcrA* gene copies per liter measured via two LAMP primer sets (*vcrA* set A and *vcrA* set B) and qPCR in the triplicate cultures. The *vcrA* gene copies steadily

increased to $\sim 9.0 \times 10^7$ gene copies/L. As VC rapidly dechlorinated to ETH, we observed that the *vcrA* gene copies increased from $\sim 9.0 \times 10^7$ gene copies/L to $\sim 1.1 \times 10^9$ gene copies/L.

The mean gene copies of *vcrA* (A), *bvcA* (B), and *tceA* (C) per L in triplicate cultures of KB-1 while growing on TCE are shown (Figure 2.5). The bars represent standard deviations from the mean values. Note, the y-axis is a log scale, which does not start at zero to illustrate the differences between *vcrA*, *bvcA*, and *tceA* concentrations. To elucidate the potential of LAMP as an alternate method to monitor *Dehalococcoides mccartyi* in commercial reductive dechlorinating cultures, the absolute quantification of each gene in KB-1 templates was compared using both methods. Figure 2.6 is a comparison of *vcrA* (A), *bvcA* (B), and *tceA* (C) mean gene copies (per L) in triplicate cultures of KB-1 while growing on TCE. The bars represent standard deviations from the mean values. For each gene, the qPCR data is plotted against the LAMP data at each time point over the growth cycle. The dashed line represents a 1:1 comparison.

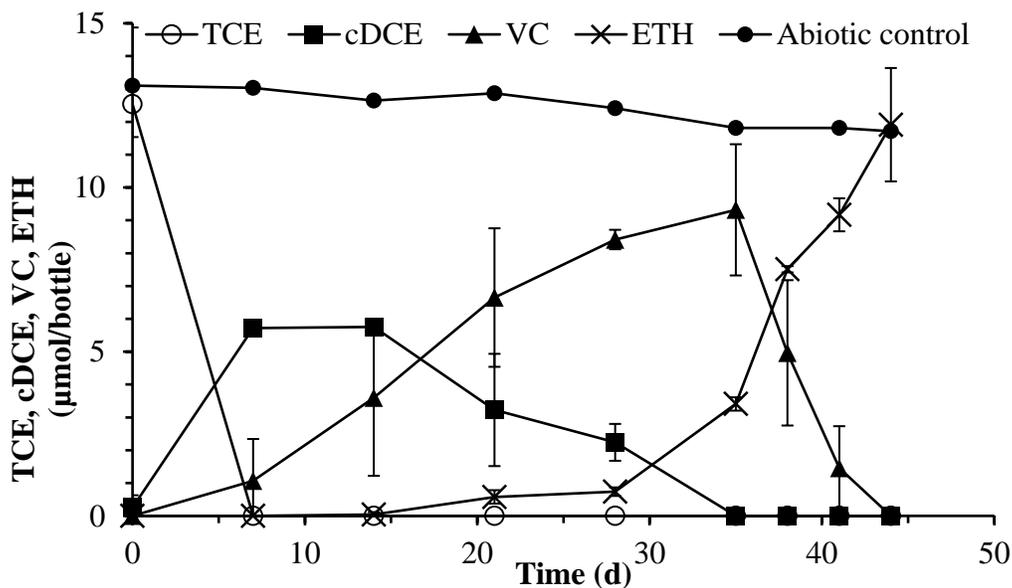


Figure 2.1 Mean mass of TCE, cDCE, VC, and ETH in triplicate KB-1 cultures and an abiotic control.

Note: The bars represent one standard deviation from the mean values

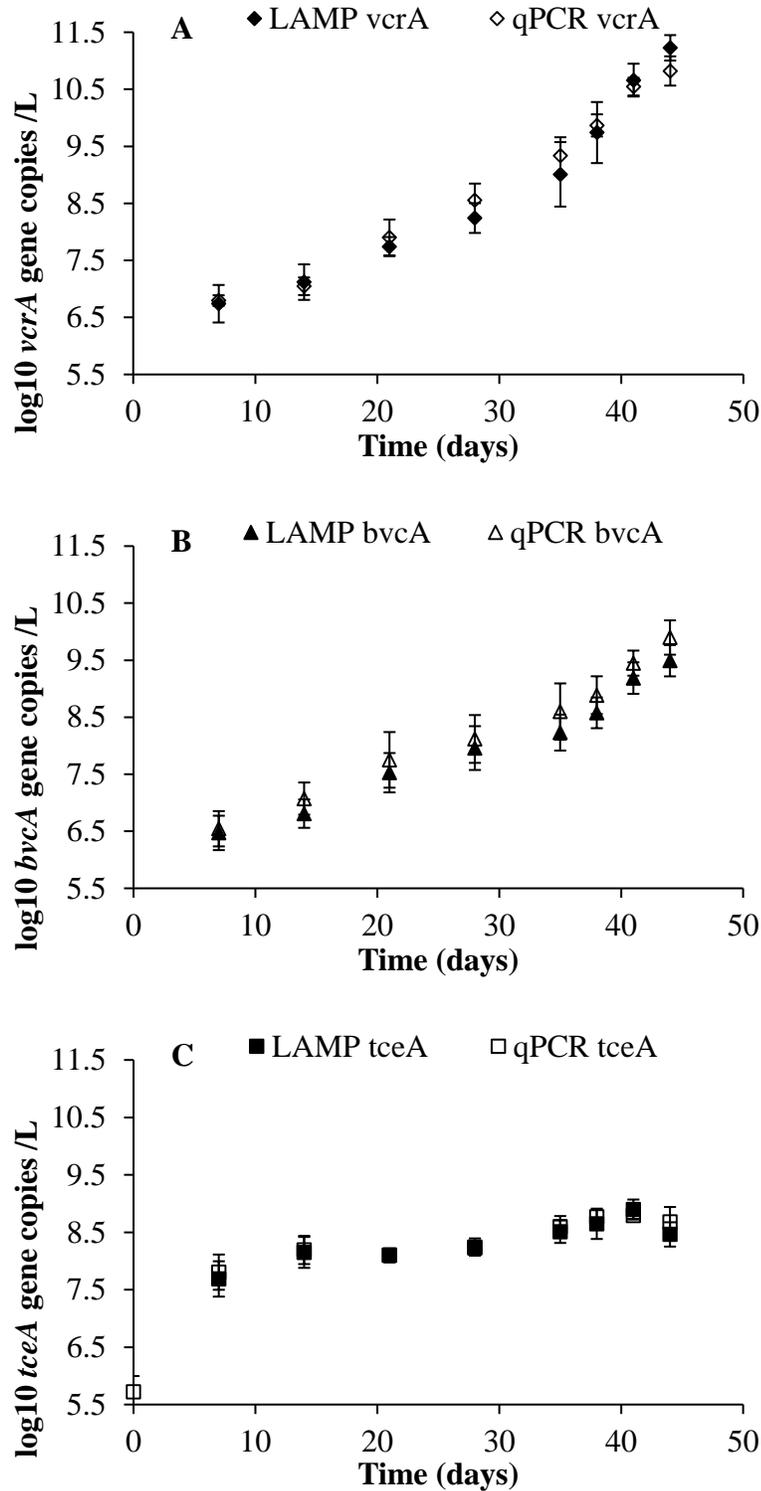


Figure 2.2 Gene copies of *vcrA* (A), *bvcA* (B), and *tceA* (C) per L in triplicate cultures of KB-1 while growing on TCE, LAMP *vcrA* set C was used to target *vcrA*

Note: The bars represent one standard deviation from the mean values

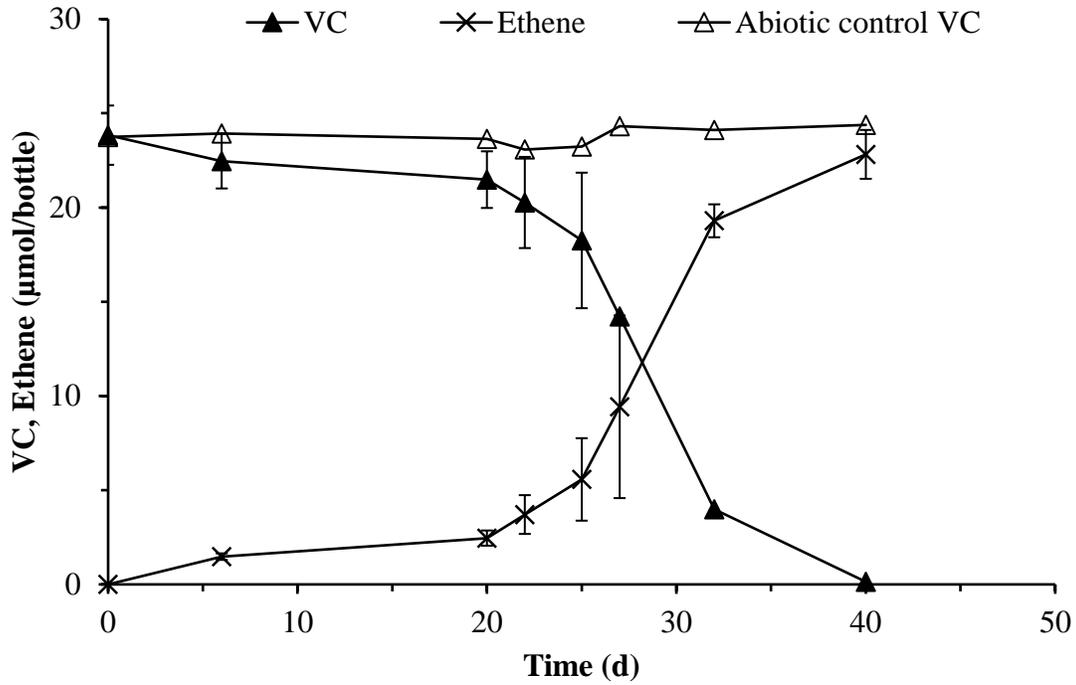


Figure 2.3 Mean mass of VC and ETH in triplicate SDC-9 cultures and an abiotic control.

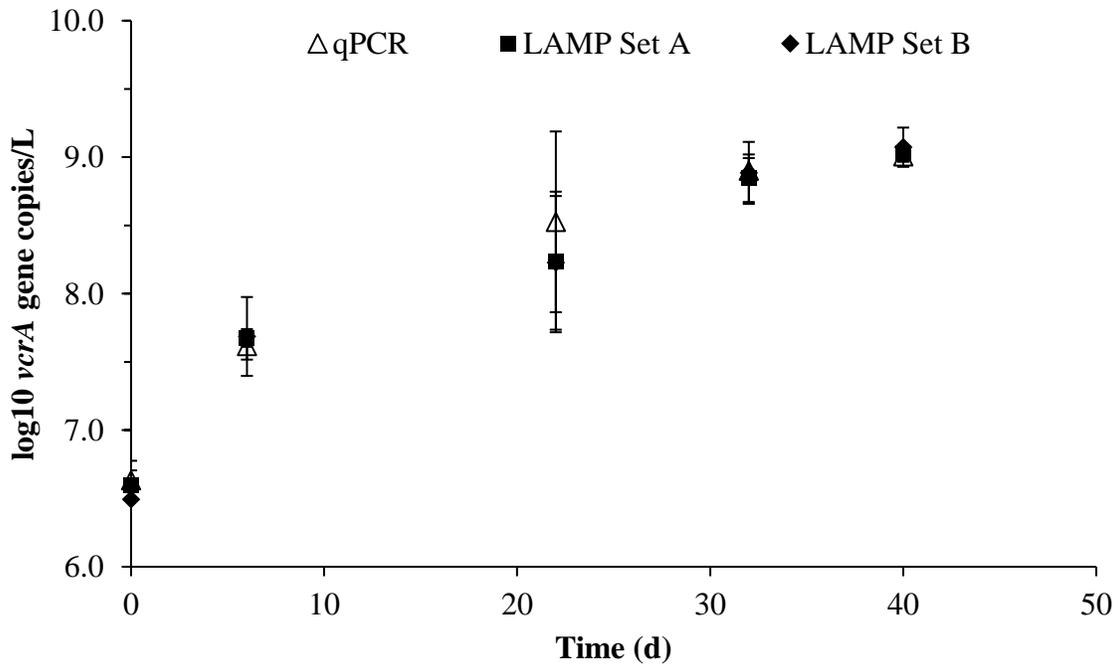


Figure 2.4 *vcrA* gene copies per L measured via qPCR and two LAMP assays (*vcrA* set A and *vcrA* set B) in triplicate cultures of SDC-9 (A, B, C) during growth of VC

Note: The bars represent one standard deviation from the mean values

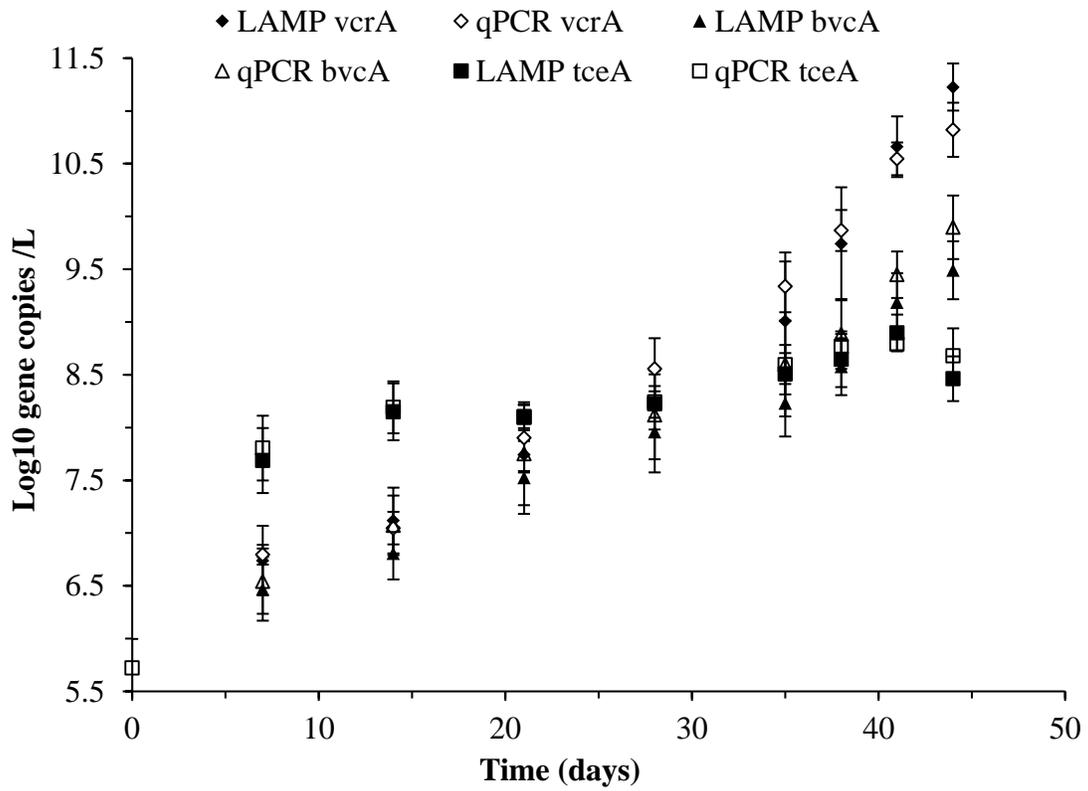


Figure 2.5 Mean mass of *vcrA* (A), *bvcA* (B), and *tceA* (C) per L in triplicate cultures of KB-1 while growing on TCE

Note: The bars represent standard deviation from the mean values. The y-axis is a log scale and does not start at zero.

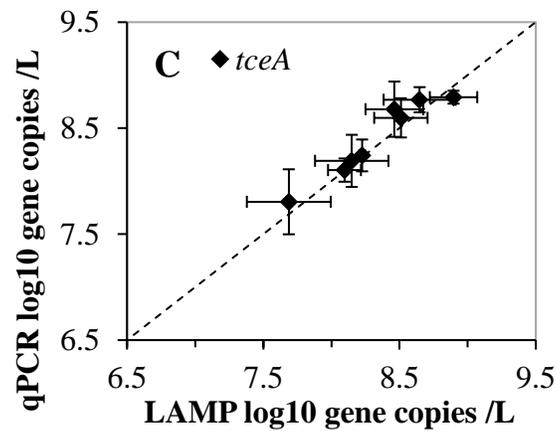
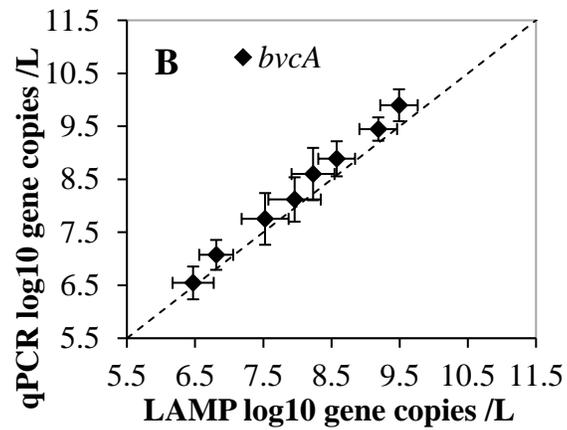
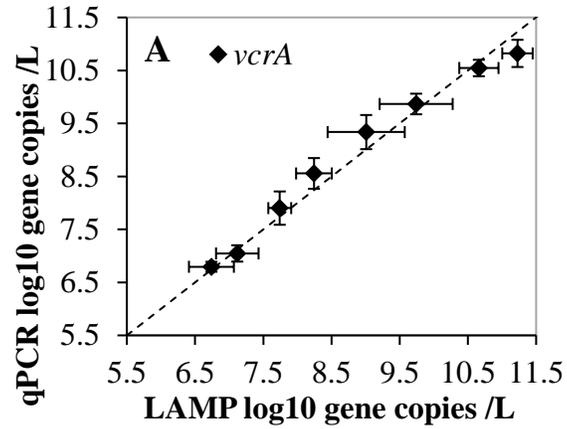


Figure 2.6 Comparison of *vcrA* (A), *bvca* (B), *tceA* (C) mean gene copies (per L) in triplicate cultures of KB-1 while growing on TCE.

Note: The bars represent standard deviations from the mean values. The dashed line represents a 1:1 comparison

2.4.3 Validation of new LAMP assays with the Gene-Z using groundwater templates

Nucleic acids extracted from groundwater from a previously bio augmented, chlorinated solvent contaminated site were used to validate the novel LAMP assays with a hand held device, the Gene-Z. The data obtained using the new LAMP assays on the Gene-Z were compared to those obtained using qPCR on a real time thermal cycler. Specifically, *vcrA* and *tceA* gene copies (per L) from four monitoring wells and four injection wells (in triplicate) were compared using the new LAMP assays and qPCR (Figure 2.7). Again, the dashed line represents 1:1 slope.

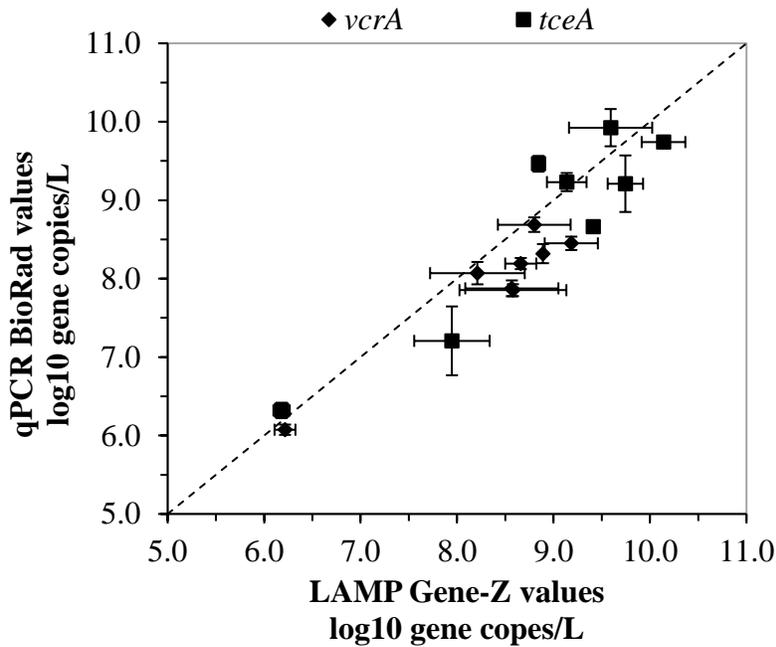


Figure 2.7 Comparison of *vcrA* and *tceA* mean gene copies (per L) in triplicates of eight different groundwater DNA templates observed using qPCR on a real time thermal cycler and the Gene-Z.

2.5 Discussion

To date, LAMP primer sets have been developed for phylogenetic or functional genes of prokaryotes, eukaryotes, and viruses to detect target sequences in templates extracted from a variety of environmental matrices such as air, water, soil, fecal matter, or blood. For example, LAMP can be used for detection of the *invA* gene in all known 89 *Salmonella* spp. strains, a food borne bacterial pathogen causing Salmonellosis (7, 16). LAMP was also applied to detect *Cryptosporidium* oocysts, which cause cryptosporidiosis, using functional gene *gp60* (17). Similarly, LAMP primer sets for detection of viral pathogens such as HIV-1 and Ebola have also been described (18, 19). Another application of LAMP has been the identification of beef contaminated with ostrich meat. The LAMP assay successfully identified ostrich meat contamination of up to 0.01% using direct cell amplification from swabs (20). These examples demonstrate the versatility of LAMP in terms of its application to human health, environmental and food microbiology. In this study, LAMP was applied for the rapid quantification of the key biomarker RDase genes, *vcrA*, *bvcA*, and *tceA*. These biomarker genes are important for monitoring the activity of *Dehalococcoides mccartyi* in groundwater during natural attenuation, biostimulation, and bioaugmentation at chlorinated solvent contaminated sites.

The growth patterns observed in this study are characteristic of cultures such as KB-1 and SDC-9 when amended with TCE and VC, respectively. KB-1 is highly enriched in a few unique *Dehalococcoides mccartyi* strains, which are capable of catabolic growth using cDCE and VC as electron acceptors for reductive dechlorination (21). Typically, the individual cells of such strains carry one copy of *vcrA* or *bvcA* genes that code for the two distinct vinyl chloride reductases (22-24). However, neither vinyl chloride reductase is capable of growth linked metabolic reduction of TCE to cDCE or VC. When amended with TCE, initial growth is

cometabolic and often slower (4, 25, 26). As a result, the increase in *vcrA* and *bvcA* gene copies observed is faster and more discernable when cDCE and VC are being dechlorinated. In contrast, the *tceA* gene codes for trichloroethene reductive dehalogenase responsible for the reductive dechlorination of TCE to cDCE and VC (27). An initial increase in the *tceA* gene copies coupled to rapid reduction of TCE to cDCE and VC was observed in all KB-1 microcosms, but, as TCE depleted, the increase in *tceA* gene copies was substantially less. This is indicative of growth of *Dehalococcoides mccartyi* strains with the *tceA* gene. However, the abundance of the *tceA* gene within the *Dehalococcoides* genus is more widespread than *vcrA* or *bvcA* genes and strains that carry the *tceA* gene may carry *vcrA* or *bvcA* genes (4, 10, 28). Additionally, TCE dechlorination may also be driven by *Geobacter* strains in KB-1 along with *Dehalococcoides mccartyi* (29), which may explain the less discernable growth pattern.

In this research, the novel LAMP assays for *vcrA* and *tceA* were validated using DNA extracted from groundwater samples. We compared the quantification i.e. gene copies/L values for both genes obtained using qPCR on a real time thermal cycler with LAMP on the Gene-Z. The Gene-Z yields slightly higher values compared to qPCR, which may be attributed to the difference in fluorescence sensing mechanisms of the two platforms. Nevertheless, similar values were obtained for each gene on both platforms, indicating quantification with LAMP on the Gene-Z is a viable alternative to qPCR. Moreover, the new LAMP primer sets were able to detect quantities below 10^7 gene copies/L, the accepted limit for natural attenuation. LAMP offers two key advantages over qPCR. First, the LAMP primer sets described here may be used with a variety of several less expensive platforms (with diverse types of detection mechanisms), which include real time turbidimeters, microfluidic chips (e.g. Gene-Z), electrochemical, or ultrasonic sensors (11, 30-33). These platforms are cheaper and more accessible alternatives to

qPCR thermal cyclers. Second, these platforms use different reaction chemistries (e.g. producing significant visible fluorescence or post reaction electrochemical changes) for the detection of amplified target sequences and thus can be more economical compared to qPCR. In time-limited studies, another potential advantage is that amplification during LAMP is faster than qPCR. With the primer sets and reaction chemistries described in this study, all LAMP reactions were complete in less than one hour, which is significantly shorter than a typical qPCR run (>1.5 h). In summary, the development of LAMP assays for the detection of the RDase genes, *vcrA*, *bvcA*, and *tceA* will enable using alternative, potentially field deployable platforms, such as the Gene-Z (11), for the rapid detection and quantification of *Dehalococcoides mccartyi* 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.

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Chapter 3:

Development and Application of a Rapid, User-Friendly and Inexpensive Method to Detect

Dehalococcoides mccartyi RDase Genes from Groundwater

This chapter is adapted from previously published work in Applied Microbiology and Biotechnology:

Kanitkar YH, Stedtfeld RD, Hatzinger PB, Hashsham SA, Cupples AM. 2017. Development and application of a rapid, user-friendly, and inexpensive method to detect *Dehalococcoides mccartyi* reductive dehalogenase genes from groundwater. Applied Microbiology and Biotechnology 101:4827-4835.

3.1 Abstract

TaqMan probe based qPCR specific to the biomarker RDase genes is a widely accepted MBT for determining the abundance of *Dehalococcoides mccartyi* in groundwater samples from chlorinated solvent contaminated sites. However, there are significant costs associated with this MBT. In this study, we describe an approach that requires only low cost laboratory equipment (a bench top centrifuge and a water bath) and requires less time and resources compared to qPCR. The method involves the concentration of biomass from groundwater, without DNA extraction, and loop mediated isothermal amplification (LAMP) of the cell templates. The amplification products are detected by a simple visual color change (orange/green). The detection limits of the assay were determined using groundwater from a contaminated site. In addition, the assay was tested with groundwater from three additional contaminated sites. The final approach to detect RDase genes, without DNA extraction or a thermal cycler, was successful to 1.8×10^5 gene

copies per L for *vcrA* and 1.3×10^5 gene copies per L for *tceA*. Both values are below the threshold recommended for effective *in situ* dechlorination.

3.2 Introduction

The remediation of chlorinated solvent contaminated sites frequently involves approaches such as biostimulation or bioaugmentation to facilitate the reductive dechlorination of these chemicals via a process known as organohalide respiration (2). Both approaches aim at increasing the population of *Dehalococcoides* cells in the subsurface. It has become common to quantify the population of these microorganisms both before and during the remediation process. TaqMan probe based qPCR specific to the biomarker RDase genes, such as *vcrA*, *bvcA*, and *tceA*, is now a widely accepted MBT for these tasks (3-7). Other methods for detecting *Dehalococcoides* cells include catalyzed reporter deposition-fluorescent in situ hybridization (CARD-FISH) and microarray based platforms (8-10). Despite being very sensitive, these MBTs use sophisticated equipment for visualization and quantification. Consequently, most remediation professionals either detect RDase genes in-house using qPCR or use the service of a commercial laboratory with expertise in qPCR. However, there are significant costs associated with both approaches. Specifically, the cost of purchasing a real time thermal cycler (~\$20K) for in-house analysis or the cost of having many samples analyzed by a commercial laboratory (typically >\$250 per sample). Given this expense and the large number of on-going projects addressing chlorinated solvent contamination, there is clearly a need for the development of a fast, cost-effective, and user-friendly approach to detect RDase genes.

Our previous research involved LAMP to detect *Dehalococcoides* and *Dehalobacter* 16S rRNA and RDase genes in groundwater using a hand-held proprietary microfluidic platform

called the Gene-Z (11, 12). Also, in 2016, additional LAMP assays for *vcrA* and *tceA* were validated using DNA extracted from two commercial cultures (KB-1 and SDC-9) commonly used in bioaugmentation (13). The latter study found that the quantification of *vcrA* and *tceA* gene copies from these cultures using LAMP was comparable on both the Gene-Z and a real time thermal cycler. The research also illustrated that the results were similar for groundwater samples, however, only groundwater from one site was examined (Tulsa, OK). In both studies, amplification was performed either in a real time thermal cycler or in the Gene-Z. The core objective of the current research was to develop an approach to detect RDase genes without either platform, so that the method could be more widely applied. Additional goals were to shorten the analysis time and decrease the cost per sample compared to qPCR.

In this study, we developed a rapid, easy to use and lower cost method to detect *vcrA* and *tceA* genes in groundwater and then tested the method with multiple groundwater samples from different chlorinated solvent sites. The first stage of the research involved a comparison of LAMP to qPCR for *tceA* and *vcrA* gene quantification using DNA extracted from numerous groundwater samples and a real time thermal cycler (to establish that the LAMP assays were comparable to methods currently used). Following this, experiments were conducted to optimize a sample concentration approach, which, unlike qPCR, does not involve DNA extraction. Finally, a method was developed which requires only a bench top centrifuge and a water bath (no Gene-Z or thermal cycler) for RDase detection.

3.3 Methods

3.3.1 Groundwater samples

Groundwater samples were collected from monitoring wells at five different chlorinated solvent sites (San Antonio TX, Tulsa OK, Edison NJ, Quantico VA, and Indian Head MD) using traditional low-flow sampling (14). The groundwater was pumped into autoclaved 1L amber bottles, which were subsequently placed on ice and shipped overnight to Michigan State University for analysis. Each of these sites was previously bioaugmented with the commercially available reductive dechlorinating culture SDC-9 (15-17), which contains the *vcrA* and *tceA* genes. A summary of the groundwater wells and sites is provided, along with the gene targets, assay type and template type used for each sample in Table 3.3

3.3.2 Preparation of DNA templates

Extracted DNA from groundwater was used in several sets of experiments. First, extracted DNA was used to enable the comparison of LAMP with qPCR. In addition, extracted DNA was used in the experiments to evaluate the gene numbers obtained using centrifuged cell templates and direct cell templates (see below). Lastly, extracted DNA was used to determine the detection thresholds for the SYBR green assay and to test the SYBR green assay with centrifuged cell templates from a number of contaminated sites. For producing DNA templates, groundwater (100 mL) was filtered through 0.22 μm filter (EMD Millipore Corp., Billerica, MA) using a vacuum pump. Membranes were cut into 5 mm strips inside a petri dish with a 15 blade (Bard Parker, catalog no. 37615) using aseptic technique and were added to 15 mL bead tubes supplied with the MO BIO Ultraclean water kit (MO BIO Laboratories Inc., Carlsbad, CA) (18). The DNA was eluted according to the instructions supplied by the manufacturer and the final template was suspended in 100 μL of dH_2O . The extracted DNA template was immediately used

for real time amplification or stored at -20 °C for future use. The entire process was repeated three times to generate triplicates of each groundwater sample.

3.3.3 Preparation of direct and centrifuged cell templates (no DNA extraction)

To concentrate *Dehalococcoides* cells from groundwater, a 5 µm nylon membrane filter (Nalgene, Rochester, NY) and a 0.22 µm Sterivex filter (EMD Millipore Corp., Billerica, MA) were placed in series to form a groundwater filtration module (to remove turbidity). Groundwater samples (100 mL) were filtered through the groundwater filtration module using a sterile 160 mL syringe. *Dehalococcoides* sp. are 0.3- 1.0 µm (19). A cell elution buffer was prepared by adding compound ST1B (MO Bio Catalog #14600-50-NF-1B) to solution ST1A (MO Bio Catalog #14600-50-NF-1A) according to manufacturer's protocol. The buffer (1000 µL) was added to the filter and the filter was capped on both ends. The whole module was vortexed for 10 min (at medium speed) to resuspend the retained biomass. The filters were cut open and the suspension was poured into a 1.5 mL tube. The resuspended cell templates will herein be referred to as "direct cells". To obtain cell templates with centrifugation (herein called "centrifuged cells"), the resuspended biomass was further centrifuged (13000 g x 15 min.) to obtain a biomass pellet by decanting the liquid content from the tube. The pellet was then resuspended in 100 µL of 1X phosphate buffer and was immediately used for amplification. Each process was repeated three times to generate triplicates of each groundwater sample for both the centrifuged cells and the direct cells.

3.3.4 LAMP specificity experiments

The specificity of each RDase LAMP SYBR green assay was examined using plasmid standards. Plasmid templates for *vcrA* and *bvcA* genes were prepared as previously described (13), while the plasmid template for the *tceA* gene was gifted from Dr. Frank Löffler's laboratory

(University of Tennessee, Knoxville). The specificity of each assay was determined using positive controls (the plasmid with the target gene) and negative controls (plasmids with other RDase genes as well as no template controls). The plasmids were present at 10^6 gene copies per reaction.

3.3.5 Amplification with LAMP and qPCR in a real time thermal cycler

qPCR and LAMP real time amplification reactions were performed using two commercially available real time thermal cyclers (Chromo 4 PCR thermal cycler or Bio-Rad Laboratories C1000 touch with CFX96 real time platform). Real time LAMP reactions were set up using the primers and protocol previously described (12, 13). Each 20 μ L LAMP reaction contained 1x isothermal amplification buffer (NEB, Catalog# B0537S), 1.4 mM dNTPs, 0.8 mM Betaine, 6.0 mM $MgSO_4$, 1.6 units of BST 2.0 Warm Start (NEB), 0.8 μ L SYTO 82 orange fluorescent dye (Life Technologies, Inc., Grand Island, NY), 0.8 μ L Pluronic (Life Technologies, Inc., Grand Island, NY), 0.8 μ L Bovine Serum Albumin, 0.25 μ M 10X Primer Mix and balance water to make up 18 μ L. The reactions were incubated at 63 $^{\circ}C$ for 60 min for amplification.

Each 20 μ L TaqMan reaction contained 10 μ L iTaq Universal super mix supplied by Bio-Rad, 1.2 μ L TaqMan probe, and balance water to make up 18 μ L. PCR amplifications were performed using cycling conditions of 95 $^{\circ}C$ for 15 s, 60 $^{\circ}C$ for 1 min, a slow ramp of 1% to 95 $^{\circ}C$ for 15 s and 60 $^{\circ}C$ for 15 s. Templates and standards were added to each LAMP and qPCR reaction as 2 μ L aliquots. All qPCR primers and probes are listed (Table 2.2). From the LAMP primers supplied in Table 2.1 vcrA set C, bvcA set A, and tceA set A were used for this study.

3.3.6 SYBR Green LAMP in a water bath

Before preparing the reactions, a calibrated water bath (Cole-Parmer, Catalog # EW-14576-04) was set to 63 $^{\circ}C$. The reactions were performed using 0.2 mL PCR tubes or a 96 well

plate. Each 50 μL reaction contained 45 μL Master mix containing 25 μL of 2X reaction mix (2X Isothermal amplification buffer, 2.3 mM dNTPs Betaine 1.6 mM Betaine, 12.0 mM MgSO_4 and balance water), 32 units (4 μL) of BST 2.0 WarmStart enzyme, 2.0 μL Pluronic, 5.0 μL Bovine serum albumin, 5.0 μL 10X primer mix for specific genes described previously (12, 13) as 2.0 μM F3 and B3, 16.0 μM FIP and BIP, 8.0 μM LF and LB, and 5.0 μL templates. After dispensing the master mix and the templates, PCR tubes were capped and placed in a polypropylene 96 well PCR tube rack. The rack was then incubated in the water bath for 1 hour after which it was removed, dried, and allowed to cool to room temperature (~ 5.0 min.). Then, 2.0 μL 0.1 X SYBR green 1 (Molecular Probes, Catalog #S7563) was added to each tube. If a 96 well plate was used, the plate was sealed using a real time PCR optical film before direct incubation in the water bath.

3.4 Results

To date, LAMP has been used with the Gene-Z, a hand-held device, to quantify the *vcrA* gene in groundwater samples spiked with known quantities of that gene (12). Recently, new LAMP primers were developed for *tceA* and *vcrA* genes and these were used to quantify the growth of *Dehalococcoides mccartyi* in the bioaugmentation cultures, SDC-9 and KB-1 (12, 13). With primers developed in that study, the goals in the current research were 1) to evaluate if quantification with LAMP was comparable to qPCR for DNA extracted from multiple groundwater samples from different chlorinated solvent sites, 2) to optimize the cell concentration approach (when DNA extraction is not used), 3) to develop a rapid, cost-effective approach for RDase detection and 4) to evaluate the detection limits for *vcrA* and *tceA* in groundwater using the novel approach.

3.4.1 Comparison of qPCR and LAMP for DNA extracts from groundwater samples

Our previous research compared LAMP and qPCR for DNA extracted from groundwater samples from a single site. The current study expands on the previous work to ascertain if the two methods produce similar results for a larger number of samples, sites, and site conditions. For this, DNA from each groundwater sample was extracted in triplicate (23 samples for *tceA* and 27 samples for *vcrA*) from five different active remediation sites. A comparison of gene copy data for all sites/wells by each technique is provided (Figure 3.1). The gene concentrations ranged from approximately 10^4 to 10^{10} gene copies/L for *tceA* and 10^5 to 10^{10} gene copies/L for *vcrA*. The data generated via LAMP and qPCR were highly correlated ($R^2 = 0.9908$, slope of 1.2129) across this wide concentration range of the two genes (Figure 3.2).

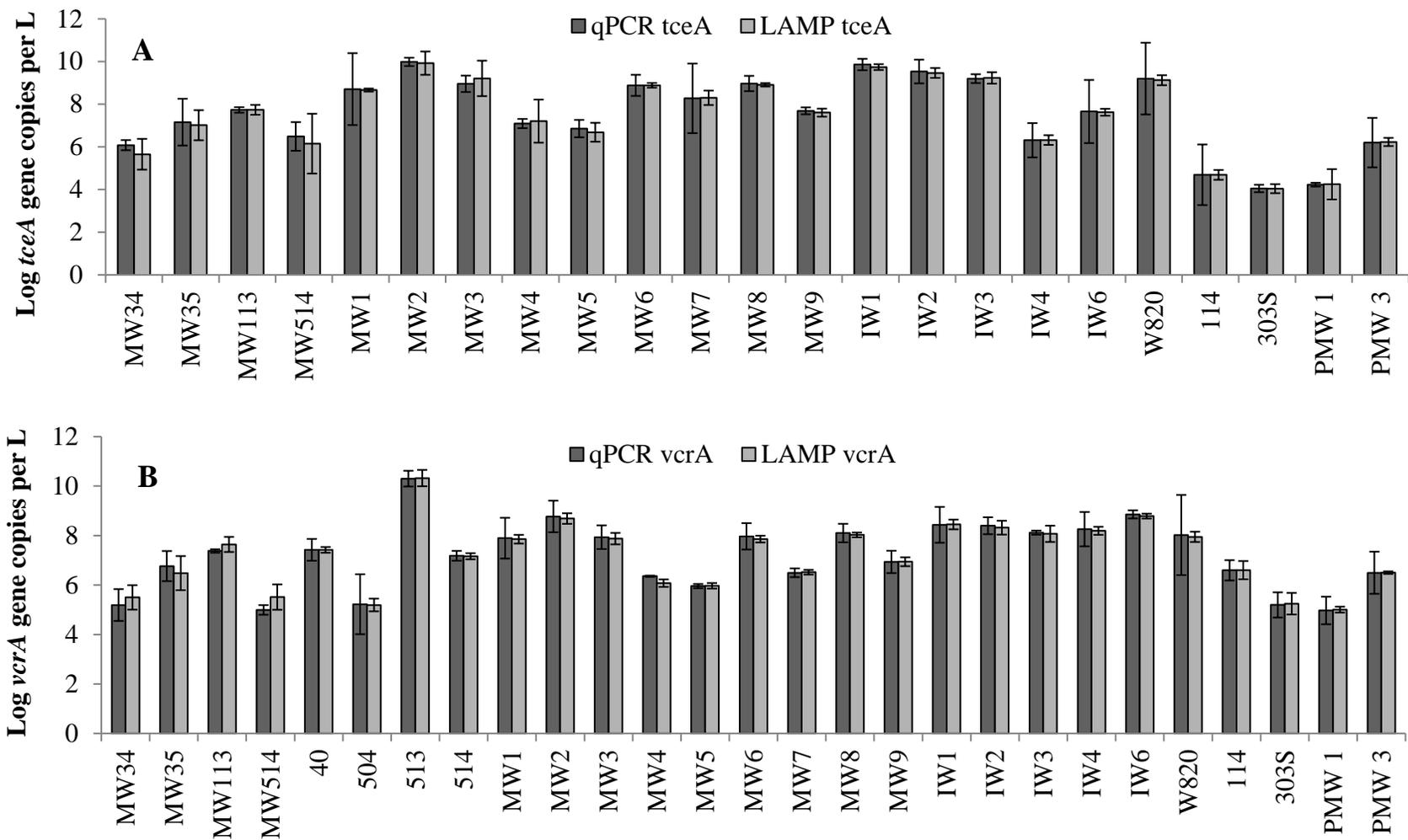


Figure 3.1 Comparison of qPCR and LAMP to quantify *tceA* (A) and *vcrA* (B) gene copies in DNA extracted from groundwater from different chlorinated solvent sites.

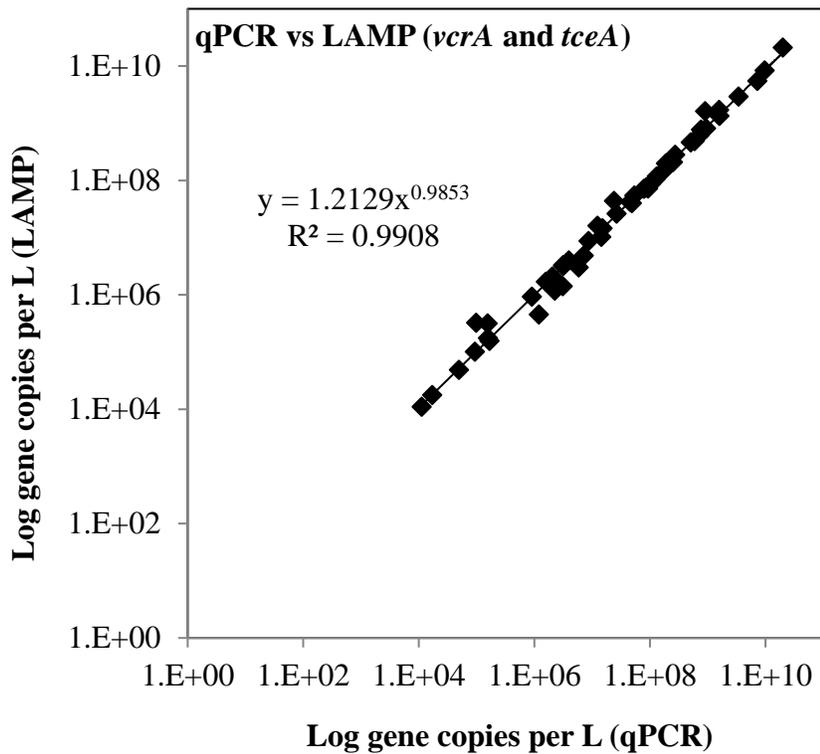


Figure 3.2 Correlation between gene concentrations (*tceA* and *vcrA*) determined via qPCR and LAMP using DNA extracted from numerous groundwater samples.

3.4.2 Optimization of cell template concentration, without DNA extraction

Following the establishment of similar results with qPCR and LAMP for *tceA* and *vcrA*, the next step was to determine if a cell concentration method could be developed to eliminate the need for DNA extraction. In previous research, direct cell amplification with LAMP was successfully used to detect 16S rRNA and *vcrA* templates (12). In this study, we optimized this approach by concentrating the direct cell templates using centrifugation. Here, we also compared gene concentrations for each sample using 1) LAMP with DNA extracts, 2) LAMP with direct amplification of cells, and 3) LAMP with amplification of centrifuged cells. This comparison was performed for groundwater samples from different sites for both *tceA* and *vcrA* (Figure 3.3)

In all samples, DNA templates yielded greater gene concentrations compared to centrifuged cells or direct cell templates. This is likely because adequate cell lysis does not occur while incubating LAMP reactions at 63°C. A visual comparison of the two cell based methods (direct and centrifuged cells, Figure 3.3) clearly illustrates the centrifuged cell method resulted in higher gene concentrations. Moreover, in some samples where LAMP was not able to detect the direct cell templates, while centrifuged cell templates produced threshold times, which were quantifiable. In many samples (indicated with an asterisk, Figure 3.3), the centrifuged cell method successfully quantified *vcrA* and *tceA* gene copies in all three replicates.

When gene concentrations determined without DNA extraction (direct or centrifuged cells) were plotted against those determined with DNA extraction, it was again clear that the centrifuged cell method produced improved data (greater gene copy numbers) over the direct cell method (Figure 3.4). There was also a better correlation between the values from centrifuged cell templates and those produced from DNA templates compared to the correlation between direct cell templates and DNA templates ($R^2 = 0.918$ vs. 0.687). Overall, centrifuged cells templates had higher quantities of *vcrA* and *tceA* genes than direct cell templates, suggesting that concentrating cell templates by centrifugation was an effective way of improving the quantification approach without DNA extraction. The high level of correlation between the values generated from centrifuged cells and those determined with DNA extracts, suggests the centrifuged method could be used to quantify *vcrA* or *tceA* genes in groundwater, saving the time and expense associated with DNA extraction. The regression equation between the two approaches (Figure 3.4) could be used on data generated from the centrifuged cell method to determine the concentration expected using DNA extracts.

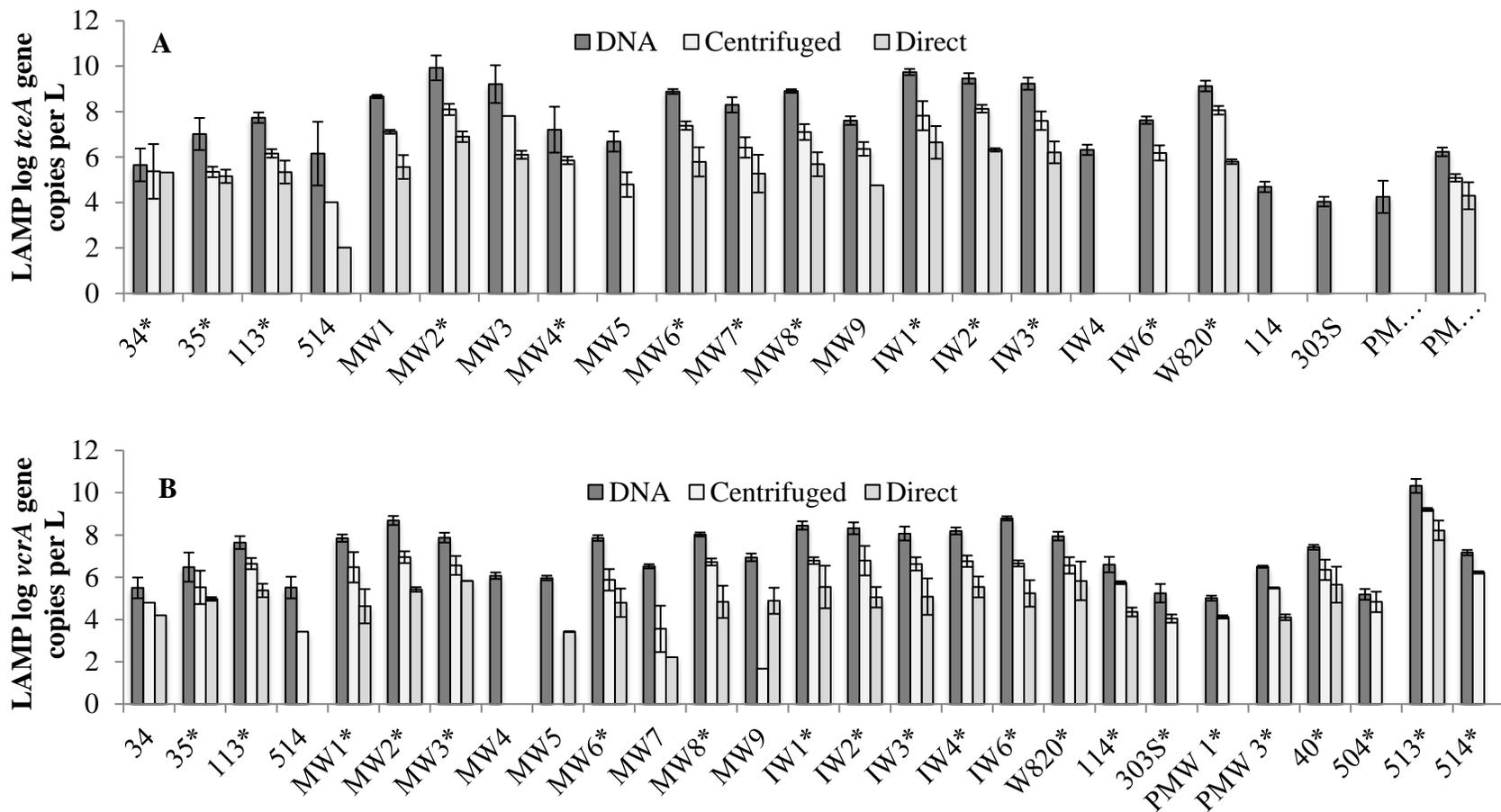


Figure 3.3 Comparison of *tceA* (A) and *vcrA* (B) gene concentrations (log gene copies per L) determined using DNA extracts, direct cells or centrifuged cells as templates.

Note: The values represent means of triplicate groundwater samples and the bars represent one standard deviation. *Templates were quantification was possible in all three replicates for centrifuged cells.

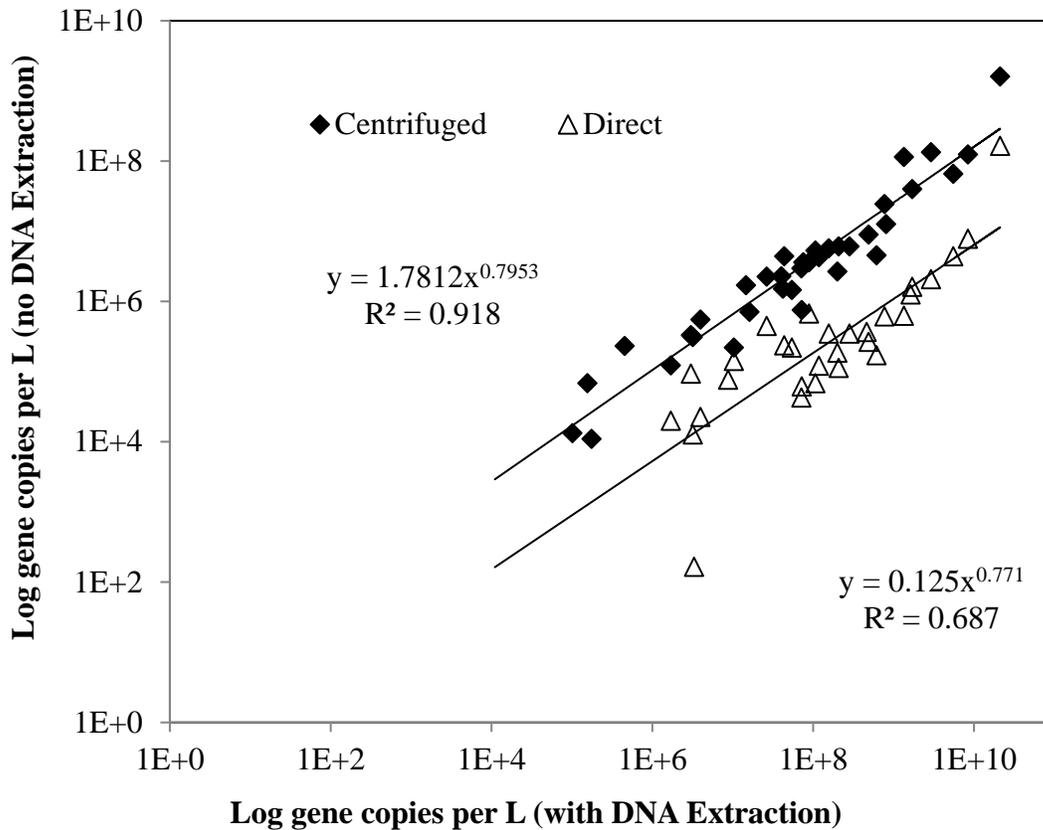


Figure 3.4 Comparison of the LAMP generated gene concentrations (*vcrA* and *tceA*) determined using DNA as a template to those values obtained using cells as a template (direct and centrifuged cells)

3.4.3 LAMP detection of RDase Genes without DNA extraction or a thermal cycler

The method described above (centrifuged cells with LAMP) will be valuable to those interested in quantifying RDase genes without the time and cost associated with DNA extraction. However, the method requires access to a real time thermal cycler. Therefore, the next step was to apply the assay without the use of a thermal cycler, using only visual detection and SYBR green dye. In other studies, for other targets, SYBR green for visual detection of LAMP amplicons has been well documented (20, 21).

To evaluate the specificity of the SYBR green LAMP assay for each RDase gene, plasmid standard templates containing the three genes (*vcrA*, *bvca*, *tceA*) were amplified (by

incubation in a water bath) and, following this, SYBR green was added to all reaction tubes. For the *vcrA* gene, target templates (plasmid standard for *vcrA* gene, 10^6 gene copies/reaction) fluoresced green, while the negative control templates (water, *bvcA* and *tceA* plasmid standards) remained orange (Figure 3.5). The same results of amplification in the positive controls (green fluorescence) but not in the negative controls (10^6 copies/reaction) occurred for both the *bvcA* and *tceA* LAMP assays. These results indicate the LAMP primers were specific using SYBR green for visual detection of *vcrA*, *tceA*, and *bvcA* genes.

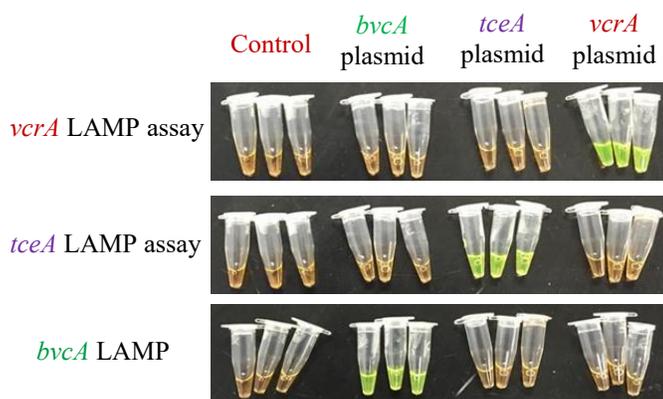


Figure 3.5 Specificity of LAMP/SYBR green assays with triplicates of plasmid standards (10^6 gene copies/reaction) containing RDase genes

3.4.4 Detection limits of visual detection

Triplicate samples of centrifuged cell templates were created from groundwater from six wells (IW5, IW7, MW38, MW40, MW41, and MW43) from the Indian Head site. To evaluate the gene copy concentration limits of visual detection with the SYBR green LAMP assays with *tceA* and *vcrA* genes, a five-fold 10X dilution series was generated for each triplicate of the centrifuged cell templates. The reactions were incubated in a water bath and SYBR green was added post amplification. The results of this analysis have been summarized (Figure 3.6 and Table 3.4). Each bar represents the *tceA* and *vcrA* gene copies expected from the four dilutions in each triplicate. The color of the bar represents the endpoint visualization of that dilution (green –

positive detection, orange – no detection). An example image of the assay is shown for two samples (MW40 and MW41) for the detection of *vcrA* gene in Figure 3.8. The five reaction tubes in each row represent amplicons created with templates produced by making the five-fold 10X dilution series of that triplicate. The tubes were arranged in a descending order of concentration for the templates starting from the undiluted template (highest concentration) to the left and lowest concentration to the right. The final row containing three tubes represents negative controls (no template, water and 10^6 gene copy plasmid standard of *tceA* gene). As with the plasmid standards, a very clear color change from orange to green was observed in templates that amplified, however, templates that did not amplify remained orange.

At higher and lower RDase gene concentrations, all three replicates turned green or remained orange. However, between these values (9×10^4 to 5×10^5 and 1×10^5 to 6×10^5 for *tceA* and *vcrA*, respectively) some replicates turned green while some remained orange. To eliminate the uncertainty associated with these ranges, we suggest that conclusions should only be made if all three triplicates produce the same result. The dilution data were examined to determine the lowest concentration where all three triplicates turned green for *vcrA* and *tceA* and guidance tables were generated to estimate the gene concentrations (Table 3.1 and Table 3.2). The guidance tables also list hypothetical gene concentrations if dilutions of the original sample are made. The approach, along with additional replicate dilutions, could be used with the MPN technique to enable the method to be used in a quantitative manner.

The accuracy of the detection thresholds was tested using the *vcrA* assay and groundwater from additional sites (Figure 3.7). As predicted, in samples containing $> 1.8 \times 10^5$ *vcrA* gene copies/L all replicates fluoresced green, while in samples below this value, all replicates were orange. In samples, PMW1, PMW3, and TW265 quantities of *vcrA* gene were

below detection limit. SYBR green LAMP on templates created with these samples remained orange.

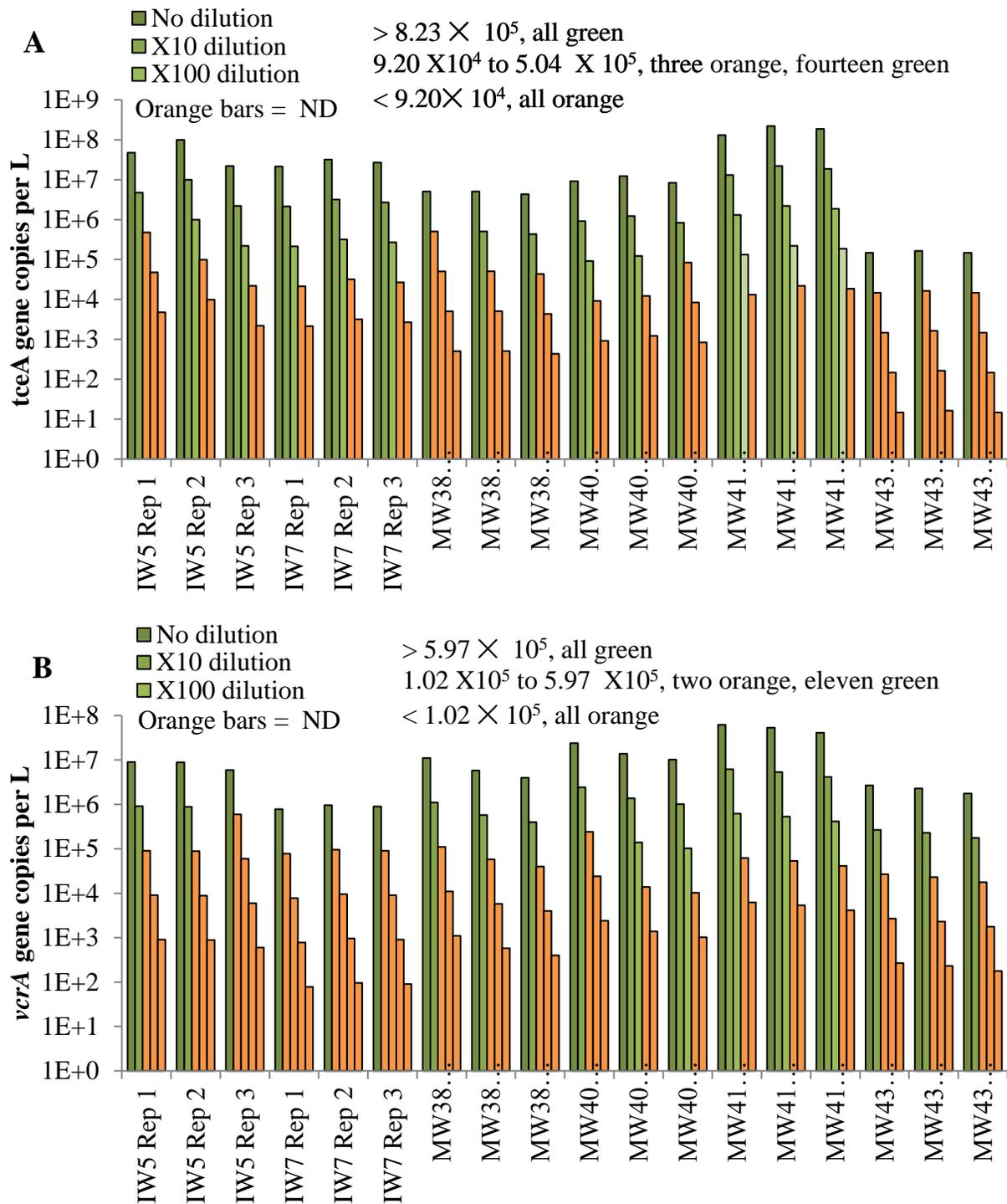


Figure 3.6 Amplification results using a 10X dilution series of centrifuged cell templates from groundwater for *tceA* (A) and *vcrA* (B).

Note: The highest value in each dilution series was measured (DNA extraction and LAMP) and the resulting dilution values are estimated from this. The green bars indicate positive gene detection.

Table 3.1 *vcrA* gene concentrations (gene copies per L) for SYBR Green assay for groundwater samples and dilutions examined in triplicates and the predicted outcome for in situ reductive dechlorination

Dilution	<i>vcrA</i>	Concentration in groundwater	Prediction
None	3 replicates = green	> 1.8 X10 ⁵ gene copies per L ^a	Possible dechlorination
None	3 replicates = orange	< 1.1 X10 ⁵ gene copies per L ^b	Possible dechlorination
<i>X 10</i>	<i>3 replicates = green</i>	<i>> 1.8 X10⁶ gene copies per L</i>	<i>Threshold for effective dechlorination</i>
X 10 ²	3 replicates = green	> 1.8 X10 ⁷ gene copies per L	Effective dechlorination
X 10 ³	3 replicates = green	> 1.8 X10 ⁸ gene copies per L	Effective dechlorination
X 10 ⁴	3 replicates = green	> 1.8 X10 ⁹ gene copies per L	Effective dechlorination
X 10 ⁵	3 replicates = green	> 1.8 X10 ¹⁰ gene copies per L	Effective dechlorination

^a Derived from the lowest copy number when all three turned green (1.77 X10⁵)

^b Derived from the highest copy number when all remained orange (10 fold dilution of 1.1 X 10⁶)

Table 3.2 *tceA* gene concentrations (gene copies per L) for SYBR Green assay for groundwater samples and dilutions examined in triplicate and the predicted outcome for in situ reductive dechlorination

Dilution	<i>tceA</i>	Concentration in groundwater	Prediction
None	3 replicates = green	> 1.3 X10 ⁵ gene copies per L ^a	Possible dechlorination
None	3 replicates = orange	< 3.2 X10 ⁴ gene copies per L ^b	Possible dechlorination
<i>X 10</i>	<i>3 replicates = green</i>	<i>> 1.3 X10⁶ gene copies per L</i>	<i>Threshold for effective dechlorination</i>
X 10 ²	3 replicates = green	> 1.3 X10 ⁷ gene copies per L	Effective dechlorination
X 10 ³	3 replicates = green	> 1.3 X10 ⁸ gene copies per L	Effective dechlorination
X 10 ⁴	3 replicates = green	> 1.3 X10 ⁹ gene copies per L	Effective dechlorination
X 10 ⁵	3 replicates = green	> 1.3 X10 ¹⁰ gene copies per L	Effective dechlorination

^a Derived from the lowest copy number when all three turned green (1.32 X10⁵)

^b Derived from the highest copy number when all remained orange (10 fold dilution of 3.19 X 10⁵)

From Lebrón, C. A., E. Petrovskis, F. Löffler & K. Henn, Jan 2011, Guidance Protocol, ER-0518

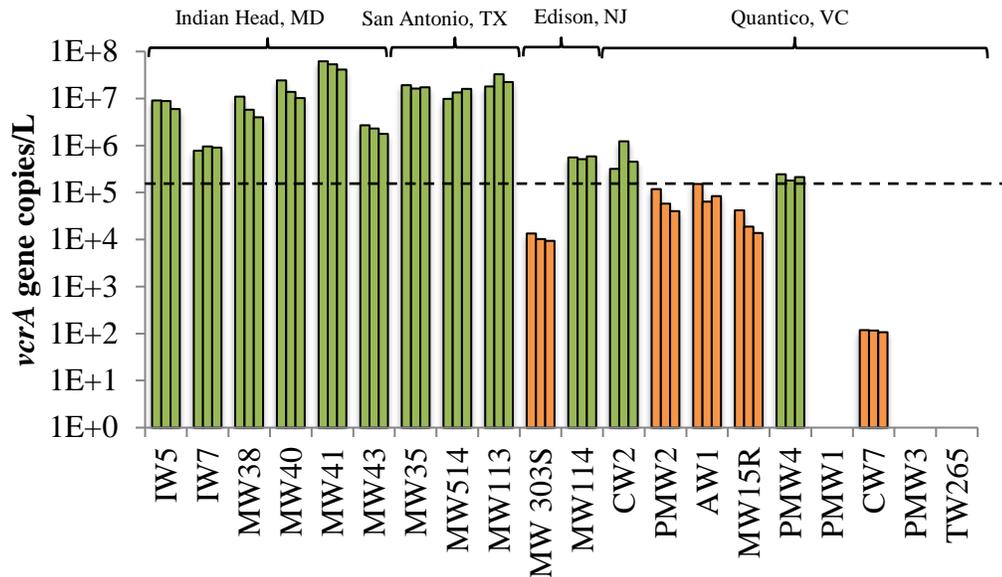


Figure 3.7 Testing of detection guidance values using groundwater from different sites

Note: The dashed line represents the determined threshold for *vcrA* gene detection. Green and orange bars represent samples with the *vcrA* gene above and below the detection threshold, respectively. Three samples (PMW1, PMW3, and TW265) contained *vcrA* genes below the detection limit.

3.5 Discussion

The correlation between the *in situ* dechlorination activity and the observed quantity of *Dehalococcoides mccartyi* in groundwater has been documented (5, 22). In groundwater samples where *Dehalococcoides mccartyi* counts are low ($<10^4$ 16S rRNA gene copies/L), efficient dechlorination and production of ethene is unlikely. Predicting whether dechlorination will occur with moderate *Dehalococcoides* sp. counts (10^4 to 10^6 16S rRNA gene copies/L), is less definitive. High *Dehalococcoides mccartyi* counts ($>10^6$ 16S rRNA gene copies/L) are often associated with high dechlorination rates and ethene generation (5). Visual detection with the LAMP and SYBR green assays using centrifuged cells detects the *vcrA* gene above 1.8×10^5 gene copies/L and the *tceA* gene above 1.3×10^5 gene copies/L. Therefore, if the assay produces

three green vials for a groundwater sample, this will indicate the site contains moderate *Dehalococcoides mccartyi* counts. Further, if the sample produces three green vials following one 10X dilution, this will indicate the cell concentration has been reached for effective dechlorination.

The developed LAMP SYBR green approach is a low cost and user-friendly alternative to qPCR for the quantitative evaluation of *Dehalococcoides 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 (Table 3.5). In addition, compared to qPCR, the LAMP assay has a shorter run time and the visualization of amplification products is immediate. Additionally, the approach has the potential for use in the field, as it requires equipment that could be easily transported on site 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 (Table 3.6). A 50 μ L LAMP reaction with centrifuged cells is slightly cheaper (~\$ 0.30) than a 20 μ L 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 to each sample (almost doubles the cost). Another key difference concerns the use of low cost laboratory equipment for LAMP (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 of a dilution series, then the gene copies can be estimated, provided the concentration is above $\sim 10^5$ gene copies per L. This value is less than the concentration required for effective dechlorination *in situ*.

Future research will focus on optimizing the overall process to achieve detection limits $< 10^4$ gene copies/L. In addition, future work will focus on the optimization of the LAMP SYBR green approach to enable quantification of RDase genes (e.g. using most probable number) and on addressing problems reported by others concerning the aerosolization of LAMP products (causing contamination between experiments).

APPENDIX

Appendix

Table 3.3 The gene targets, assay type and template type used for each well and site.

Site	Well Name	Date	DNA Templates						Cells					
			qPCR DNA <i>tceA</i>	LAMP DNA <i>tceA</i>	qPCR DNA <i>vcrA</i>	LAMP DNA <i>vcrA</i>	qPCR DNA <i>bvcA</i>	LAMP DNA <i>bvcA</i>	Direct cells <i>tceA</i>	Direct cells <i>vcrA</i>	Direct cells <i>bvcA</i>	Centrifuged cells <i>tceA</i>	Centrifuged cells <i>vcrA</i>	Centrifuged cells <i>bvcA</i>
San Antonio, TX	34 ^{B1}	03/03/15	✓D	✓D	✓D	✓D	✓ND	X	✓D	✓D	X	✓D	✓D	X
	35 ^{B1}	03/03/15	✓D	✓D	✓D	✓D	✓ND	X	✓D	✓D	X	✓D	✓D	X
	40 ^{B1}	04/13/15	✓ND	✓ND	✓D	✓D	✓ND	X	X	✓D	X	X	✓D	X
	113 ^{B1}	04/13/15	✓D	✓D	✓D	✓D	✓ND	X	✓D	✓D	X	✓D	✓D	X
	504 ^{B1}	12/1/15	✓ND	✓ND	✓D	✓D	✓ND	X	X	✓D	X	X	✓D	X
	513 ^{B1}	12/1/15	✓ND	✓ND	✓D	✓D	✓ND	X	X	✓D	X	X	✓D	X
	514 ^{B1}	12/1/15	✓D	✓D	✓D	✓D	✓ND	X	✓D	✓ND	X	✓D	✓D	X
	35 ^{B2}	08/06/16	X	✓D	X	✓D	✓ND	X	✓ND	✓ND	X	✓D	✓D	X
	113 ^{B2}	08/06/16	X	✓D	X	✓D	✓ND	X	✓ND	✓ND	X	✓D	✓D	X
514 ^{B2}	08/06/16	X	✓D	X	✓D	✓ND	X	✓ND	✓D	X	✓D	✓D	X	
Tulsa, OK	MW1	06/11/15	✓D	✓D	✓D	✓D	✓ND	X	✓D	✓D	X	✓D	✓D	X
	MW2	06/11/15	✓D	✓D	✓D	✓D	✓ND	X	✓D	✓D	X	✓D	✓D	X
	MW3	06/11/15	✓D	✓D	✓D	✓D	✓ND	X	✓D	✓D	X	✓D	✓D	X
	MW4	06/11/15	✓D	✓D	✓D	✓D	✓ND	X	✓ND	✓ND	X	✓D	✓ND	X
	MW5	06/11/15	✓D	✓D	✓D	✓D	✓ND	X	✓ND	✓D	X	✓D	✓D	X
	MW6	06/11/15	✓D	✓D	✓D	✓D	✓ND	X	✓D	✓D	X	✓D	✓D	X
	MW7	06/11/15	✓D	✓D	✓D	✓D	✓ND	X	✓D	✓D	X	✓D	✓D	X
	MW8	06/11/15	✓D	✓D	✓D	✓D	✓ND	X	✓D	✓D	X	✓D	✓D	X
	MW9	06/11/15	✓D	✓D	✓D	✓D	✓ND	X	✓D	✓D	X	✓D	✓D	X
	IW1	06/11/15	✓D	✓D	✓D	✓D	✓ND	X	✓D	✓D	X	✓D	✓D	X
	IW2	06/11/15	✓D	✓D	✓D	✓D	✓ND	X	✓D	✓D	X	✓D	✓D	X
	IW3	06/11/15	✓D	✓D	✓D	✓D	✓ND	X	✓D	✓D	X	✓D	✓D	X
	IW4	06/11/15	✓D	✓D	✓D	✓D	✓ND	X	✓ND	✓D	X	✓ND	✓D	X
	IW5	06/11/15	✓ND	✓ND	✓ND	✓ND	✓ND	X	X	X	X	X	X	X
	IW6	06/11/15	✓D	✓D	✓D	✓D	✓ND	X	✓ND	✓D	X	✓D	✓D	X
W820	06/11/15	✓D	✓D	✓D	✓D	✓ND	X	✓D	✓D	X	✓D	✓D	X	

Table 3.3 (cont'd)

Quantico, VA	PMW1 ^{B1}	11/10/15	✓D	✓D	✓D	✓D	✓ND	X	✓ND	✓ND	X	✓ND	✓D	X
	PMW3 ^{B1}	11/10/15	✓D	✓D	✓D	✓D	✓ND	X	✓ND	✓ND	X	✓ND	✓D	X
	CW2	11/16/15	✓D	✓D	✓D	✓D	✓ND	X	✓ND	✓ND	X	✓ND	✓D	X
	PMW2	11/16/15	✓D	✓D	✓D	✓D	✓ND	X	✓ND	✓ND	X	✓ND	✓ND	X
	AW1	11/16/15	✓D	✓D	✓D	✓D	✓ND	X	✓ND	✓ND	X	✓ND	✓ND	X
	MW 15R	11/16/15	✓D	✓D	✓D	✓D	✓ND	X	✓ND	✓ND	X	✓ND	✓ND	X
	PMW4	11/16/15	✓D	✓D	✓D	✓D	✓ND	X	✓ND	✓ND	X	✓ND	✓ND	X
	PMW1 ^{B2}	11/16/15	✓ND	✓ND	✓ND	✓ND	✓ND	X	✓ND	✓ND	X	✓ND	✓ND	X
	CW7	11/16/15	✓ND	✓ND	✓D	✓D	✓ND	X	✓ND	✓ND	X	✓ND	✓D	X
	PMW3 ^{B2}	11/16/15	✓ND	✓ND	✓ND	✓ND	✓ND	X	✓ND	✓ND	X	✓ND	✓ND	X
TW265	11/16/15	✓ND	✓ND	✓ND	✓ND	✓ND	X	✓ND	✓ND	X	✓ND	✓ND	X	
Edison, NJ	114	11/10/15	✓D	✓D	✓D	✓D	✓ND	X	✓ND	✓D	X	✓ND	✓D	X
	303S	11/10/15	✓D	✓D	✓D	✓D	✓ND	X	✓ND	✓ND	X	✓ND	✓D	X
Indian Head, MD	IW5	06/24/16	X	✓D	X	✓D	✓ND	X	X	X	X	✓D	✓D	X
	IW7	06/24/16	X	✓D	X	✓D	✓ND	X	X	X	X	✓D	✓D	X
	MW38	06/24/16	X	✓D	X	✓D	✓ND	X	X	X	X	✓D	✓D	X
	MW40	06/24/16	X	✓D	X	✓D	✓ND	X	X	X	X	✓D	✓D	X
	MW41	06/24/16	X	✓D	X	✓D	✓ND	X	X	X	X	✓D	✓D	X
MW43	06/24/16	X	✓D	X	✓D	✓ND	X	X	X	X	✓D	✓D	X	

D = template was detected
 ✓ = Assay was performed

ND = template was not detected
 X = Assay was not performed

B1 = Batch 1
 B2 = Batch 2

Table 3.4 Gene concentrations (*vcrA* and *tceA* per L) and estimated gene concentration in serial dilutions of cell templates from groundwater from six monitoring wells (Indian Head site)

Sample	Replicate #	<i>tceA</i> gene copies/L					<i>vcrA</i> gene copies/L				
		No Dilution	10X Dilution	100X Dilution	1000X Dilution	10000X Dilution	No Dilution	10X Dilution	100X Dilution	1000X Dilution	10000X Dilution
IW5	1	4.77 X10 ⁷	4.77 X10 ⁶	ND	ND	ND	9.04 X10 ⁶	9.04 X10 ⁵	ND	ND	ND
	2	9.88 X10 ⁷	9.88 X10 ⁶	9.88 X10 ⁵	ND	ND	8.84 X10 ⁶	8.84 X10 ⁵	ND	ND	ND
	3	2.20 X10 ⁷	2.20 X10 ⁶	2.20 X10 ⁵	ND	ND	5.97 X10 ⁶	ND	ND	ND	ND
IW7	1	2.13 X10 ⁷	2.13 X10 ⁶	2.13 X10 ⁵	ND	ND	7.77 X10 ⁵	ND	ND	ND	ND
	2	3.19 X10 ⁷	3.19 X10 ⁶	3.19 X10 ⁵	ND	ND	9.56 X10 ⁵	ND	ND	ND	ND
	3	2.68 X10 ⁷	2.68 X10 ⁶	2.68 X10 ⁵	ND	ND	9.04 X10 ⁵	ND	ND	ND	ND
MW38	1	5.04 X10 ⁶	ND	ND	ND	ND	1.10 X10 ⁷	1.10 X10 ⁶	ND	ND	ND
	2	5.07 X10 ⁶	5.07 X10 ⁵	ND	ND	ND	5.78 X10 ⁶	5.78 X10 ⁵	ND	ND	ND
	3	4.34 X10 ⁶	4.34 X10 ⁵	ND	ND	ND	3.99 X10 ⁶	3.99 X10 ⁵	ND	ND	ND
MW40	1	9.20 X10 ⁶	9.20 X10 ⁵	9.20 X10 ⁴	ND	ND	2.41 X10 ⁷	2.41 X10 ⁶	ND	ND	ND
	2	1.23 X10 ⁷	1.23 X10 ⁶	1.23 X10 ⁵	ND	ND	1.38 X10 ⁷	1.38 X10 ⁶	1.38 X10 ⁵	ND	ND
	3	8.40 X10 ⁶	8.40 X10 ⁵	ND	ND	ND	1.02 X10 ⁷	1.02 X10 ⁶	1.02 X10 ⁵	ND	ND
MW41	1	1.32 X10 ⁸	1.32 X10 ⁷	1.32 X10 ⁶	1.32 X10 ⁵	ND	6.22 X10 ⁷	6.22 X10 ⁶	6.22 X10 ⁵	ND	ND
	2	2.20 X10 ⁸	2.20 X10 ⁷	2.20 X10 ⁶	2.20 X10 ⁵	ND	5.34 X10 ⁷	5.34 X10 ⁶	5.34 X10 ⁵	ND	ND
	3	1.86 X10 ⁸	1.86 X10 ⁷	1.86 X10 ⁶	1.86 X10 ⁵	ND	4.13 X10 ⁷	4.13 X10 ⁶	4.13 X10 ⁵	ND	ND
MW43	1	1.47 X10 ⁵	ND	ND	ND	ND	2.68 X10 ⁶	2.68 X10 ⁵	ND	ND	ND
	2	1.64 X10 ⁵	ND	ND	ND	ND	2.30 X10 ⁶	2.30 X10 ⁵	ND	ND	ND
	3	1.47 X10 ⁵	ND	ND	ND	ND	1.77 X10 ⁶	1.77 X10 ⁵	ND	ND	ND

ND = template

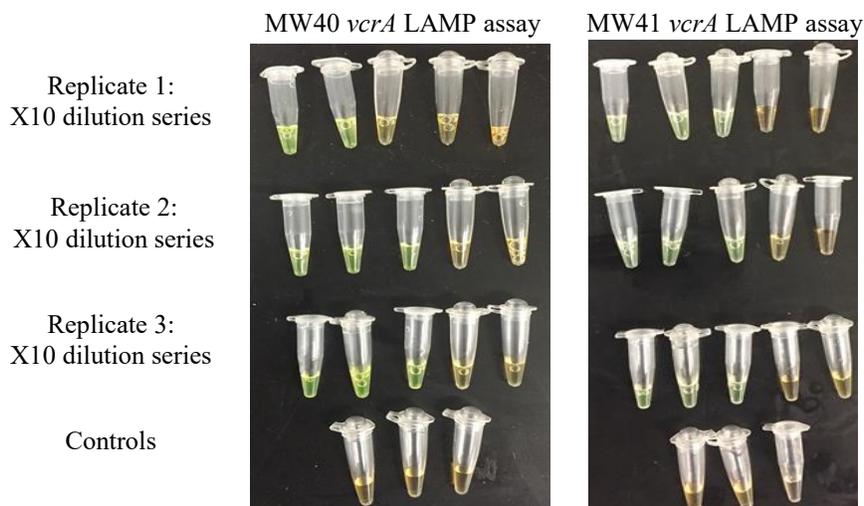


Figure 3.8 Examples of SYBR green LAMP assays for *vcrA* with triplicates of 10X dilutions for centrifuged cells from groundwater from the Indian Head site

Note: The dilution levels increase to the right.

Table 3.5 Time based comparison of qPCR and SYBR green LAMP assays

Process	qPCR	SYBR Green LAMP
Filtering	5-25 min. per sample	5-25 min. per sample
DNA extraction	35-45 min per sample	No DNA extraction
Centrifugation and template elution	No centrifugation or sample elution	<15 min per sample
Master Mix Preparation	<5 min. if commercial master mix is used.	20-30 min.
Analysis	90 -120 min	<1.5 hr. (1 hr. in the water bath and <0.5 hr. for adding SYBR green ^a)
Total	135-195 min	115-160 min

^a Value based on a 96 well plate. The value is significantly lower if fewer samples are processed (1 hour in the water bath and < 5 min for adding the SYBR green)

Table 3.6 Cost based comparison of qPCR and SYBR green LAMP assays

Process	qPCR (20µL reaction)	SYBR Green LAMP (50 µL reaction)
DNA extraction	\$ 9.30 per sample ^a	No cost
Consumables + reagents	\$ 12.70 per sample	\$ 12.38 per sample
Instrument costs	~\$ 20,000	~\$395 (water bath) ~\$300 (centrifuge)

^aMO BIO PowerWater DNA Isolation Kit (\$465 for 50 preps)

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REFERENCES

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Chapter 4:

The Application of Most Probable Number Method with Visual Detection LAMP for Quantitative Estimation of RDase Gene Concentrations in Groundwater Samples

The data presented in this chapter is being compiled for preparation of a manuscript.

4.1 Abstract

Recently, a LAMP based assay, which allows for the visual detection of RDase genes from groundwater, was described. In that study, *Dehalococcoides mccartyi* cells from bioaugmented groundwater samples were concentrated using filtration and centrifugation and were added to LAMP reactions as templates for amplification. The reactions were incubated in a water bath, avoiding the use of an expensive thermal cycler, and amplification was visualized by the addition of dilute SYBR Green dye (post incubation). Despite having a detection limit ($\sim 1.18 \times 10^5$ gene copies/L) lower than 10^7 gene copies/L (the threshold for effective in situ remediation), the application of the assay was limited because of the semi-quantitative nature of the data generated. Moreover, the assay was prone to false positives due to the aerosolization of amplicons. In this study, a dUTP-UNG system was incorporated in the reaction mix of SYBR Green LAMP assay to reduce the probability of false positives due to carry over contamination. Optimization experiments revealed a UNG concentration of 0.2 units per reaction is adequate for degrading trace levels of AUGC contamination of $\sim 1.2 \times 10^4$ gene copy reaction without significant increases in the detection limit of ~ 100 gene copies/reaction of template. Additionally, the optimized SYBR Green LAMP assay was used with the MPN method to provide quantitative estimates of RDase gene concentrations in seven groundwater samples obtained from a chlorinated solvent waste site. Concentrations obtained using this approach were

significantly correlated to concentrations obtained using qPCR and DNA templates prepared from the same samples for both *vcrA* ($\rho = 0.952$, p-value = 0.0329) and *tceA* ($\rho = 0.994$, p-value = 0.0484) genes. Although the SYBR Green LAMP assay under-estimated the concentrations of *vcrA* and *tceA* genes, this study demonstrates a strong correlation between the two data sets and could be used to calibrate the SYBR Green LAMP method for quantification by using the slopes of the trend lines for *vcrA* (10.386) and *tceA* (11.873) genes as response factors.

4.2 Introduction

Subsurface injections of commercial microbial mixed cultures and various amendments to remediate chlorinated solvent plumes at hazardous waste sites aim at facilitating biological reductive dechlorination of these chemicals by a process known as organohalide respiration. These approaches aim at increasing the population of *Dehalococcoides* cells in the contaminated groundwater aquifers (1). It has become standard practice to monitor the concentrations of these microorganisms both before and during the remediation process using TaqMan probe based quantitative polymerase chain reaction since high *Dehalococcoides mccartyi* counts ($>10^6$ 16S rRNA gene copies/L) are often associated with high dechlorination rates and ethene generation (2). Consequently, qPCR primers and probes highly specific to the biomarker RDase genes, *vcrA*, *bvcA*, and *tceA*, are now widely used for monitoring the in situ growth of *Dehalococcoides mccartyi* (2, 3). Many remediation professionals either carry out qPCR in-house or use the service of a commercial laboratory with expertise in qPCR. Both approaches involve significant costs like the cost of purchasing a real time thermal cycler (~\$20K) for in-house analysis or the cost of having many samples analyzed by a commercial laboratory (typically >\$250 per sample). Given the economic constraints, there is a need for fast, cost-effective quantification assays for RDase genes.

LAMP is a sensitive, specific, and one-step isothermal amplification method, which is often used as an alternative point-of-care diagnostic tool to PCR for a wide variety of applications (4-6). For example, many LAMP assays have been developed for testing food borne bacterial pathogens and fungal contaminants (7, 8). LAMP assays for the quantification of *Dehalococcoides mccartyi* 16S rRNA and *vcrA* genes without DNA extraction on a proprietary microfluidic platform, the Gene-Z, are also available (9, 10). Kanitkar, et al. (2016) developed new LAMP primer sets for *Dehalococcoides mccartyi* RDase genes and validated the use of LAMP as a quantification tool by monitoring the growth of commercial dechlorinating cultures (SDC-9 and KB-1), and comparing the concentrations obtained using LAMP to qPCR with DNA templates extracted at several time points over one growth cycle. Additionally, DNA templates extracted from groundwater samples were used to compare concentrations obtained using LAMP on the Gene-Z to qPCR. Similar values were obtained for each RDase gene on both platforms, indicating quantification with LAMP is a viable alternative to qPCR when DNA templates were used.

In the first phase of the research described in this chapter, the reaction chemistry of the SYBR Green LAMP assay, developed in chapter three, was optimized to incorporate dUTP and UNG based enzymatic control for prevention of false positives due to carry over contamination without a significant increase in the detection limits of the SYBR Green LAMP assay. Replacing dTTP with dUTP in the reaction mixture produces LAMP amplicons containing uracil. Before initiating LAMP, the reaction mixture is treated with UNG to destroy carry over contamination from the previous set of experiments. This strategy is often used to prevent carry-over contamination in qPCR. In 2015, this strategy of an altered reaction mixture with a dTTP to dUTP substitution was tested along with the addition of UNG to the LAMP reaction. The

modified LAMP method successfully prevented carry over contamination issues while detecting plant viruses (12). Here, we have optimized the amount of UNG required to destroy lab relevant cross contamination of $\sim 1.23 \times 10^4$ gene copies while preserving the detection limit of SYBR Green LAMP of $\sim 10^2$ gene copies/reaction.

Recently, Ahmad, et al. (2017) coupled the LAMP amplification on a microfluidic chip similar to the Gene-Z to MPN to create MPN-LAMP technique for the quantification of gram negative and gram positive water borne pathogens. In the second phase, we extend the application of this technique with the optimized SYBR Green LAMP assay for quantitative estimation of *Dehalococcoides mccartyi* RDase gene concentrations in groundwater samples without DNA extraction.

4.3 Methods

4.3.1 Integration of the dUTP-UNG contamination control system in to the SYBR Green LAMP assay

The reaction mix recipe described by Kanitkar et al. (2017) for visually detecting centrifuged cell templates was used as a basis for LAMP experiments described below. The dNTPs mix used in that recipe were replaced with PCR Nucleotide Mix plus containing sodium salts of dATP, dCTP, dGTP, each at a concentration of 10 mM, and dUTP at a concentration of 30 mM in PCR grade water (Roche Diagnostics Inc. Catalog number #11888412001) to obtain a final concentration of 1.15 mM. The quantities of each reaction component were calculated for a reaction volume of 25 μ L to make up a reaction mix volume of 22 μ L per reaction. To determine the precise amount of UNG (Thermo Fischer, Catalog# EN0361) required for eliminating carry over contamination, reaction mixes with variable concentrations of UNG (1.0, 0.8, 0.6, 0.4, 0.2

units per reaction) were prepared. The reaction mixes were then incubated for 1.5 min at room temperature after adding UNG. Finally, 3.0 μ L templates were dispensed to each tube.

4.3.2 Optimized SYBR Green LAMP amplification

SYBR Green LAMP reactions were set up to have a final volume of 25 μ L with 22 μ L master mix and 3 μ L template. Master mix for each reaction comprised of 12.5 μ L of 2X reaction mix (2X Isothermal amplification buffer, 2.3 mM dUTP-dNTPs mix, 1.6 mM Betaine, 12.0 mM MgSO₄ and balance water), 32 units (2 μ L) of BST 2.0 WarmStart enzyme, 1.0 μ L Pluronic, 2.5 μ L Bovine serum albumin, 2.5 μ L 10X primer mix for specific gene described previously (11, 14) as 2.0 μ M F3 and B3, 16.0 μ M FIP and BIP, 8.0 μ M LF and LB. UNG was added to the master mix, such that each reaction received 0.2 units (1unit/ μ L) to make up a final volume of 22.0 μ L. After adding UNG, the master mix was incubated at room temperature for 1.5 min before adding templates. After dispensing the templates, PCR tubes were capped and placed in a polypropylene 96 well PCR tube rack. The rack was then incubated in the water bath for 1 hour after which it was removed, dried, and allowed to cool to room temperature (~5.0 min.). Then, 2.0 μ L 0.1 X SYBR green 1 (Molecular Probes, Catalog #S7563) was added to each tube. From the LAMP primers supplied in Table 2.1 vcrA set C, bvcA set A, and tceA set A were used for this study.

4.3.3 Preparation of dilution series for MPN analysis from centrifuged cell templates of groundwater samples.

Centrifuged cell templates were prepared from all groundwater samples listed in Table 4.3 using a protocol described previously (14). Briefly, a 5 μ m nylon membrane filter (Nalgene, Rochester, NY) and a 0.22 μ m Sterivex filter (EMD Millipore Corp., Billerica, MA) were placed in series to form a groundwater filtration module (to remove turbidity). Groundwater samples

(150 -250 mL) were filtered through the groundwater filtration module using a sterile 160 mL syringe. *Dehalococcoides mccartyi* are 0.3- 1.0 μm (15) and are thus expected to pass through the 5 μm filter but be retained on the 0.22 μm Sterivex filter. A cell elution buffer was prepared by adding compound ST1B (MO Bio Catalog #14600-50-NF-1B) to solution ST1A (MO Bio Catalog #14600-50-NF-1A) according to manufacturer's protocol. The buffer (1000 μL) was added to the filter and the filter was capped on both ends. The whole module was vortexed for 10 min (at medium speed) to resuspend the retained biomass. The suspension was extracted using a 1 mL syringe. The resuspended biomass was further centrifuged (13000 g x 15 min.) to obtain a biomass pellet by decanting the liquid content from the tube. The pellet was then resuspended in 100 μL of 1X phosphate buffer to obtain centrifuged cells. Using these centrifuged cells, a seven fold 10X dilution series was created. Six such replicate dilution series were created for each groundwater sample and used as templates for SYBR Green LAMP amplification.

4.3.4 Quantification with MPN LAMP

Pictures of the six replicate dilution series templates for each groundwater sample showing the endpoint color were captured using a cell phone camera (iPhone 6) and used to create data tables for MPN analysis. If the endpoint color in a single reaction tube was green, it was denoted with a value of one while that of orange was denoted with zero. Table 4.1 and Table 4.2 show the representative data recorded for MPN analysis of *vcrA* and *tceA* genes in groundwater sample MW100 using SYBR Green LAMP method. Concentrations of *Dehalococcoides* cells in centrifuged cell templates were then determined using the statistical method of result rejection (Equation 1) developed by Hurley and Roscoe (1983). Six replicates of each dilution were used for the MPN calculations.

Table 4.1 Data table denoting the endpoint color change in six replicates of seven fold 10X dilution series templates for MPN analysis of *vcrA* gene in groundwater sample MW100

Replicate	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	Dilution 6	Dilution 7
Replicate #1	1	1	1	1	1	1	1
Replicate #2	1	1	1	1	0	1	0
Replicate #3	1	1	1	1	1	0	0
Replicate #4	1	1	1	1	1	1	0
Replicate #5	1	1	1	1	0	0	0
Replicate #6	1	1	1	1	1	0	0
Number of positives (p_i)	6	6	6	6	4	3	1

Table 4.2 Data table denoting the endpoint color change in six replicates of seven fold 10X dilution series templates for MPN analysis of *tceA* gene in groundwater sample MW100

Replicate	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	Dilution 6	Dilution 7
Replicate #1	1	1	1	1	1	1	1
Replicate #2	1	1	1	1	1	1	0
Replicate #3	1	1	1	1	1	1	1
Replicate #4	1	1	1	1	1	1	1
Replicate #5	1	1	1	1	0	0	0
Replicate #6	1	1	1	1	1	1	0
Number of positives (p_i)	6	6	6	6	5	5	3

Equation 1 The equation described by Hurley and Roscoe for MPN analysis:

$$\sum_{i=1}^k \frac{v_i d_i p_i}{1 - e^{-v_i d_i x}} = \sum_{i=1}^k v_i d_i n_i$$

Here, k is the dilution level, n_i is the number of subsamples, p_i is the number of positive subsamples, d_i is the dilution factor, v_i is the volume of each subsample, and x is the estimated concentration of the RDase gene. A numerical spreadsheet was set up using Microsoft Excel 2016 was used to iterate the value of x .

Table 4.4 Concentrations of *vcrA* and *tceA* genes obtained using SYBR Green LAMP coupled to MPN method with centrifuged cell templates and qPCR with DNA templates.

Samples	Concentrations in gene copy/L				Standard deviation for qPCR	
	MPN <i>vcrA</i>	MPN <i>tceA</i>	qPCR <i>vcrA</i>	qPCR <i>tceA</i>	<i>vcrA</i>	<i>tceA</i>
MW100	4.00E+05	9.60E+05	2.24E+06	7.28E+06	9.36E+05	1.10E+06
IW5 ^{B1}	3.60E+06	6.00E+06	6.06E+07	2.01E+07	1.08E+07	4.07E+06
IW6 ^{B1}	4.30E+06	1.20E+07	5.47E+07	7.78E+07	5.71E+06	1.09E+07
IW5 ^{B2}	6.68E+06	6.44E+06	3.94E+07	4.86E+07	6.92E+06	7.96E+06
IW6 ^{B2}	3.20E+07	2.90E+07	5.50E+07	8.85E+07	2.09E+07	2.87E+07
MW113	1.02E+06	7.84E+05	3.13E+06	2.41E+06	1.65E+06	9.90E+05
MW514	4.35E+05	4.98E+05	1.46E+06	2.96E+06	4.97E+05	3.88E+05

Table 4.5 and Table 4.6 show the sample calculations used in the iterative evaluation of *vcrA* and *tceA* gene concentrations (i.e., x) in groundwater sample MW100. We assumed that the *Dehalococcoides* cells were colloidal, and had a uniform clustering in the template added. Moreover, MPN values at the microliter scale were assumed consistent with milliliter and liter volumes. If the dilution series provided all positive results, then the calculation was performed using the most dilute samples. On the other hand, if dilutions provided all negative results, then MPN values were calculated using the most concentrated dilutions. These assumption are similar to those made by Ahmad, et al. (2017) for their MPN-LAMP method.

4.3.5 Preparation of DNA templates from groundwater samples for qPCR

For producing DNA templates, groundwater (100 - 250 mL) was filtered through 0.22 μm filter (EMD Millipore Corp., Billerica, MA) using a vacuum pump. Membranes were cut into 5 mm strips inside a petri dish with a 15 blade (Bard Parker, catalog no. 37615) using aseptic technique and were added to 15 mL bead tubes supplied with the MO BIO Ultraclean water kit (MO BIO Laboratories Inc., Carlsbad, CA) (17). The DNA was eluted according to the instructions supplied by the manufacturer and the final template was suspended in 100 μL of dH_2O . The extracted DNA template was immediately used for qPCR or stored at $-20\text{ }^\circ\text{C}$ for future use. The entire process was repeated three times to generate triplicates of each groundwater sample.

Each 20 μL TaqMan reaction contained 10 μL iTaq Universal super mix supplied by Bio-Rad, 1.2 μL TaqMan probe, and balance water to make up 18 μL . PCR amplifications were performed using cycling conditions of $95\text{ }^\circ\text{C}$ for 15 s, $60\text{ }^\circ\text{C}$ for 1 min, a slow ramp of 1% to $95\text{ }^\circ\text{C}$ for 15 s and $60\text{ }^\circ\text{C}$ for 15 s. Templates and standards were added to qPCR reaction as 2 μL

aliquots. From the primers listed in Table 2.2, the primers for *vcrA* and *tceA* gene were used for qPCR in this study.

4.4 Results

Kanitkar, et al. (2017) described a novel assay for the visual detection of *Dehalococcoides mccartyi* RDase gene in groundwater samples without DNA extraction. Figure 4.1 provides a brief outline of the protocol for that assay. In this study, the protocol was altered with three key changes. First, the reaction volume was reduced from 50 μL to 25 μL . The original protocol involved adding 5 μL templates to the 45 μL master mix, while the current protocol adds 3 μL templates to a 22 μL master mix effectively increasing the availability of the template from 10% in the original protocol to 12% in the current method. Second, reaction mix was altered to include this dUTP-UNG contamination control system, which involves using dNTPs mix with dUTP instead of dTTP for producing amplicons containing uracil. For all subsequent experiments involving LAMP, trace levels of carry over contamination resulting from aerosolization of templates were destroyed using UNG. Both changes required using a master mix with lower water content compared to the original recipe to accommodate a larger template volume as well as UNG. Finally, greater volumes (100-250 mL) of groundwater were filtered in order to avoid false negatives, which might result from the addition of UNG.

In the second set of experiments, the optimized SYBR Green LAMP assay was coupled to the MPN technique and quantitative concentration estimates of *vcrA* and *tceA* genes in groundwater samples listed in Table 4.3 were obtained using centrifuged cell templates prepared from each sample. Then, qPCR with DNA templates was used to precisely determine the concentration of *vcrA* and *tceA* genes in each sample. Finally, concentration estimates obtained using the SYBR Green LAMP method and centrifuged cell templates, were compared to the

concentrations obtained using qPCR with DNA templates. The goal of these experiments was to determine if the SYBR Green LAMP assay can be used for quantification of RDase genes from groundwater samples without DNA extraction.

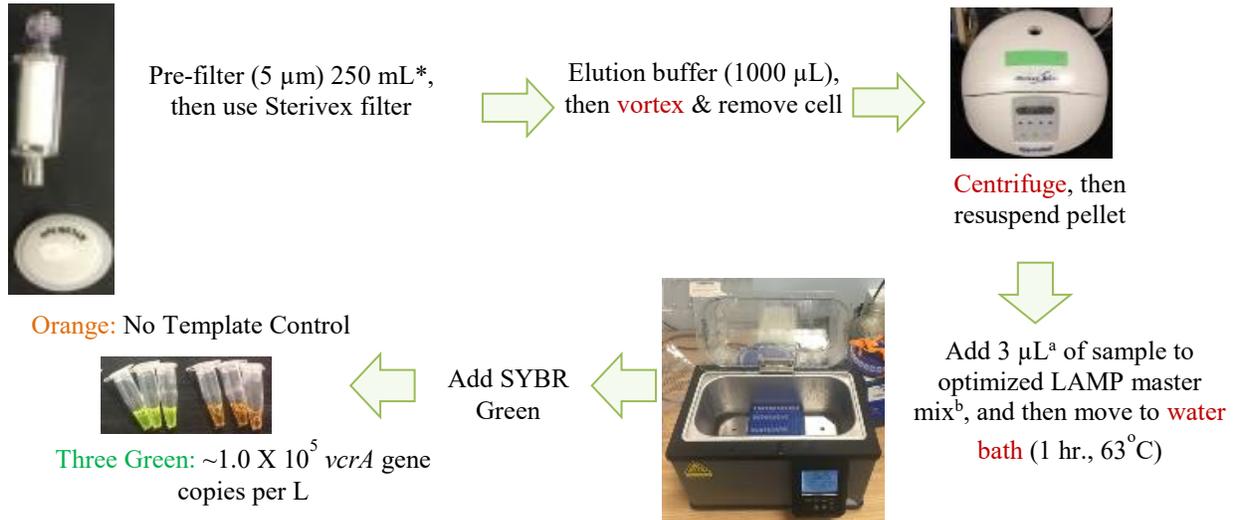


Figure 4.1 Schematic of the SYBR Green LAMP method for detection of RDase genes from groundwater samples (14).

*Greater volumes of groundwater samples were filtered to avoid a significant increase in detection limit of the SYBR Green LAMP assay resulting from incorporation of dUTP-UNG contamination control system.

^a The optimized assay also delivered greater volume of template to the final reaction while reducing the reaction volume from 50 µL to 25 µL.

^b LAMP master mix was incubated with UNG at room temperature before addition of templates.

4.4.1 Optimization of SYBR Green LAMP assay with dUTP-UNG contamination control system (DNA templates)

The primary goal of this set of experiments was to determine the precise amount of UNG required for degrading trace levels of carry over contamination of *vcrA* gene while preserving the amplification resulting from four fold 10X dilution series templates of *vcrA* plasmid. To do this, the master mix described above was deliberately contaminated with a *vcrA* AUGC LAMP amplicon such that each tube received 1.44×10^4 *vcrA* gene copies. The contaminated master

mix was then treated with UNG and incubated at room temperature for 1.5 min before dispensing the plasmid standard templates. Figure 4.2 shows the results of that experiment. The endpoint color of the reaction is denoted on the y-axis. If the endpoint color a reaction was green (positive detection), it was plotted with a value of one. On the other hand, if the endpoint color of the reaction was orange, it was plotted with a value of zero. The gene copies of ATGC template of the *vcrA* plasmid DNA added per reaction is plotted on the x-axis while the amount of UNG in the LAMP master-mix is plotted on the depth axis. Amplification from the four fold 10X dilution series as well as the negative controls with water templates using the spiked master mix at different amount of UNG is represented with bars. The false positives in negative controls are marked with an asterisk.

At high levels of UNG (1.0 and 0.8 units per reaction) amplification in all templates was inhibited indicating that these levels were excessive, while 0.6 units of UNG produced false negatives in tubes containing the 1.04×10^3 and 1.04×10^2 gene copies of plasmid standard template. Similarly, 0.4 units of UNG produced a false negative in tube containing 1.04×10^2 gene copies of plasmid standard template. At 0.2 units of UNG, all the tubes containing plasmid standard template produced a green color while the negative control fluoresced orange. On the other hand, if the level of UNG was reduced below 0.2 units, contamination persisted and the negative control fluoresced green.

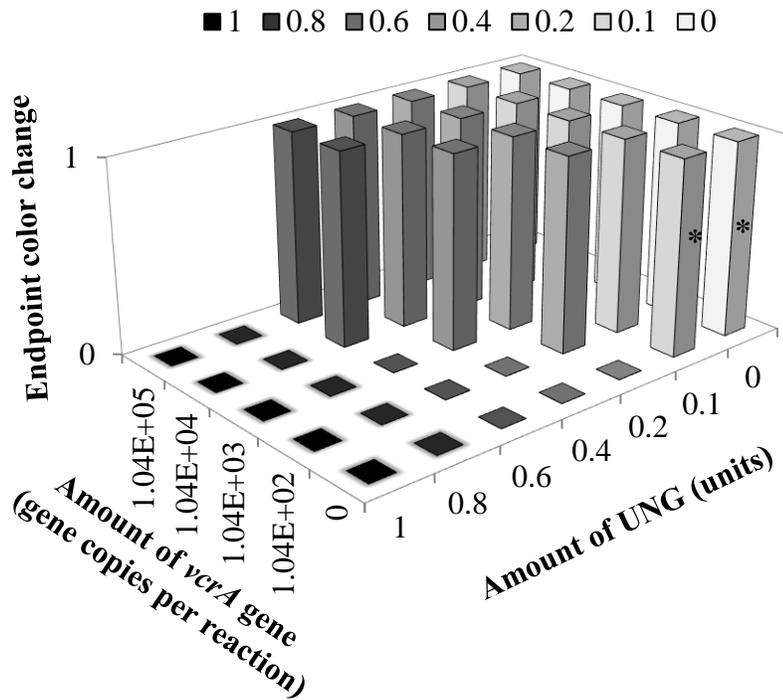


Figure 4.2 Optimization of amount of UNG required to destroy a *vcrA* gene AUGC contamination of 1.44×10^4 gene copies per reaction while preserving amplification resulting from ATGC *vcrA* plasmid DNA template from up to ~100 gene copies per reaction.

Note, if the endpoint color change was green, it was denoted with a value of one while that of orange was denoted with zero. No template controls not represented in the figure remained orange.

*Amplification observed in negative controls (water + contaminant template)

4.4.2 Optimization of SYBR Green LAMP assay with dUTP-UNG contamination control system (centrifuged cell templates)

The detection limit of SYBR Green LAMP assay was tested with seven fold 10X dilution series templates prepared from the SDC-9 culture and 0.4, 0.3 and 0.2 units of UNG. Figure 4.3 shows the results of that study. The endpoint color of the reaction is denoted on the y-axis. If the endpoint color in a replicate of reaction was green, it was plotted with a value of one. On the other hand, if the endpoint color of the reaction was orange, it was plotted with a value of zero. Since each dilution had triplicates, the maximum and the minimum values on y-axis can be three and zero respectively. qPCR estimated gene copies in the seven fold 10X dilution series of centrifuged cell templates are plotted on x-axis while the amount of UNG in the LAMP master-mix is plotted on the depth axis. At 0.4 units of UNG, the SYBR Green LAMP assay failed to produce any amplification below 8.4×10^6 gene copies. Moreover, at 8.4×10^6 gene copies only one replicate fluoresced green. With 0.3 units of UNG, the detection limit was reduced to 8.4×10^3 gene copies. In this case, two replicates each of dilutions containing 8.4×10^3 and 8.4×10^2 gene copies fluoresced green. On the other hand, at 0.2 units of UNG, detection limit was closer to ~84 gene copies which is similar to the detection limit with DNA templates. This detection limit translates to a hypothetical *vcrA* gene concentration of 1.12×10^4 gene copies per liter.

These results indicate that the inclusion of dUTP and UNG into the SYBR green LAMP successfully prevents false positives due to carry over contamination. With 0.2 units of UNG per reaction the hypothetical detection limit of SYBR Green LAMP assay is 1.12×10^4 gene copies per liter, which is lower than acceptable limit for monitored natural attenuation (2, 17).

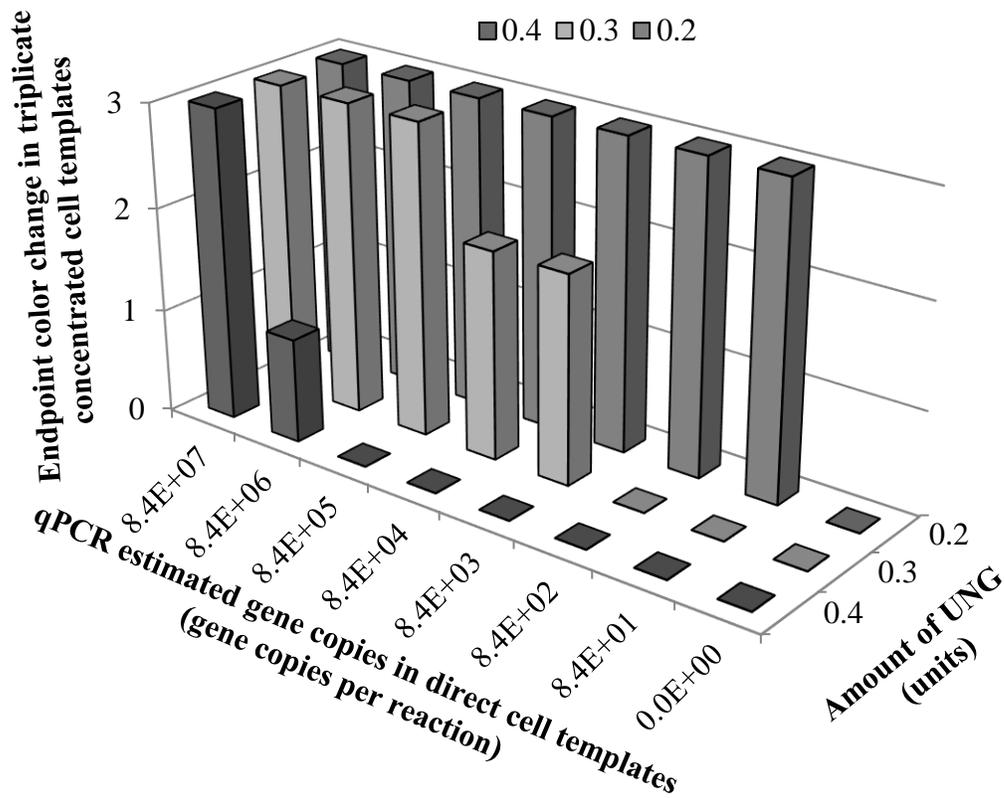


Figure 4.3 Optimization of UNG with concentrated cell templates prepared from groundwater spiked with known quantities SDC-9 culture

Note, if the endpoint color change in a replicate was green, it was denoted with a value of 1 while that of orange was denoted with 0. No template controls not represented in the figure remained orange.

4.4.3 Quantitative estimation of *vcrA* and *tceA* genes with SYBR Green LAMP using MPN

Centrifuged cell templates were prepared from groundwater samples listed in Table 4.3. Quantitative estimates of *vcrA* and *tceA* genes in these samples were obtained using SYBR Green LAMP with MPN technique according to previously described methods (13, 14, 16). Figure 4.4 shows the endpoint color observed in a six replicates of seven fold 10X dilution series prepared from a single centrifuged cell template of groundwater sample MW100 for *vcrA* gene. Picture A has the first set of triplicates, and Picture B has the second set. No template controls for each replicate are on the right hand side. If the endpoint color change was green, it was denoted with a value of one while that of orange was denoted with zero in Table 4.1.

qPCR with DNA templates from the same sample set were used to validate these concentrations of *vcrA* and *tceA* genes. Figure 4.5 shows the concentrations of *vcrA* (A) and *tceA* (B) genes obtained using qPCR with DNA templates and SYBR Green LAMP coupled to MPN technique with centrifuged cell templates. The sample names are plotted on the x-axis and the log₁₀ concentrations for both assays are plotted on the y-axis. The concentrations obtained using qPCR and SYBR Green LAMP, are represented with grey and black bars respectively. The error bars with qPCR represent one standard deviation from the mean. Samples from batch 1 and batch 2 are marked B1 and B2 respectively and represent samples collected at two different sampling events from the same well.

Between different groundwater samples, the maximum concentration of *vcrA* gene obtained using qPCR and DNA templates was 6.06×10^7 gene copies/L while that for *tceA* gene was 8.85×10^7 gene copies/L. The minimum concentration of *vcrA* gene obtained using qPCR and DNA templates in these samples was 1.4×10^6 gene copies/L while that for *tceA* gene was

2.41×10^6 gene copies/L. Over all, concentrations obtained using qPCR were greater than concentrations obtained using SYBR Green LAMP (See Table 4.4). The maximum concentration of *vcrA* gene obtained using SYBR Green LAMP and centrifuged cell templates was 3.2×10^7 gene copies/L while that for *tceA* gene was 2.9×10^7 gene copies/L. The minimum concentration of *vcrA* gene obtained using SYBR Green LAMP and centrifuged cell templates in these samples was 4.0×10^5 gene copies/L while that for *tceA* gene was 4.98×10^5 gene copies/L.

Concentrations obtained using the SYBR green LAMP assay coupled to the MPN method with centrifuged cell templates, were compared to qPCR with DNA templates. Figure 4.6 shows a correlation between concentrations of *vcrA* and *tceA* genes obtained using qPCR and SYBR Green LAMP on linear scaled axes with log₁₀ values (A) and log scaled axes (B). The concentrations obtained using qPCR are plotted on the y-axis and the concentrations obtained using SYBR Green LAMP are plotted on the x-axis. The concentrations of *vcrA* gene are plotted with closed markers while the concentrations of *tceA* gene are plotted with open markers.

A strong correlation was observed between concentrations obtained using the SYBR Green LAMP and qPCR. It is worthwhile to note that the SYBR Green LAMP assay used centrifuged cell templates while the qPCR assay used DNA templates. For the *vcrA* gene the Spearman's coefficient (ρ) of 0.952 and the p-value of 0.0329 was observed while for the *tceA* gene these values were 0.994 and 0.0484 respectively. The correlation between qPCR and SYBR Green LAMP concentrations is plotted with solid line for the *vcrA* gene and a dashed line for the *tceA*. On linear axes, the slope and the intercept of the trend line for *vcrA* gene were 0.9549 and 1.0165 ($R^2 = 0.78096$) while for the *tceA* gene, these values were 0.9413 and 1.0745 ($R^2 = 0.92501$) respectively. On log axes, for a trend line passing through origin the slope and the

exponent values for *vcrA* gene were 10.386 and 0.9549. Similarly, these values for the *tceA* gene were 11.873 and 0.9413.

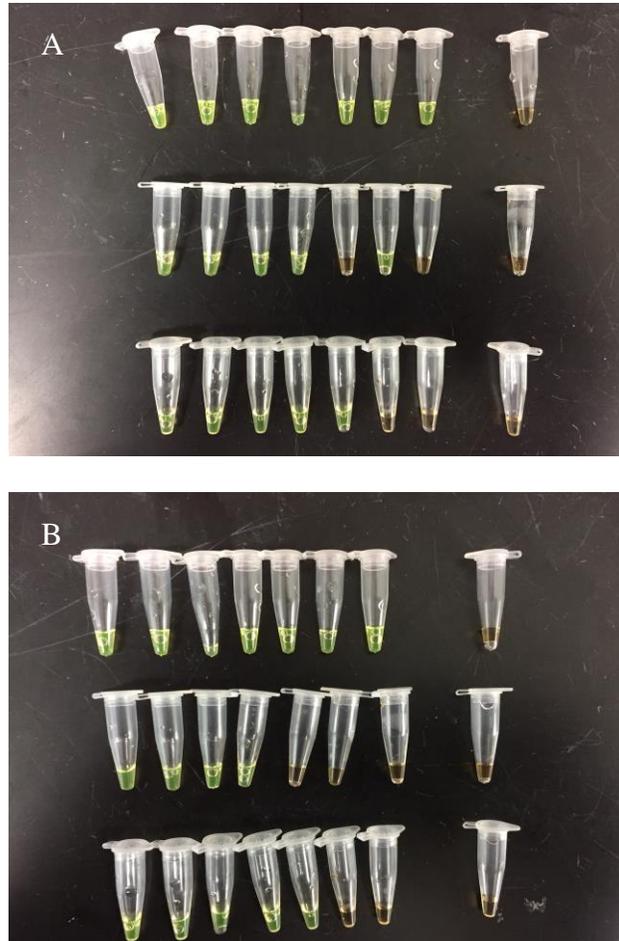


Figure 4.4 Endpoint color observed in a six replicate seven fold 10X dilution series prepared from a single centrifuged cell template of groundwater sample MW100 for *vcrA* gene.

Note: Picture A has the first set of triplicates, and Picture B has the second set. No template controls for each replicate are on the right hand side. If the endpoint color change was green, it was denoted with a value of one while that of orange was denoted with zero in Table 4.1

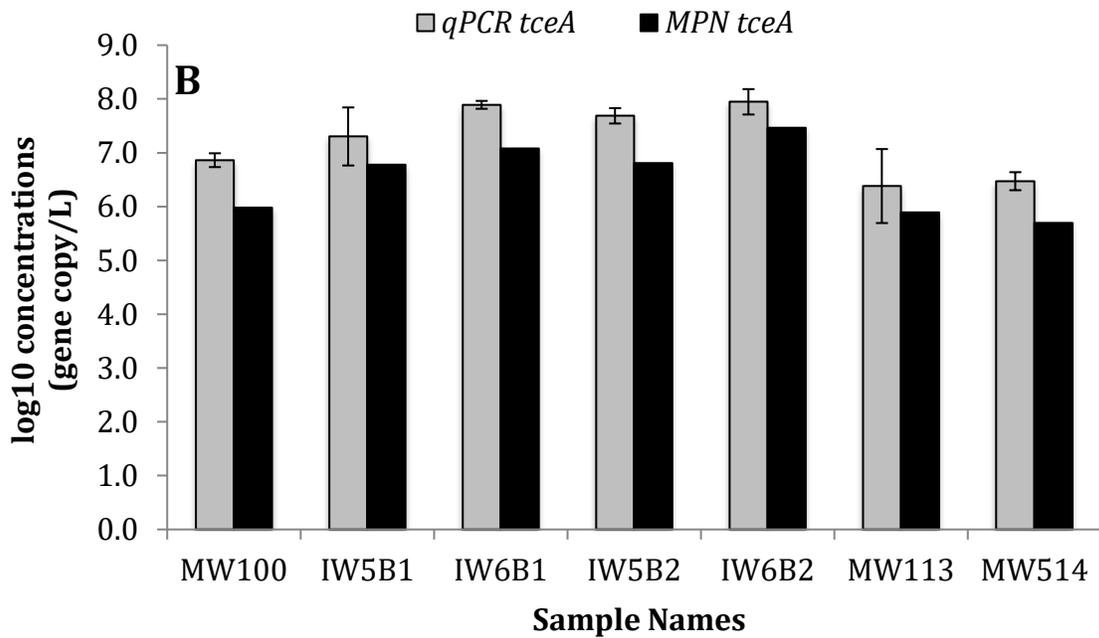
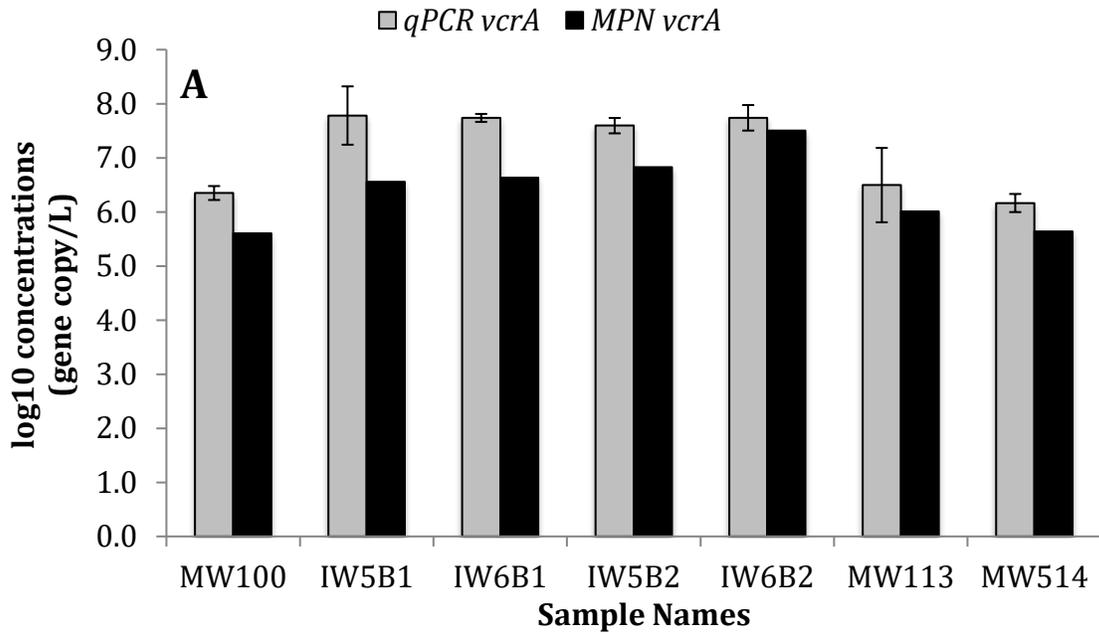


Figure 4.5 log₁₀ concentrations obtained using qPCR with DNA templates (grey) and MPN coupled to SYBR Green LAMP with centrifuged cell templates (black) for *vcrA* (A) and *tceA* (B) genes.

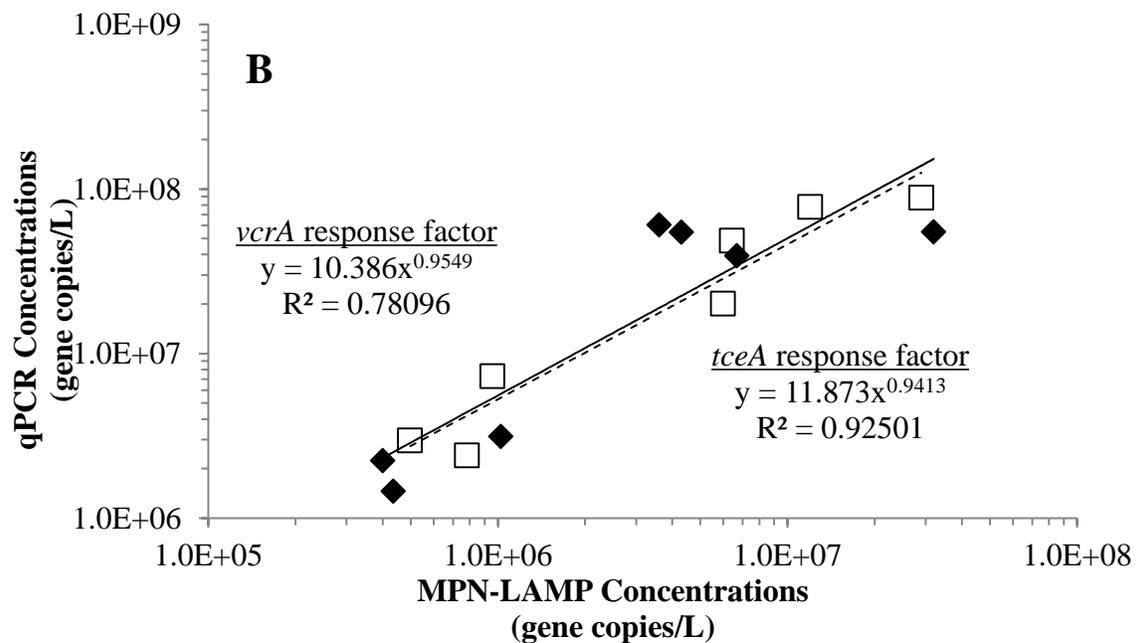
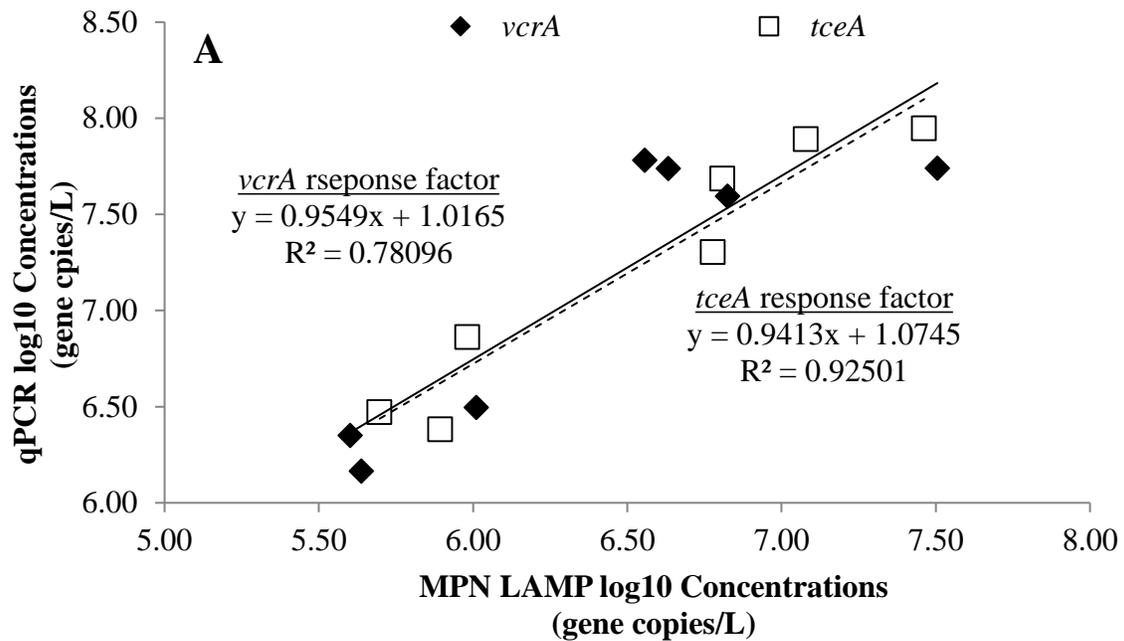


Figure 4.6 Correlation between concentrations of *vcrA* and *tceA* genes obtained using qPCR with DNA templates and MPN coupled to SYBR Green LAMP with centrifuged cell templates on linear scaled axes with log₁₀ values (A) and log scaled axes (B)

4.5 Discussion

The use of dUTP and UNG to control carry over contamination in qPCR is ubiquitous (18-21). In fact, several commercial master mixes for qPCR with varying concentrations of UNG (e.g. Applied Biosystems SYBR Green PCR Master Mix, Catalog #4309155 or QuantiTect SYBR Green PCR, Catalog # 204141) are available. However, the application of dUTP-UNG system to LAMP is relatively new. In 2011, He and Xu (2011) developed a LAMP reaction recipe for simultaneous detection of white spot syndrome virus and infectious hypodermal and hematopoietic necrosis virus in penaeid shrimp. To date, there are three other examples of application of dUTP-UNG to LAMP exist (12, 23, 24). In this study, the SYBR Green LAMP assay developed for the visual detection of RDase genes was optimized to include the dUTP UNG system. Plasmid DNA and centrifuged cell templates containing known quantities of *vcrA* gene were used to determine the concentration of UNG required to degrade a trace AUGC contamination of $\sim 10^4$ gene copies while preserving fluorescence resulting from amplification of ATGC templates for a detection limit of 10^2 gene copies. Between 1.0 to 0.3 units of UNG, the SYBR Green LAMP assay produced several false negatives at lower concentrations of *vcrA* gene. At UNG concentrations below 0.2 units, amplification was observed in negative controls resulting from carry over contamination. This data suggests that the addition of 0.2 units of UNG per reaction suppresses amplification due to carry over contamination of up to 10^4 gene copies/reaction. A detection limit of ~ 84 gene copies per reaction was observed with this optimized reaction chemistry for both plasmid DNA templates as well as centrifuged cell templates. With a groundwater filtration volume of 100-250 mL, a detection limit of 84 gene copies per reaction corresponds to a concentration of 1.12×10^4 gene copies/L. Never the less, Kanitkar et al. (2017) observed a detection limit of $\sim 1.18 \times 10^5$ gene copies/L for the *vcrA* gene

and 5.0×10^5 gene copies/L for *tceA* gene. This increase in detection limit may be attributed to the effect of humic substances of the amplification efficiency on LAMP (25) as well as the smaller filtration volumes used in that study.

Ahmad, et al. (2017) demonstrated the use of MPN based LAMP approach on a microfluidic platform for quantification of representative gram-negative and gram-positive water borne pathogens, *Escherichia coli* and *Enterococcus faecalis*, respectively. In that study, quantification with direct cell templates in samples containing < 10 CFU was observed. Here, the application of MPN to the SYBR Green LAMP assay previously described is extended for quantitative estimation of *vcrA* and *tceA* genes from seven different groundwater samples obtained from the former Kelly Air Force Base site bioaugmented with SDC-9 culture. Quantitative estimates of *vcrA* gene concentrations determined using SYBR Green LAMP and centrifuged cell templates with MPN in these samples were strongly correlated to concentrations obtained using qPCR and DNA templates ($\rho = 0.952$, p-value = 0.0329). However, the value of the slope (10.386) of the trend line indicates that concentrations obtained using SYBR Green LAMP were at least an order of magnitude smaller compared to concentrations obtained using qPCR. Similar result was observed with *tceA* gene. The *tceA* gene concentrations determined using SYBR Green LAMP and centrifuged cell templates with MPN in these samples were also strongly correlated to concentrations obtained using qPCR and DNA templates ($\rho = 0.994$, p-value = 0.0484). In this case, the slope of the trend line was 11.783. These slope and intercept values can be used as response factors to determine the concentrations of *vcrA* and *tceA* genes in groundwater samples for future applications of SYBR Green LAMP for quantification of RDase genes in groundwater samples.

In conclusion, SYBR Green LAMP approach is a low cost and user-friendly alternative to qPCR for the quantitative evaluation of *Dehalococcoides mccartyi* RDase genes in groundwater samples. It offers three key advantages compared to existing methods: time, potential *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. Moreover, the dUTP-UNG system reduces the probability of false positives due to carry over contamination and increases the overall robustness of visual detection with SYBR Green LAMP. The response factors generated for SYBR Green LAMP assay with MPN technique can be used to calibrate the assay and demonstrate its potential for use in the field.

APPENDIX

Appendix

Table 4.3 List of groundwater samples used to prepare DNA and centrifuged cell templates.

Well name	Date of analysis	Concentration of DNA templates (ng/μL)
IW5 ^{B1}	06/02/17	33.17
IW6 ^{B1}	06/02/17	63.58
MW100	06/02/17	20.22
IW5 ^{B2}	06/20/17	45.69
IW6 ^{B2}	06/20/17	75.07
MW514	06/20/17	44.8
MW113	06/20/17	93.6

B1 = Batch 1

B2 = Batch 2

Table 4.4 Concentrations of *vcrA* and *tceA* genes obtained using SYBR Green LAMP coupled to MPN method with centrifuged cell templates and qPCR with DNA templates.

Samples	Concentrations in gene copy/L				Standard deviation for qPCR	
	MPN <i>vcrA</i>	MPN <i>tceA</i>	qPCR <i>vcrA</i>	qPCR <i>tceA</i>	<i>vcrA</i>	<i>tceA</i>
MW100	4.00E+05	9.60E+05	2.24E+06	7.28E+06	9.36E+05	1.10E+06
IW5 ^{B1}	3.60E+06	6.00E+06	6.06E+07	2.01E+07	1.08E+07	4.07E+06
IW6 ^{B1}	4.30E+06	1.20E+07	5.47E+07	7.78E+07	5.71E+06	1.09E+07
IW5 ^{B2}	6.68E+06	6.44E+06	3.94E+07	4.86E+07	6.92E+06	7.96E+06
IW6 ^{B2}	3.20E+07	2.90E+07	5.50E+07	8.85E+07	2.09E+07	2.87E+07
MW113	1.02E+06	7.84E+05	3.13E+06	2.41E+06	1.65E+06	9.90E+05
MW514	4.35E+05	4.98E+05	1.46E+06	2.96E+06	4.97E+05	3.88E+05

Table 4.5 Representative calculation table for MPN analysis of *vcrA* gene in groundwater sample MW100 based on the outcomes of the SYBR Green LAMP assay performed on dilutions of centrifuged cell template.

Dilution level	1	2	3	4	5	6	7
Dilution factor (d_i)	1	0.1	0.01	0.001	0.0001	0.00001	0.000001
Number of subsamples (n_i)	6	6	6	6	6	6	6
Volume of subsample in μL (v_i)	25	25	25	25	25	25	25
Number of positive subsamples (p_i)	6	6	6	6	4	3	1
$v_i \times d_i \times n_i$	150	15	1.5	0.15	0.015	0.0015	0.00015
$v_i \times d_i \times p_i$	150	15	1.5	0.15	0.01	0.00075	0.000025
$v_i \times d_i$	25	2.5	0.25	0.025	0.0025	0.00025	0.000025

Table 4.6 Representative calculation table for MPN analysis of *tceA* gene in groundwater sample MW100 based on the outcomes of the SYBR Green LAMP assay performed on dilutions of centrifuged cell template.

Dilution level	1	2	3	4	5	6	7
Dilution factor (d_i)	1	0.1	0.01	0.001	0.0001	0.00001	0.000001
Number of subsamples (n_i)	6	6	6	6	6	6	6
Volume of subsample in μL (v_i)	25	25	25	25	25	25	25
Number of positive subsamples (p_i)	6	6	6	6	5	5	3
$v_i \times d_i \times n_i$	150	15	1.5	0.15	0.015	0.0015	0.00015
$v_i \times d_i \times p_i$	150	15	1.5	0.15	0.0125	0.00125	0.000075
$v_i \times d_i$	25	2.5	0.25	0.025	0.0025	0.00025	0.000025

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Chapter 5: Conclusions

LAMP is a rapid, user friendly, and a cost effective method for quantification of *Dehalococcoides mccartyi* RDase genes *vcrA*, *bvcA*, and *tceA*. The highly specific LAMP primers developed for these genes can be used to monitor the in-situ growth of *Dehalococcoides* cells in commercial reductive dechlorinating cultures SDC-9 and KB-1 using DNA templates. Since quantification with LAMP was comparable to qPCR over a wide concentration range, assays can be performed on real time thermal cycler or proprietary microfluidic platforms like the Gene-Z (Chapter 2). To date the growth of SDC-9 and KB-1 is routinely monitored using qPCR. Platforms like the Gene-Z are cheaper and more accessible alternatives to qPCR thermal cyclers. Since 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 are more economical compared to qPCR. In time-limited studies, another potential advantage is that amplification during LAMP is faster than qPCR. With the primer sets and reaction chemistries described in this study, all LAMP reactions were complete in less than one hour, which is significantly shorter than a typical qPCR run (>1.5 h).

The SYBR Green LAMP assay is a user-friendly assay and can be used for quantitative evaluation of *Dehalococcoides mccartyi* RDase gene concentrations in groundwater samples. A key feature of this assay is the use of low cost laboratory equipment for LAMP (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. With the detection limits of $\sim 10^5$ gene copies/L, the assay can potentially be applied

to track the growth of *Dehalococcoides mccartyi* at sites where monitored natural attenuation of chlorinated solvents is used as a clean-up strategy (Chapter 3).

The optimization of SYBR Green LAMP assay by reducing the reaction volume further reduces the cost per reaction. The dUTP – UNG contamination control system increases the overall robustness of the assay by reducing the probability of false positives while retaining a detection limit of $\sim 10^2$ gene copies per reaction. The application of MPN to the SYBR Green LAMP assay previously described by Dr. Hashsham's laboratory is extended for quantitative estimation of *vcrA* and *tceA* genes from seven different groundwater samples obtained from the former Kelly Air Force Base site bioaugmented with SDC-9 culture. Overall, the estimates of concentration obtained using MPN with SYBR Green LAMP assay on centrifuged cell templates showed a significant correlation to the concentrations obtained using qPCR and DNA templates. However, the SYBR Green LAMP assay underestimated the concentration of *vcrA* and *tceA* genes compared to qPCR. To account for the difference in concentrations between the two assays, response factors for correlating the MPN SYBR Green LAMP data to actual concentrations of *vcrA* and *tceA* genes in groundwater samples were developed (Chapter 4).

In summary, the SYBR green LAMP method can potentially be used for on-site quantification of *Dehalococcoides mccartyi* RDase genes in groundwater samples without DNA extraction. Since the method is fast, user-friendly, and inexpensive, large number of replicates can be run for every sample to increase the confidence level of the quantification obtained using MPN LAMP at a lower cost compared to qPCR. The response factors developed here could then be used as a guide to evaluate the actual concentration of RDase genes in each sample. Moreover, because SYBR Green LAMP assay uses ubiquitously available laboratory equipment

it could free remediation engineers from relying on services of commercial laboratories with expertise in qPCR

5.1 Future Work

- Development and commercialization of a LAMP master mix – A significant portion of time associated with setting up SYBR Green LAMP assay is consumed in mixing the different ingredients of the master mix and adding LAMP primers of each gene. Additionally, this step is prone to pipetting errors, which can affect the outcome of experiment. Commercialization of the SYBR Green LAMP assay would therefore require supplying a premade master mix for each RDase gene.
- Chip based LAMP and multiplexing with probe based chemistries – Chip based platforms could be developed to allow for detection of multiple RDase genes in a single tube. This can be achieved by designing unincorporated amplification signal reporters complimentary to the FIPs of existing primer sets and then labeling the respective FIPs with probes, which fluoresce at different wavelengths.
- LAMP primers for other genes relevant for bioremediation – With the onset of high throughput sequencing data, novel putative reductive dehalogenases (e.g. *cerA*, *mbrA*) and several other monooxygenases (e.g. *etnC*, *etnE*, *pmoA*) have become relevant to the bioremediation of chlorinated solvents. Specific LAMP primers for these genes need to be designed and tested with bioaugmenting cultures and groundwater samples from various sites.