FINAL REPORT

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

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14. ABSTRACT
Nucleic acid amplification of biomarkers is increasingly used to measure microbial activity and predict remedial performance in sites with trichloroethene (TCE) contamination. Field-based genetic quantification of microorganisms associated with bioremediation may help increase accuracy that is diminished through transport and processing of groundwater samples. Sterivex™ cartridges and a previously undescribed mechanism for eluting biomass was used to concentrate cells. DNA extraction-free loop mediated isothermal amplification (LAMP) was monitored in real-time with a point of use device (termed Gene-Z).

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Supplementary Figure 7.27. Comparison between *vcrA* gene copies (per L) determined via qPCR and shotgun sequencing (normalized relative abundance, %, MG-RAST). The results from two shotgun sequencing quantification methods are shown (as discussed in the text).

List of Acronyms

ATCC – American Type Culture Collection
cDCE – *cis*- dichloroethene
C_t – Cycle threshold
CV – Coefficient of variation
DIAMOND – Double Index Alignment of Next-Generation Sequencing Data
DHC – *Dehalococcoides*
DFA – Direct filter amplification
cATP – Deoxyadenine triphosphate
dCTP – Deoxycytosine triphosphate
dGTP – Deoxyguanine triphosphate
dTTP – Deoxythymidine triphosphate
dUTP – Deoxyuridine triphosphate
EDA – Elution direct amplification
FunGene – Functional Gene Pipeline and Repository
GC-FID – Gas Chromatograph- Flame Ionization Detector
gDNA – Genomic deoxyribonucleic acids
HTOC – High organic carbon
LAMP – Loop Mediated Isothermal Amplification
LOD – Limit of Detection
LTOC – Low organic carbon
Luc gene – Luciferase gene
MG-RAST – Meta Genome Rapid Annotation using Subsystem Technology
MIAC – Microbial Internal Amplification Control
MPN – Most Probable Number
NCBI – National Center for Biotechnology Information
NCBI BLAST – Basic Local Alignment Search Tool
NGS – Next Generation Sequencing
PBS – Phosphate buffered saline
PCE – Tetrachloroethene

PCR – Polymerase chain reaction
qPCR – Quantitative polymerase chain reaction
pMMO – Particulate methane monoxygenase
RDase – Reductive dehalogenase
RDP – Ribosomal Database Project
sMMO– Soluble methane monoxygenase
SNR – Signal to noise ratio
TCE – Trichloroethene
T_t – Threshold time
UNG – Uracil DNA glycosylase
VC – Vinyl chloride
1,1,1-TCA – 1,1,1-Trichloroethane
1,1-DCA - 1,1-Dichloroethane

Keywords

Dehalococcoides, Dehalobacter, Dehalogenimonas, LAMP, qPCR,

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Executive Summary

Introduction

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

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

Objectives

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

2. To use LAMP 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 APTIM) using DNA templates (rather than extracted DNA).
3. To compare quantification (i.e. gene copies/L values) between LAMP and qPCR to evaluate the effectiveness of LAMP as a tool to monitor the growth of *Dehalococcoides* spp. in KB-1 and SDC-9.
4. To validate the developed assays using DNA templates isolated from groundwater samples using LAMP with the Gene-Z (a hand-held device)[18].
5. To concentrate biomass in reaction wells of disposable Gene-Z chips for direct filter amplification (DFA) for use with LAMP assays targeting a *Dehalobacter* 16S rRNA gene and a putative 1,2-DCA reductive dehalogenase gene (*rdhA* gene)[19].
6. To compare DFA LAMP with qPCR including: i) spiking reactions with various concentrations of humic acid, ii) spiking multiple groundwater samples with *Dehalobacter* (from a commercially available bioaugmentation culture, TCA-20) following a concentration step, and iii) comparing methods for field-able target enrichment followed by direct isothermal amplification or DFA.
7. To develop visual based SYBR green LAMP assays (requiring on a bench top centrifuge and a water bath) for quantifying *vcrA* and *tceA* genes with groundwater from contaminated sites, without DNA extraction.
8. To quantify chlorinated solvent and 1,4-dioxane degrading microorganisms in contaminated site groundwater using shotgun sequencing and both taxonomic and functional analyses.

Technical Approach

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

In another study, six LAMP primers were designed for three RDase genes (*vcrA, bvcA, tceA*) using Primer Explorer V4. The LAMP assays were compared to qPCR assays using

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

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

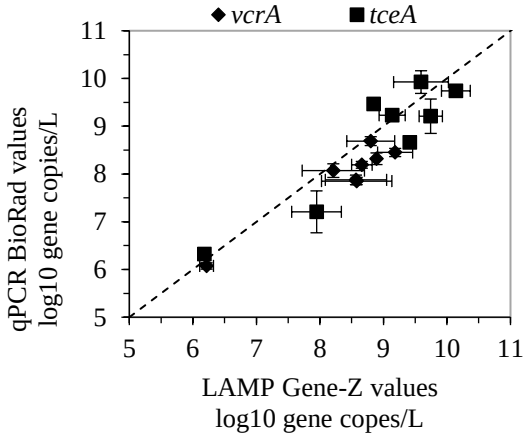
An approach for quantifying RDase genes was developed requiring only low cost laboratory equipment (a bench top centrifuge and a water bath) and less time and resources compared to qPCR. This involved the concentration of biomass from groundwater, without DNA extraction, and LAMP amplification of the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

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

Results and Discussion

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

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

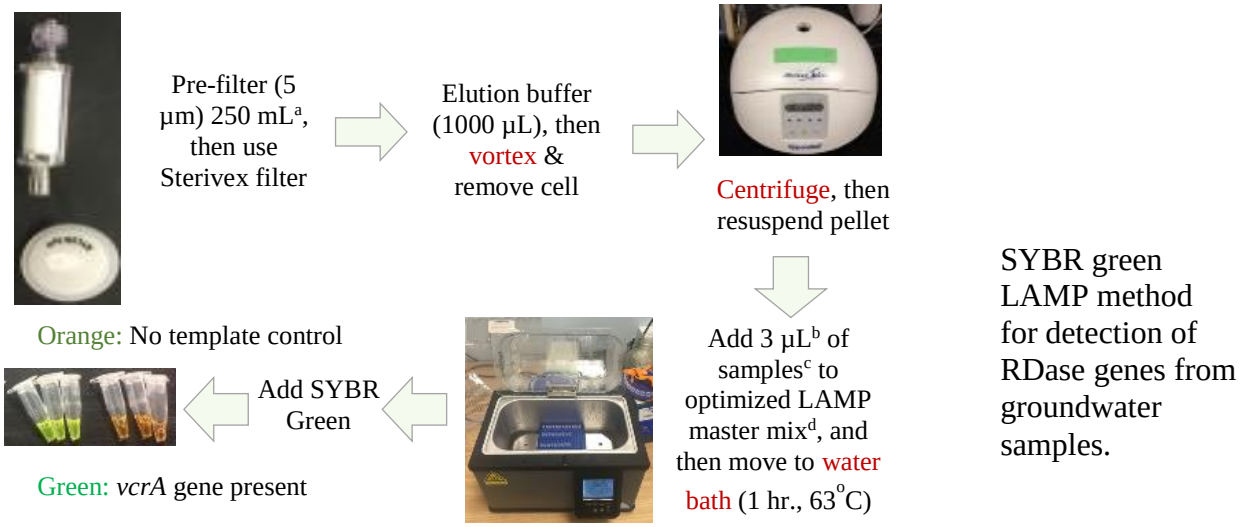


Comparison of *vcrA* and *tceA* mean gene copies (per L) in triplicates or eight different groundwater DNA templates observed using qPCR on real time thermal cycler and the Gene-Z. The dashed line represents 1:1 comparison.

The LAMP assays were less susceptible to inhibition by humic acid than qPCR, amplifying with up to 100 mg per L of humic acid per LAMP reaction compared to only 1 mg per L for qPCR. Direct LAMP of *Dehalobacter* assays was less influenced by inhibition in groundwater samples (collected from remediation sites) compared to qPCR. For the DFA experiments, amplification was observed down to 10^2 *Dehalobacter* cells spiked into 100 mL.

The DFA method was also tested to enrich for low target concentrations in environmental waters. While tested with different microbial targets (e.g. *Dehalococcoides* versus *Dehalobacter*), LAMP assays for both targets had similar sensitivity (10 to 100 gene copies per reaction depending on the assay tested). Placement of filters directly onto Gene-Z chips also reduced the time required for an elution step (10-15 min), which required an elution buffer stored at 4 ^{o}C and a means to vortex the Sterivex cartridges. Thus, DFA may provide a field-able alternative for biomass enrichment of water for genetic diagnostics.

A visual based SYBR green LAMP- MPN approach was developed, offering a low cost and user-friendly alternative to qPCR for quantifying RDase genes in groundwater samples. It offers three key advantages compared to existing methods: time, cost and the potential *in situ* application. The use of centrifuged cells, instead of DNA, reduces the time and cost required for sample preparation (no DNA extraction). Also, compared to qPCR, the LAMP assay has a shorter run time and the visualization of amplification products is immediate. The assay requires only basic laboratory equipment (benchtop centrifuge and water bath), does not require an expensive real time thermal cycler. With additional development and validation, it is possible that the method could be applied in the field. Additionally, the dUTP-UNG system reduces the probability of false positives due to carry over contamination and increases the overall robustness of visual detection with SYBR green LAMP. The regression equations generated for SYBR green LAMP assay with MPN technique can be used to calibrate the assay to relate the data to traditional qPCR data.



SYBR green LAMP method for detection of RDase genes from groundwater samples.

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

Implications for Future Research and Benefits

LAMP offers two key advantages over qPCR. First, the LAMP primer sets developed in this research may be used with a variety of 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 [18, 21-24]. 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 research, 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 [18] , for the rapid detection and quantification of *Dehalococcoides* spp. in groundwater from contaminated solvent sites. Further, the development of LAMP assays specific to two commonly used commercially available cultures will facilitate specific detection of these RDase genes at sites subject to bioaugmentation.

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

The developed visual based 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. Also, 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. A 50 µL LAMP reaction with centrifuged cells is slightly cheaper (~$ 0.30) than a 20 µL qPCR reaction when consumables and reagents are considered. However, commercially available master mixes are used for qPCR, whereas reagents are mixed manually for LAMP. When commercial master mixes for LAMP become available, this will further decrease the time and cost associated with LAMP. More importantly, qPCR requires DNA extraction, which adds approximately $9 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 in a dilution series, then the gene copies can be estimated,

providing the concentration is above ~10^5 gene copies per L. This value is less than the concentration required for effective dechlorination *in situ*.

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

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Chapter 1 - Abstract

This chapter provides an overview of the work in the form of an abstract. The remaining chapters each represent separate publications derived from the research.

Abstract

Background and Introduction: The chlorinated solvents are prevalent and problematic groundwater contaminants because of their tendency to form large, dissolved-phase plumes, their recalcitrant nature and the subsequent risk to human health. Remediation approaches are frequently based on bioremediation, which relies on establishing and maintaining significant populations of microorganisms capable of reductive dehalogenation. The majority of monitoring methods involve quantitative PCR (qPCR), which is performed in the laboratory on an expensive thermal cycler. The objective of this work was to explore other quantification methods, which could potentially be more specific, less expensive, less sensitive to inhibition, be field deployable or enable a larger selection of functional genes to be quantified.

Technical Approach: The work involved the development of loop mediated isothermal amplification (LAMP) to quantify microorganisms (16S rRNA gene & reductive dehalogenase genes) associated with reductive dehalogenation. LAMP assays for three RDase genes (*vcrA, bvcA, tceA*) were compared to qPCR assays using two commercially available bioaugmentation cultures (KB-1, SDC-9). The developed LAMP assays for *vcrA* and *tceA* genes were validated by

comparing quantification on the hand held platform, the Gene-Z, and a real time thermal cycler using DNA isolated from groundwater samples from a SDC-9 bioaugmented site. Other experiments tested DNA amplification from *Dehalobacter* with and without crude lysis and varying concentrations of humic acid. A new technique was developed where filters were amplified directly within disposable Gene-Z chips (direct filter amplification, DFA). Another approach for quantifying RDase genes (without DNA extraction) was developed requiring only low cost laboratory equipment (a bench top centrifuge and a water bath) and less time and resources compared to qPCR. Shotgun sequencing was used for the quantification of taxonomic and functional biomarkers associated with chlorinated solvent and 1,4-dioxane bioremediation.

Results: The comparison of LAMP primers and qPCR indicated quantification was similar over a large range of gene concentrations. Also, the quantitative increase in gene concentrations over one growth cycle of KB-1 and SDC-9 using LAMP primers was comparable to that of qPCR. The developed LAMP assays for *vcrA* and *tceA* genes were validated by comparing quantification the Gene-Z and a real time thermal cycler using DNA isolated from eight groundwater samples obtained from a SDC-9 bioaugmented site. A visual based SYBR green LAMP- MPN approach was developed, offering a low cost and user-friendly alternative to qPCR for quantifying RDase genes in groundwater samples.

The taxonomic analysis of the shotgun sequencing data revealed numerous genera previously linked to chlorinated solvent degradation, including *Dehalococcoides, Desulfitobacterium* and *Dehalogenimonas*. Reads aligning with both aerobic and anaerobic biomarkers were observed across all sites. Aerobic solvent degradation genes, *etnC* or *etnE,* were detected in at least one sample from each site, as were *pmoA* and *mmoX*. The most abundant 1,4-dioxane biomarker detected was *Methylosinus trichosporium OB3b mmoX.* Reads aligning to *thmA* or *Pseudonocardia* were not found.

Benefits: The visual based SYBR green LAMP- MPN approach offers three key advantages compared to existing methods: time, cost and the potential *in situ* application. The use of centrifuged cells, instead of DNA, reduces the time and cost required for sample preparation (no DNA extraction). Also, compared to qPCR, the LAMP assay has a shorter run time and the visualization of amplification products is immediate. The assay requires only basic laboratory equipment (benchtop centrifuge and water bath), does not require an expensive real time thermal cycler. With additional development and validation, it is possible that the method could be applied in the field. Additionally, the dUTP-UNG system reduces the probability of false positives due to carry over contamination and increases the overall robustness of visual detection with SYBR green LAMP. The regression equations generated for SYBR green LAMP assay with MPN technique can be used to calibrate the assay to relate the data to traditional qPCR data.

The work illustrates the importance of shotgun sequencing to provide a more complete picture of the functional abilities of microbial communities. The approach is advantageous over current methods because an unlimited number of functional genes can be quantified. Additional work should focus on RDase detection limits for shotgun sequencing data and comparisons to data generated with qPCR.

In summary, LAMP would be beneficial at sites containing groundwater with higher humic acid contents, as LAMP amplification is less sensitive to inhibition, compared to qPCR. LAMP would be beneficial if funds for monitoring were limited, as the only equipment needed include an incubator and a waterbath. However, the individuals performing the assays would still need basic skills in microbiology/molecular methods. In comparison, qPCR requires an expensive thermal cycler. Further, LAMP can be performed without DNA extraction, which also reduces costs.

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Chapter 2

Stedtfeld, R., Stedtfeld, T., Kronlein, M., Seyrig, G., Steffan, R., Cupples, A.M. and S. A. Hashsham. 2014. DNA extraction-free quantification of *Dehalococcoides* spp. in groundwater using a hand-held device. Environmental Science and Technology, 48: 13855-13863.

1. Abstract

Nucleic acid amplification of biomarkers is increasingly used to measure microbial activity and predict remedial performance in sites with trichloroethene (TCE) contamination. Field-based genetic quantification of microorganisms associated with bioremediation may help increase accuracy that is diminished through transport and processing of groundwater samples. Sterivex™ cartridges and a previously undescribed mechanism for eluting biomass was used to concentrate cells. DNA extraction-free loop mediated isothermal amplification (LAMP) was monitored in real-time with a point of use device (termed Gene-Z). A detection limit of 10^5 cells L^{-1} was obtained, corresponding to sensitivity between 10 to 100 genomic copies per reaction for assays targeting the *Dehalococcoides* spp. specific 16S rRNA gene and *vcrA* gene, respectively. The quantity of *Dehalococcoides* spp. genomic copies measured from two TCE contaminated groundwater samples with conventional means of quantification including filtration, DNA extraction, purification, and qPCR was comparable to the field ready technique. Overall, this method of measuring *Dehalococcoides* spp. and *vcrA* genes in groundwater via direct amplification without intentional DNA extraction and purification is demonstrated, which may provide a more accurate mechanism of predicting remediation rates.

2. Introduction

Trichloroethene (TCE) is a contaminant at more than 3,000 US Department of Energy and Department of Defense sites.[1] In medium sized anaerobic plumes, bioremediation of TCE relies on the use of halorespiring microbial populations, for example *Dehalococcoides* spp. (DHC), and *Dehalobacter* spp.[2-7] Nucleic acid amplification of biomarkers is a promising technique to determine microbial activity in these sites and predict remedial performance.[8-10] For TCE bioremediation, 10^7 cells of DHC L^{-1} correlates with strong degradation activity while less than 10^4 cells L^{-1} requires active intervention. Critical aspects that could greatly increase the accuracy of quantitative molecular testing and ultimately provide a greater level of reliability in predicting rates of dechlorination and ethene production include: 1) on-site analysis that does not rely on

sample storage, 2) amplification that is less influenced by sample preparation protocols such as DNA extraction and purification and inhibitory substances (e.g. dissolved organic carbon), and 3) parallel detection of multiple functional genes and species specific markers related to vinyl chloride reductive dehalogenases.[11, 12]

For amplification based quantification of biomarkers, groundwater samples are typically collected in 0.5 to 1 L glass jars and shipped to an analytical lab for quantitative (qPCR). Shipping the samples to an off-site laboratory is cumbersome, costly, and requires proper disposal influencing the number of replicates that are generally analyzed. Transport of samples also causes chances for leaking/contamination, and increased storage time has the potential to alter the integrity of unstable biomarkers. Quantification of genomic copies via qPCR can be biased due to cell lysis, DNA purification, and inhibitors,[13, 14] causing 1) false negatives,[15] and 2) inaccurate quantification and perception of low microbial activity, leading to superfluous intervention. As such, alternative approaches that alleviate some of the above problems are of interest.

This study demonstrates a field-deployable approach for cell concentration followed by rapid direct amplification and quantification without cell lysis and DNA purification. While Sterivex™ membranes have recently been described for on-site bacterial cell concentration, the methods for biomass collection involve either removing the membrane from the housing and washing, or using lysate mixtures directly added to the filter and subsequent incubation at 37 ºC for 1-3 hours.[10, 16] We report a different mechanism for removing biomass solely by eluting biomass, which is then used for direct amplification (**e**lution-**d**irect-**a**mplification, EDA) that reduces hand-on tasks and incubation time. For amplification and quantification of the recovered cells, loop mediated isothermal amplification (LAMP) assays targeting the *vcrA* gene and DHC specific sequences from the 16S rRNA gene were designed. Assays were tested using the Gene-Z platform which is a hand-held, battery-operated, wireless and automated device that monitors fluorescence in a 64-well microfluidic card for parallel detection of multiple assays.[17] LAMP is an established technique but has not previously been reported for DHC.[18-22] DNA extraction-free protocols using LAMP have been demonstrated for many different targets (bacterial, viral, human, fungal), and varied sample types including blood,[19, 23-26] stool,[27] urine samples,[22, 28] and 32% nasal swabs,[29] but to our knowledge have not been previously described for groundwater or environmental samples. Overall, the EDA method

has the potential to eliminate sample transport, increase accuracy, and simplify the analysis of DHC.

3. Methods

3.1. DHC Consortium

A consortium of DHC (SDC-9^{TM,} CB&I, APTIM)[30] was used to establish the sensitivity of the described EDA method. Based on qPCR of the 16S rRNA gene specifically targeting DHC, the concentration of the consortium was determined to be $8.0 \pm 1.8 \times 10^{11}$ copies L^{-1}. Dilutions were prepared based on the qPCR determined concentration and it was assumed that 1 copy of 16S rRNA gene was the equivalent of 1 cell (DHC has one 16S rRNA gene per cell, previously determined[31] as described[31]). Dilutions (10-fold) of 8.0×10^{11} to 8.0×10^{4} cells L^{-1} (reported herein as 10^{12} to 10^{5} cells L^{-1}) were prepared. Serial dilutions of the DHC were either filtered with Sterivex™ cartridges followed by EDA, or added directly to the amplification reaction as described below.

3.2. Sample Concentration

Sample filtration experiments were performed using Sterivex™ cartridges (SVGPL10RC, Millipore, Billerica, MA). Dilutions of the DHC consortium and groundwater at the specified volume (100 mL, 1 L, and 4L) were pushed through the filter using sterile 140 mL syringes with luer lock fittings. After filtration, concentrated cells were released from the filter by adding 0.9 mL of elution buffer (14600-50-NF-1A and 14600-50-NF-1B, cell elution buffers sold separately or as part of the PowerWater® Sterivex™ DNA Isolation Kit, MoBio), capping with autoclaved luer fittings (Part numbers 72-1430, 72-1431, Harvard Apparatus) and vortexing the filters at minimum speed for 10 min. Sample was eluted as described in the MoBio protocol manual, however, sample lyses and purification steps were not performed. Briefly, the Sterivex™ cartridges were held vertically while 1 mL of air was pushed into the bottom of the filter via a 3 mL sterile syringe. The syringe was pressed until there was slight resistance, the plunger was then released and pulled back to remove as much of the elution sample from the cartridge as possible. The syringe was then detached from the Sterivex™ filter and the eluted volume (0.7 to 0.9 mL) was dispensed into an microfuge tube and used directly for EDA in both the Gene-Z device (4 µL of sample per column) and the Chromo4^{TM} (1 µL of sample per microfuge tube) thermal cycler (at an isothermal temperate) as described below.

3.3. Assay and Primer Design

Based on their importance as potential biomarkers for remediation of chlorinated solvents,[32, 33] 16S rRNA and *vcrA* gene sequences of DHC (SDC-9^{TM}, CB&I)[30] were selected for this study. Sequences used for primer design were selected from *Dehalococcoides ethenogenes* 195 (CP000027.1) for the 16S rRNA gene, and from Bacterium VS vinyl-chloride reductive dehalogenase operon (AY322364.1) for the *vcrA* gene. Six specific LAMP primers (F3, B3, FIP, BIP, LF and LB) were designed using Primer Explorer V4 (Table 2.1.), and synthesized by Integrated DNA Technologies (IDT, Coralville, IA) with standard desalting purification. To theoretically verify primer specificity, ProbeMatch search with the RDP was used for primers targeting the 16S rRNA gene and a BLAST search against the GenBank database was used for primers targeting the *vcrA* gene. In total, 26 *vcrA* sequences were targeted in GenBank database and between 83 and 552 DHC 16S rRNA gene sequences were targeted in the RDP (dependent on the primer sequence).

3.4. LAMP Reaction

For all experiments tested with disposable cards in the Gene-Z device, 4 µL of sample (filtered or unfiltered) were mixed with 36 µL of LAMP master mix and loaded into one column (n=16 reaction wells) on the card. Reagent concentration, conditions for LAMP, and calculation of time to threshold (Tt) have been described previously.[17] Briefly, primers were dispensed and dehydrated in the reaction wells (during card assembly) to yield 1.6 µM each of FIP and BIP primers, 800 nM each of LB and FB primers, and 200 nM each of F3 and B3 primers, after dissolution of sample. Reaction mixtures loaded in the card, after assembly, contained 800 mM betaine (Sigma Aldrich), 1.4 mM of each dNTP (Invitrogen), 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 8 mM $MgSO_4$, 8 mM Triton X-100, 0.64 unit μL^{-1} of Bst DNA polymerase (New England Biolabs), 20 µM of SYTO-81 fluorescent dye (Invitrogen), 0.2% Pluronic F-68 (Invitrogen), 1 mg mL^{-1} of bovine serum albumin (BSA; New England Biolabs, Beverly, MA). All experiments were performed at 63 °C.

For all experiments performed in cards with the Gene-Z, primers were dehydrated in the card during assembly so that all wells in a column (n=16) had the same set of primers. After card assembly, the composition of added samples consisted of a different dilution (filtered or unfiltered) of DHC, and a no template control was used in the fourth column of each card. For samples tested on the Chromo4^{TM} Real-time PCR detector (BioRad Laboratories, Hercules, CA),

1 µL of sample and 9 µL of LAMP master mix were used per reaction vial. The Chromo4™ uses standard microfuge vials, thus replicate reactions were not fluidically connected and primers were not dried in the tubes. Based on the tested cell concentrations (Supplementary Table 2.1.), three different processing schemes were adopted (Figure 2.1.).

3.5. Card Operation

The disposable card was fabricated as described previously.[17] Briefly, the card consisted of four columns each with 16 interconnected reaction wells for a total of 64 reaction wells. Cards prepared for this study had four access ports to load a different sample into each column of 16 wells. However, the number of samples per card (load ports) can be modified. The reaction wells had a volume of roughly 1 µL each. The assembled card contained hydrophobic membranes placed downstream of each reaction well that 1) allowed air inside the microchannels to be purged, and 2) prevented liquid from exiting/overflowing from the card during sample dispensing.

A pipette was used to load the card using an access hole that fits 200 µL pipette tips. Equal distribution of sample volume was visually observed, and since previous experiments demonstrated lack of cross contamination between connected reaction wells,[17] each well is considered a technical replicate. After loading, the inlet port and air vents were sealed with tape to prevent contamination and evaporation. The total volume of sample loaded per column was 40 µL.

3.6. Gene-Z Prototype

A fully functional and packaged prototype of the Gene-Z was assembled as described previously.[17] The device incubates the card at a user specified temperature and time and monitors fluorescence in the individual reaction wells of the card in real time. The iPod Touch, which serves as a user interface and performs automated data analysis and reporting, was programmed for assays targeting DHC (Figure 2.1.). Replication among multiple cards and multiple wells within the same card were reported previously with the Gene-Z device.[17]

3.7. Contaminated Groundwater Sample Collection and LAMP

Two liters of contaminated groundwater sample were collected and separated into two 1 L amber glass bottles with screw caps. Bottles were packed in bubble wrap and ziplock bags and shipped overnight on ice. One liter was shipped to CB&I for qPCR analysis, and one liter was shipped to

Michigan State University for testing with the LAMP assays. Upon arrival, groundwater samples were placed at 4 °C. The two samples could be characterized by the high organic content (HTOC) of 269 mg L^{-1} and low organic content (LTOC) of 5.3 mg L^{-1}. Both HTOC and LTOC groundwater samples were collected from contaminated sites prior to enhanced bioremediation (additional site information is provided in Supplementary Table 2.2.).

For both the LTOC and the HTOC samples, LAMP reactions were prepared with concentrated sample from the Sterivex™ cartridges after filtering 100 mL. Filtered samples were tested on the Chromo4™ and the Gene-Z device for the *vcrA* gene, and the 16S rRNA gene assay was tested on the Chromo4™.

3.8. qPCR of Contaminated Groundwater Samples

One liter groundwater samples were filtered through a 0.2 µm pore membrane using a filter flask. To remove biomass, the filter was placed into a 15 mL centrifuge tube, 3 mL of 1 x PBS was added, the tube was vortexed for 2 minutes at high speed, the liquid sample was decanted into 2 separate 1.5 mL micro-centrifuge tubes, tubes were centrifuged for 10 minutes at 10,000 rpm, liquid was decanted, and the pellet was resuspended. DNA was extracted using the Idaho Technologies 1-2-3 DNA Isolation Kit. Briefly, resuspended samples were placed into bead beating tubes, lysed using a vortex Gene 2 with adaptor for 5 min at maximum speed, and DNA was captured and purified using centrifuges and spin filters. Quantitative PCR reactions consisted of 2 µL of the sample extract, 1 µL of LightCycler Fast Start Enzyme (Cat#: 03003248001, Roche), final concentration of 0.2 µM of each probe, 0.5 µM of both forward and reverse primer (Table 2.1.), and 2 mM $MgCl_2$, for a total 10 µL reaction volume. Duplicate reactions of each sample were performed in the LightCycler instrument using temperature cycling parameters described previously.[34]

3.9. LAMP Inhibition in Contaminated Groundwater Samples

To test for inhibition that could be caused by constitutes in the groundwater or cell elution buffer, *Legionella pneumophila*, which was not expected to be present in anaerobic plumes, was spiked into groundwater samples before and after filtration. *Legionella pneumophila* (ATCC# 33215), was grown as suggested by supplier in ATCC Medium 1099 Charcoal Yeast Extract buffered medium at 37 ºC for 1 week on plates, scraped and suspended in 1 x PBS, and dilution plated to determine cellular concentration. Dilutions were stored at 4 ºC for no longer than one

week prior to testing. A primer set targeting the *cadA* gene in *L. pneumophila* (Supplementary Table 2.3.) was selected as it had a level of sensitivity similar to the *vcrA* gene (10 and 100 cells per reaction). Inhibition experiments consisted of spiking 1 µL of serial dilutions of *L. pneumophila* cells into 9 µL of both concentrated groundwater samples (e.g. after filtering 100 mL), the HTOC groundwater samples before filtration, and PCR grade water. One µL of spiked samples was placed into the reaction mixture to yield a final amount of 8, 80, 800, 8000 cells per reaction. A negative control consisting of groundwater sample but no *L. pneumophila* was also tested for both samples. All experiments to test inhibition in the two groundwater samples were run on the Chromo4^{TM}. Lower dilutions of cells per reaction were tested (as opposed to higher dilutions) as they would be more likely to show influence of inhibition.

3.10. Data Analysis

Raw data obtained with the Gene-Z device was automatically sorted using the iPod Touch for each reaction well and emailed to a personal computer for further analysis. Data was processed as follows: 1) the baseline signal intensity was calculated as the median of all measurements recorded during the first 6 min, 2) baseline signal was subtracted from raw signals (ΔRn), 3) ΔRn curves were smoothed using average signal from 20 consecutive points, and 4) curves were normalized by dividing ΔRn by the maximum ΔRn. The time to threshold fluorescence (Tt) was then calculated as time at which the normalized ΔRn exceeds an arbitrary cut-off of 0.1. Standard curves that were generated from DHC consortium sample mixtures were compared with average Tt observed with groundwater samples to estimate copy numbers in the groundwater samples. For statistical comparison between tested assays and treatments, a two-tailed inference about differences in population means for independent samples was performed with a 95% confidence interval.

4. Results and Discussion

The goal of this study was to develop and demonstrate a strategy that could potentially be used for on-site quantification of genetic biomarkers to monitor bioremediation of chlorinated solvents. Experiments were performed to: 1) evaluate the limit of detection (LOD) on a consortium of DHC using the developed strategy, 2) ensure that sensitivity is not sacrificed using LAMP without deliberate DNA extraction/purification in groundwater samples, and 3) to quantitatively compare the EDA technique with conventional qPCR.

4.1. LOD with DHC Consortium

***Range 1: Greater than 10^7 cells L^{-1}*:** A dilution series prepared from the DHC SDC-9™ consortium was directly amplified using both the *vcrA* and 16S rRNA gene primer sets in the Gene-Z and Chromo4™. The detection limit, based on amplification of three out of three replicates on the Chromo4™ and three or more wells showing amplification in the microfluidic card in the Gene-Z, was 10^7 cells L^{-1} with the 16S rRNA gene (Figure 2.2.) and 10^8 cells L^{-1} for the *vcrA* gene on both devices (Figure 2.3.). Lower dilutions and no template control showed no amplification. Concentrations between the 10-fold serial dilutions were not tested.

LOD with the Gene-Z and Chromo4™ were comparable. For the *vcrA* gene, amplification occurred in 6 of the 16 reaction wells at 10^8 cells L^{-1} with the Gene-Z, and all tubes in Chromo4™. One µl of sample dilution was added to 9 µl of amplification mixture for tests in the Chromo4™. Thus, 10^7 cells L^{-1} dilution equates to approximately 10 cells per reaction tube in the Chromo4™. For the microfluidic card run on the Gene-Z, 4 µL of sample was added to a master-mix of reagents for a total volume of 40 µL, and this volume was added to one column (n=16 wells) on the card. It is assumed that an equal concentration of biomass is displaced into each of the 1 µL reaction wells, and solution remaining in channels is unaccounted for. Thus, a dilution of 10^7 cells L^{-1} equates to approximately 1 cell per reaction well in the Gene-Z card.

As a rule of thumb, acceptable rates of in situ chlorinated solvent degradation are estimated when DHC concentrations are equal to or greater than 10^7 cells L^{-1}.[35] With the primer set targeting the 16S rRNA gene, the method described here approaches the simplicity of "sample in-results out" because there is no sample concentration or deliberate DNA extraction and purification. Four microliters of the non filtered sample is added to LAMP reagents and loaded into the card for amplification. The card is then placed in the Gene-Z device and the reaction proceeds in less than 50 minutes. The difference in sensitivity between the 16S rRNA and *vcrA* gene assays is common and highly dependent on primer set and amount of genes/organisms present in the consortium.

Range 2: 10^6-10^7 cells L^{-1}: For detecting a lower range of cells, the EDA protocol was tested by filtering 100 mL of varying cell dilutions (10^5 to 10^{10} cells L^{-1} of the SDC-9™ consortium) through Sterivex™ cartridges. After filtration, concentrated biomass was released from the cartridge by adding 0.9 mL of elution buffer, vortexing the filters for 10 min, and eluting into a syringe. Both filtered and non-filtered solutions were tested in the microfluidic cards (Figure

2.3.). Only the *vcrA* gene target was tested using the Range 2 protocol (100 mL filtered). For non-filtered samples, amplification was observed in all wells (16/16) for dilutions from 10^{12} to 10^{9} cells L^{-1} (Supplementary Table 2.1.). For the non-filtered dilution of 10^{8} cells L^{-1}, only six reaction wells showed amplification. However, using the filtration protocol, amplification was observed in all 16 wells down to 10^{6} cells L^{-1}.

Quantification results between the Gene-Z and Chromo4™ were comparable. Using the average time to threshold (Tt) for all filtered dilutions run on the Gene-Z and Chromo4™, a correlation of $R^2 = 0.9256$ was observed (Figure 2.3. insert). This indicates that quantification is not compromised using the hand-held device instead of the conventional bench-top thermocycer. The threshold time was less reproducible at lower concentrations, which might be resolved with a slight variation in primer sequences or by optimizing reaction constitutes.[36]

Range 3: Less than 10^6 cells L^{-1}: For detection of concentrations less than 10^{6} cells L^{-1}, filtration of an additional volume of sample may be necessary. An additional experiment was performed by filtering 1 L and 4 L of water spiked with 10^{5} cells L^{-1} of SDC-9™ consortium. All of the reaction wells displayed amplification with 4 L of filtered DHC consortium, and only two of the 16 reaction wells displayed amplification with 1 L. Thus, filtration of the larger volume increased the LOD to 10^{5} cells L^{-1}.

Ideally, a 100-fold increase in biomass should have been obtained when filtering 100 mL and eluting in ~ 0.9 mL of cell elution buffer. But this was not observed, especially at lower concentrations. For example, the threshold time for a concentration of 10^{10} cells L^{-1} added directly to the card and 10^{8} cells L^{-1} added after 100 mL of filtration was 12.6 and 20 min, respectively. With a 100-fold increase in biomass, the threshold time with these two samples should be the same. It is also clear from the results that sensitivity is lost when greater volumes are filtered (i.e. only detected positive amplification in 2 of 16 reaction wells when 10^{5} cells L^{-1} from 1 L filtered volume, whereas 100 mL volume of 10^{6} cells L^{-1} was detected in all reaction wells). The loss in sensitivity may be due to biomass that is not recovered from the Sterivex™ cartridge, or extracellular DNA that is amplified in the non-filtered sample, but passes through the cartridge when the sample is filtered. Positive control experiments, described in more detail below, show that the cell elution buffer does not appear to inhibit the amplification reaction. As such, further optimization of filtration/elution protocols will be performed to increase concentration efficiency.

Additional protocols are also being explored to increase the limit of detection to 10^3 cells L^{-1}, which is the concentration of cells generally thought to require active intervention. Considering a detection limit of 10^6 cells L^{-1} was observed when one liter is filtered, we have estimated that the detection limit can be improved by two to three orders of magnitude via one or more of the following strategies: 1) developing a more sensitive primer set (i.e. 1 to 10 copies per reaction), 2) add reverse transcriptase for RT-LAMP to target RNA for detecting more cells per liter.[37] For example, the number of ribosomes per bacterial cell described for *Escherichia coli*, is 6,700 to 71,000 depending on the growth and physiological state.[38] Even if the efficiency of the reverse transcription is low,[39, 40] this may result in at least a one fold increase in the number of copies per cell, and thus the number of cells detected per liter. 3) Place eluted sample directly into lyophilized reagents so sample is not diluted prior to distribution into the card (as mentioned, 4 μL sample was placed into 40 μL of reagents for experiments described), 4) concentrate the 0.9 ml elute using an additional mechanism such as centrifugation or Erbium,[41] or 5) optimizing a protocol for removing biomass from the Sterivex filters with an elution volume that is less than 0.9 mL.

For the current EDA protocol, the total time required to compete all steps from sample filtration to results on the iPod is approximately 1 hour. The time required to complete the sample filtration protocol is 12 to 15 min (for 100 mL), however, this time will be dependent on the volume filtered and the mechanism for pushing sample through the Sterivex™ cartridge. The time required to manually push groundwater through the cartridge using a syringe is approximately 60 mL/min, and should not exceed 100 mL/ min[10] when a pump is used. Sample elution including vortexing takes 10 minutes, and in less than two additional minutes the sample can be eluted into premixed reagents and loaded into the card. The card is then placed in the Gene-Z for 45 to 50 min reaction time. It should be noted that all filtration experiments were performed via sterile syringes, which can be cumbersome and undesirably time consuming for filtering more than 0.1 L of sample. For on-site filtration of larger volumes with Sterivex™ cartridges, a peristaltic pump and sterile silicon tubing (e.g. Cole Parmer C-Flex) can be used for up to 4 L volumes of groundwater as described by Ritalahti and coauthors.[10] Correct implementation of the EDA procedure in the field should include filtering and vortexing at speeds that provide high capture efficiency, and the use of sterile luer-lock caps and syringes to minimize sample cross contamination.

In addition, we are exploring the use of multiple filters in series, for removal of larger debris prior to biomass concentration.

4.2. LAMP in TCE Contaminated Groundwater Sample

Two contaminated groundwater samples, with varying amounts of total organic carbon were spiked with a dilution series of cells as a positive control to determine if: 1) the cell elution solution used to remove biomass from Sterivex™ cartridges influenced amplification, and if 2) the groundwater samples inhibited amplification. For these experiments, dilutions of *L. pneumophila* cells were spiked directly into previously filtered groundwater samples, unfiltered groundwater sample, and PCR grade water. Amplification was not observed in negative controls of groundwater samples not spiked with *L. pneumophila* cells. Results showed no significant difference in threshold time (95% confidence interval considering mean difference) or sensitivity between cells spiked in the filtered samples and cells spiked into PCR grade water (Figure 2.4.).

The Sterivex™ cartridges were originally chosen as a field-able means to concentrate biomass, however, they might also serve to remove chlorinated solvents and soluble molecules such as humic acids that cause inhibition. Cells spiked in non-filtered HTOC sample showed a slight increase in the time to threshold (significant with ~ 10^3 and 10^4 cells reaction^{-1}), which may be caused by suspended inhibitors in the sample that are removed via filtration. These results also suggest that the cell elution buffer from MoBio appears to have no noticeable inhibition on the sample when the eluted sample volume is 10% of the amplification reaction.

Previous studies have described accurate quantification of LAMP with both real and mock samples containing common inhibitors. For example, a study by Tani and coauthors examined accurate quantification with increasing concentrations of humic acid and urea, reporting no qPCR amplification with higher concentration of humic acids, positive amplification in all tested concentration ($\leq$ 8 mg L^{-1}) with a shift in threshold time for turbidity monitored LAMP, and amplification in all wells with no shift in threshold time with fluorescent monitored LAMP.[42] In another study, 56% of spiked environmental water samples failed to show amplification for PCR, while all of the spiked samples showed amplification with LAMP.[15] With concentration of humic acids in groundwater ranging from <0.001 (20 of 35 groundwater wells) to over 10 mg L^{-1} (7 of 35 groundwater wells),[43, 44] the utility of a method that is less influenced by inhibitors and sample processing can be used to increase accuracy for quantitative genetic testing.

4.3. QPCR and EDA of DHC in TCE Contaminated Groundwater Sample

Experiments were performed with the two contaminated groundwater samples to determine if the described strategy was comparable to conventional methods, which typically include filtration to concentrate cells, DNA extraction/purification, and qPCR. The concentration of cells measured with the DHC specific 16S rRNA gene via qPCR and LAMP assays differed significantly for the HTOC sample and was not significantly different for the LTOC sample (95% confidence interval considering mean difference, Table 2.2.). The *vcrA* gene targeted LAMP assay was also tested for both samples, and had a non-detectable concentration in the LTOC (Table 2.2.).

For the HTOC and LTOC samples, 16S rRNA gene counts with the LAMP assay was approximately 28% and 23% of the estimated counts with the qPCR assays, respectively. This result could be due to many factors including primer converge. It should be noted that only 29 known 16S rRNA gene sequences were targeted with the combined set of six LAMP primers (targeting eight regions of the 16S rRNA gene) and 84 sequences were targeted with the combined set of two primers and two probes for the qPCR assay (Supplementary Figure 2.1.). A phylogenetic tree of all sequences targeted by the 16S rRNA gene qPCR primers shows that a large branch/group of sequences is not covered by the LAMP primer set. Modifications to the 16S rRNA gene LAMP assay, such as including additional primers or degenerate bases, can be performed to increase the number of targeted sequences for the combined set of primers.

While the EDA mechanisms without intentional lysing are unclear (i.e. amplification from extracellular DNA or lyses during vortex steps), the described strategy is comparable to qPCR for the two contaminated samples. It is assumed that a majority of extracellular DNA would pass through Sterivex™ cartridges, and only intact cells are placed in the amplification reaction. Perhaps the smaller 67 kDa *Bst* polymerase compared to 94 kDa *Taq* DNA polymerase[45] has a higher rate of passage into intact bacterial cells, or cells become porous at the amplification reaction temperature of 63 ^{o}C.

The lower number of *vcrA* gene copies observed in the aquifer samples, compared to DHC counts, has been previously described. In one qPCR-based study, the sum of the three functional genes (*vcrA*, *bvcA*, and *tceA*) was less than the number of copies estimated by DHC specific 16S rRNA gene assays.[12] As such, it is thought that unidentified genes associated with vinyl chloride reductive dehalogenase will need to be simultaneously targeted. Recent studies have also described the limitations of solely targeting the 16S rRNA gene, as quantitative

measurements do not always coincide with ethene formation in contaminated sites.[46] With the simultaneous detection of previously described genes including total bacterial 16S rRNA, species specific 16S rRNA gene (*Dhc*), *tceA*, *bvcA* , *vcrA*,[12] and the addition of assays from DHC, in which sequences are currently being explored,[47, 48] a multiple target assay that can target all potential markers may provide a greater level of reliability in predicting rates of dechlorination and ethene production. Additional primers can also be developed for obtaining a LOD of 1 to 10 copies per reaction[22, 49-51] with higher levels of reproducibility in terms of threshold time at lower cell concentrations.

Eventually, customized assays should only target genes that provide decision influencing actions, however, such customized assays will only be possible after a larger number of potential biomarker genes are tested with multiple samples. With an in-depth validation study to ensure specificity and optimization for accurate quantification, a multiple target assay would provide a practical impact to the field. Thus, perspective studies will focus on developing additional assays, and validation of specificity and quantitative accuracy using multiple groundwater samples under field conditions.

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5. Tables and Figures

Table 2.1. LAMP primers designed to target 16S rRNA and *vcrA* genes from dechlorinating culture SDC-9.

LAMP primers sequence (5'->3') for DHC 16S rRNA gene in SDC-9™		
Primer	Sequences targeted	Sequence
F3	209	CACACGCTACAATGGACAGA
B3	141	GGCTTTCATGACGTGACGG
FIP	552-83	CAGCCTGCAATCCGAACTGAGG-AGGTTGCAACAGTGTGAACT
BIP	223-590	ACCCGCCTGCATGAAGTTGG-GTGTACAAGGCCCGAGAAC
LF	171	CAGCTTTGGGGATTAGCTCC
LB	200	AGTTGCTAGTAACCGCATATCAGC
LAMP primers for DHC *vcrA* gene in SDC-9™		
F3	26	ACTAATATATAAGAAAGCTCAGCC
B3	26	TCTTATTGAGTTCTTGTGGTTG
FIP	26	GGTCAGGAACCTTGGGATAAATTTTGATGACTCTAGGAAAAGGAACA
BIP	26	AACTTTAAGGAAGCGGATTATAGCTATGGATTCACACTTTGTTGG
LF	26	CCTGGTCCACCTATTTCACTGTA
LB	26	ACTACAATGATGCAGAGTGGGTTA
qPCR primers for DHC 16S rRNA gene[34] in SDC-9™		
Forward	196	GAAGTAGTGAACCGAAAGG
Reverse	209	TCTGTCCATTGTAGCGTC
P-fitc	200	AGCGAGACTGCCCCG
P-640	530	AACGGGGAGGAAGGTGGG

Table 2.2. DHC cells measured with LAMP and qPCR in contaminated groundwater samples. The values are means and standard deviations of replicate reactions.

Target	Cells L^{-1} HTOC	Cells L^{-1} LTOC
qPCR 16S rRNA gene	6.4×10^9 (stdev 1.2×10^8)	4.4×10^7 (stdev 1.2×10^8)
LAMP 16S rRNA gene	1.8×10^9 (stdev 3.0×10^8)	1.1×10^7 (stdev 3.4×10^6)
LAMP vcrA gene	8.0×10^6 (stdev 4.7×10^6)	Not detected

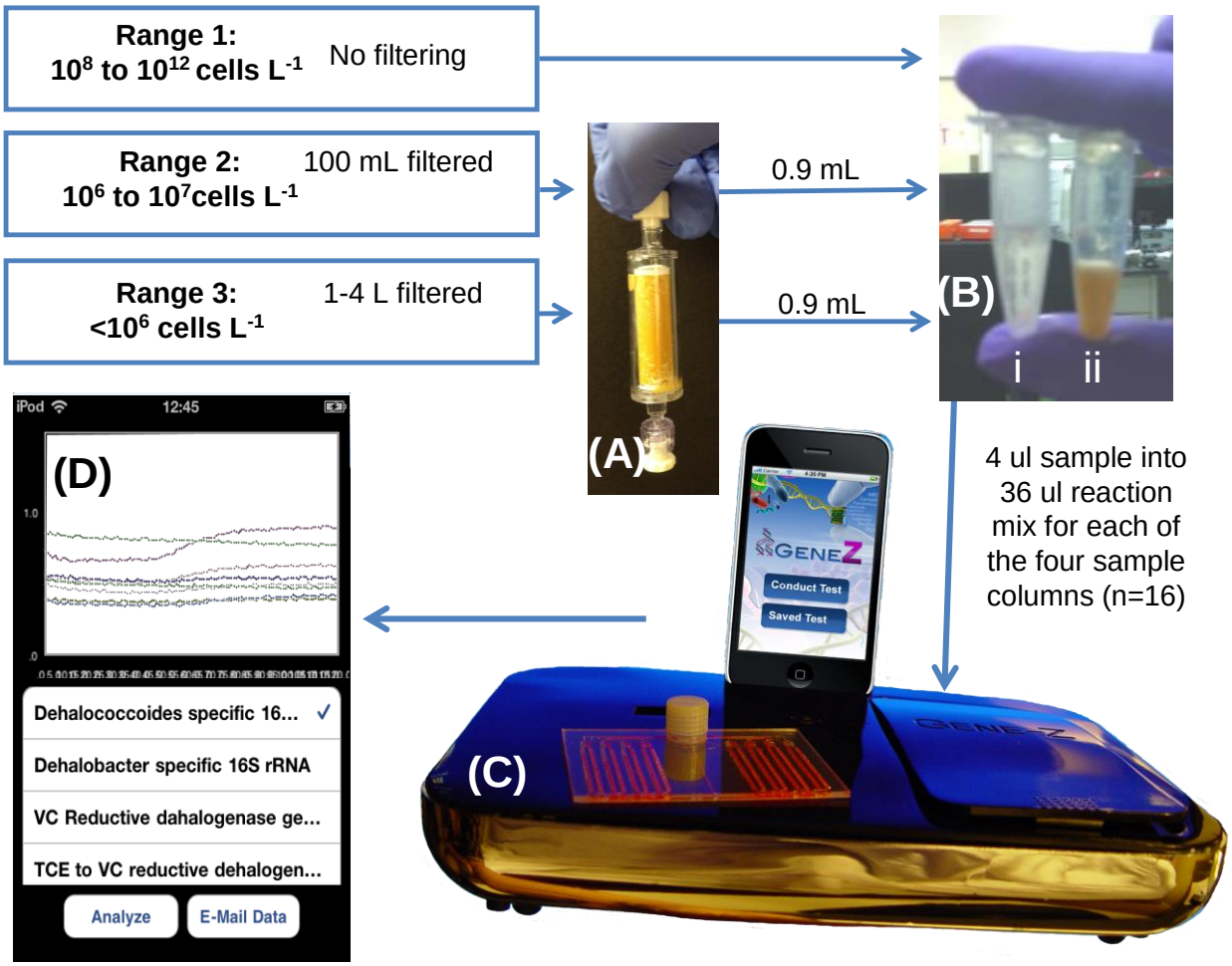

Figure 2.1. Range of concentrations detected using DHC consortium with and without A) Sterivex™ filter (pictured after filtering 100 mL of groundwater containing 269 mg L^{-1} of TOC), B) picture of HTOC sample (i) before and (ii) eluent after 100 mL of filtration, C) tested with the Gene-Z device prototype, and D) real time amplification profiles displayed on the iPod.

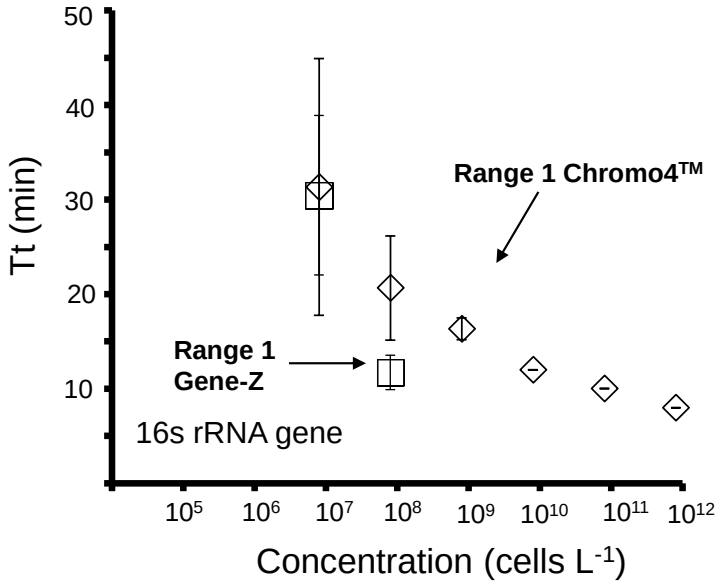

Figure 2.2. Threshold time for DHC dilutions tested with the 16S rRNA gene LAMP assay with the Chromo4™ (◊) and Gene-Z prototype (□). Concentrations lower than 10^7 cells L^{-1} did not amplify on both devices, and a concentration higher than 10^9 cells L^{-1} was not tested on Gene-Z device. Data represents average time to threshold for three or more wells and error bars represent standard deviation.

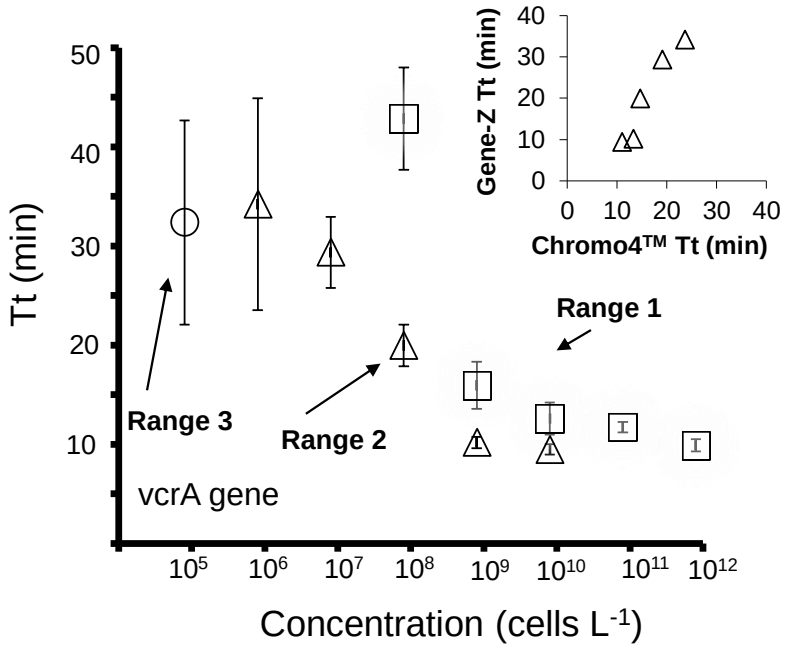

Figure 2.3. Cell concentration (cells L^{-1}) versus time to threshold (Tt) for varying dilutions of DHC cells tested for direct amplification with primers targeting the *vcrA* gene on the Gene-Z device. Tested samples included Range 1: not filtered (□), Range 2: 100 mL concentrated with Sterivex™ filters (Δ), and Range 3: 4 L concentrated with Sterivex™ filters (O). Error bars represent standard deviation of replicates. The inset shows Tt between the Gene-Z and Chromo4™ device for all tested Range 2 concentrations.

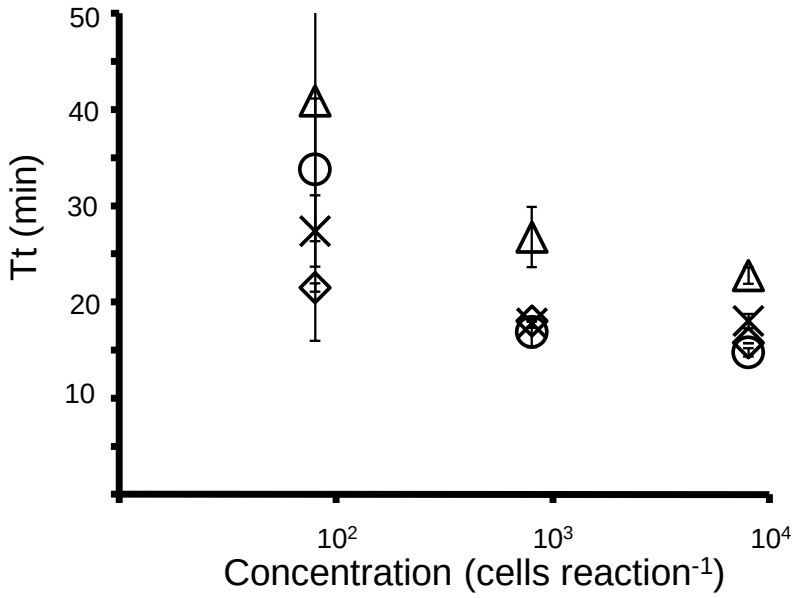

Figure 2.4. LAMP with *L. pneumophila* cells spiked directly into different water matrices including PCR grade water (X), filtered (passing 100 mL through Sterivex™ filter) HTOC groundwater sample (◊), unfiltered HTOC groundwater samples (Δ), and filtered LTOC groundwater sample (O). Error bars represent standard deviation of three replicates.

Chapter 3

Kanitkar, Y. H., Stedtfeld, R. D., Steffan, R. J., Hashsham, S. A. and A. M. Cupples. 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.

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* spp. in groundwater from chlorinated solvent contaminated sites. In this study, loop mediated isothermal amplification (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. Six 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, two commercially available bioaugmentation cultures, KB-1 and SDC-9 (both contain *Dehalococcoides* spp.). 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 hand held platform, the Gene-Z, and a real time thermal cycler using DNA isolated from eight groundwater samples obtained from a SDC-9 bioaugmented site (Tulsa, OK). These assays will be particularly useful at sites subject to bioaugmentation with these two commonly used *Dehalococcoides* spp.-containing cultures.

2. Introduction

Microbially mediated reductive dechlorination plays a vital role in the bioremediation of the chlorinated ethenes, tetrachloroethene (PCE) and trichloroethene (TCE). Under the appropriate

conditions, PCE and TCE undergo sequential reductive dechlorination via hydrogenolysis to *cis*-1,2-dichloroethene (cDCE) and vinyl chloride (VC), finally forming the non-toxic end product, ethene (ETH) [1]. When reductive dechlorination is linked to growth, it is called organohalide respiration; a metabolism commonly associated with microbial taxa like *Dehalococcoides* spp, and *Dehalobacter* spp. [2-9]. Commercially available reductive dechlorinating mixed cultures (e.g. KB-1 and SDC-9) containing such strains are frequently used for bioaugmenting contaminated groundwater aquifers [10-12]. In fact, in 2009 it was estimated that bioaugmentation with *Dehalococcoides* spp. had been used at several hundred sites in the US [13]. The growth of these strains in the field and in the laboratory is commonly monitored using real time quantitative PCR (qPCR) targeting the genes *vcrA, bvcA,* and *tceA,* which encode for distinct reductive dehalogenases (RDases) implicated in organohalide respiration [14]. To date, a number of qPCR protocols with DNA binding dyes or TaqMan probes to quantify *vcrA, bvcA,* and *tceA* genes have been developed [2, 15-17]. Although qPCR has been successful for monitoring reductive dechlorination, alternative methods would be advantageous for laboratories or practitioners without access to a real time thermal cycler. Also, any method that is more economical and faster compared to qPCR would be beneficial.

Loop mediated isothermal amplification (LAMP) is a novel molecular method recently developed for the specific detection of nucleic acids [18]. LAMP is a one step amplification reaction that amplifies a target DNA sequence using four to six primers. The *Bst* large fragment DNA polymerase has strand displacement activity and helicase-like activity allowing it to unwind and amplify DNA strands in the 60-65 ºC temperature range [18]. 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 (for review see [19]). 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* [20-22]. 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 [23, 24]**.**

In 2014, LAMP primer sets were developed for *vcrA* and the *Dehalococcoides* spp. 16S rRNA gene [25]. In that study, a field deployable approach for harvesting biomass from samples of groundwater bioaugmented 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). However, larger volumes of groundwater were required when *Dehalococcoides* spp. numbers were less than 10^4 gene copies/L**.**

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* spp. in KB-1 and SDC-9. Further, we used DNA templates isolated from eight groundwater samples to validate quantification of the developed assays with LAMP on the Gene-Z [25]. The data generated from the groundwater samples were also compared to data obtained using a real time thermal cycler.

3. Methods

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% CO_2/70% N_2 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 [1]. 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.

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 ºC 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 µL of 5 M NaCl and 3.0 mL of absolute ice-cold ethanol and incubating for 30 min at 4 ºC. Following centrifugation (14000 x g, 20 min, room temperature), the DNA pellet was rinsed with 70% ethanol, air dried and suspended in 100 µL of dH_2O. The extracted DNA was immediately used for amplification or stored at -20 ºC for future use.

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 [17]. Amplified templates were cloned into *E. coli* DH5α 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 ºC. 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 [17]. Serial dilutions of plasmid inserts from 3.16×10^8 plasmids to ~316 plasmids per µL for *vcrA*, 2.65×10^9 plasmids

to ~265 plasmids per µL for *bvcA*, and 1.41×10^{10} to 141 plasmids per µL 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.

3.4. Design of LAMP Primers

The LAMP primer sets used for this study are listed in Table 3.1. FASTA files for the functional RDase genes *vcrA* (Accesssion#NC_013552.1, region 1187298-1188857), *bvcA* (Accession# NC_009455.1 region 834959 to 836509), and *tceA* (Accession# AY165309.1) of *Dehalococcoides spp.* 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 [25]. 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}

3.5. qPCR and LAMP Amplification

Each 20µL 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 µL SYTO 82 orange fluorescent dye (Life Technologies, Inc., Grand Island, NY), 0.8 µL Pluronic (Life Technologies, Inc., Grand Island, NY), 0.8 µL Bovine Serum Albumin, 0.25 µM 10X Primer Mix and balance water to make up 18 µL. Reactions were incubated at 63 ºC for 60 min for amplification. All TaqMan assays were set up as 20 µL reactions. Each 20 µL reaction contained 10 µL iTaq Universal super mix supplied by Bio-Rad, 1.2 µL TaqMan probe described previously [15, 17, 26, 27], and balance water to make up 18 µL. 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 µL aliquots. All qPCR primers and probes used in this study are listed in

Table 3.2. 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.

3.6. 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 [28]. 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 CO_2 laser (Full Spectrum). Etched chips were cleaned and prepared as previously described [29]. Briefly, chips were cleaned with distilled water, soaked in 70% ethanol for 10 min, and dried for 10 min at 70 ^{o}C. Once dry, primers were dispensed and dried in wells at 70 ^{o}C for 5 min. Wells were enclosed with optical adhesive film (MicroAmp, Applied Biosystems) and chips were stored at -20 ^{o}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 μL 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.

4. Results

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 [25]. 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 compared to vcrA set B. For example, with $10^{3.5}$ gene copy templates, threshold times were 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* spp. 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 the specificity of the new primer sets were not evaluated experimentally, LAMP reactions requires six different primers for amplification are thus are unlikely to produce false positives.

4.2. Monitoring *Dehalococcoides* spp. 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 3.1.). 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* spp. 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 3.2. 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 ~5.8×10^6 gene copies/L on day 7 to ~6.4×10^9 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 ~6.4×10^9 gene copies/L to ~1.1×10^{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 ~1.4×10^8 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 ~3.8×10^8 gene copies/L on day 44.

Similarly, the growth of *Dehalococcoides* spp. 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 3.3. The bars represent standard deviation from the mean values. Rapid reductive dechlorination of ~24 µmol VC from day 20 to day 40 was coupled to the stoichiometric accumulation of ETH. Figure 3.4. 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 ~ 9.0×10^7 gene copies/L. As VC rapidly dechlorinated to ETH, we observed that the *vcrA* gene copies increased from ~ 9.0×10^7 gene copies/L to ~1.1×10^9 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 3.5.). 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* spp. in commercial reductive dechlorinating cultures, the absolute quantification of each gene in KB-1 templates was compared using both methods. Figure 3.6. 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.

4.3. Validation of New LAMP assays with the Gene-Z using Groundwater Templates

Nucleic acids extracted from groundwater from a previously bioaugmented, 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 3.7.). Again, the dashed line represents 1:1 slope.

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 [30, 31]. LAMP was also applied to detect *Cryptosporidium* oocysts, which cause cryptosporidiosis, using functional gene *gp60* [32]. Similarly, LAMP primer sets for detection of viral pathogens such as HIV-1 and Ebola have also been described [23, 24]. 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 [33]. 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* spp. 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 [34]. Typically, the individual cells of such strains carry one copy of *vcrA* or *bvcA* genes that code for the two distinct vinyl chloride reductases [35-37]. 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, 6, 9]. 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 [38]. 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 also carry *vcrA* or *bvcA* genes [6, 8, 17]. Additionally, TCE dechlorination may also be driven by *Geobacter* strains in KB-1 along with *Dehalococcoides spp.* [39], 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.

Overall, similar values were obtained for each gene on both platforms, indicating quantification with LAMP on the Gene-Z is a viable alternative to qPCR. For some data points, the Gene-Z yielded slightly higher values compared to qPCR, which may be attributed to the difference in fluorescence sensing mechanisms of the two platforms. This issue will be examined in more detail in future studies. 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 [25, 40-43]. 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 [25] , for the rapid detection and quantification of *Dehalococcoides* spp. in groundwater from contaminated solvent sites. Further, the development of LAMP assays specific to two commonly used commercially available cultures will facilitate specific detection of these RDase genes at sites subject to bioaugmentation.

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6. Tables and Figures

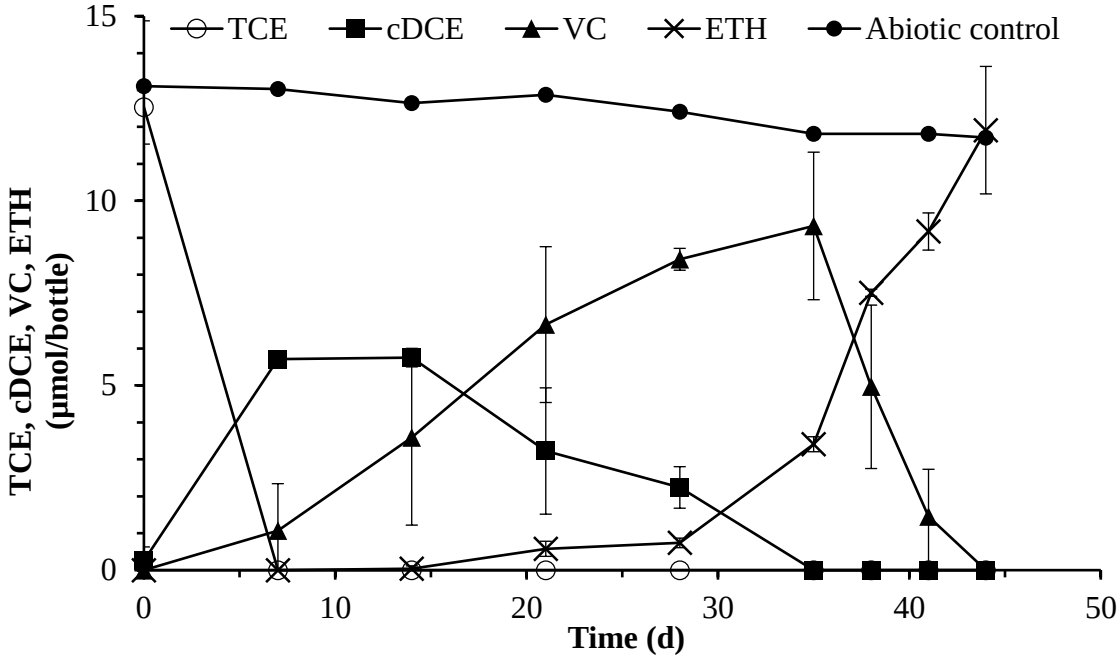


Figure 3.1. Mean mass of TCE, cDCE, VC, and ETH in triplicate KB-1 cultures and an abiotic control. The bars represent standard deviation from the mean values.

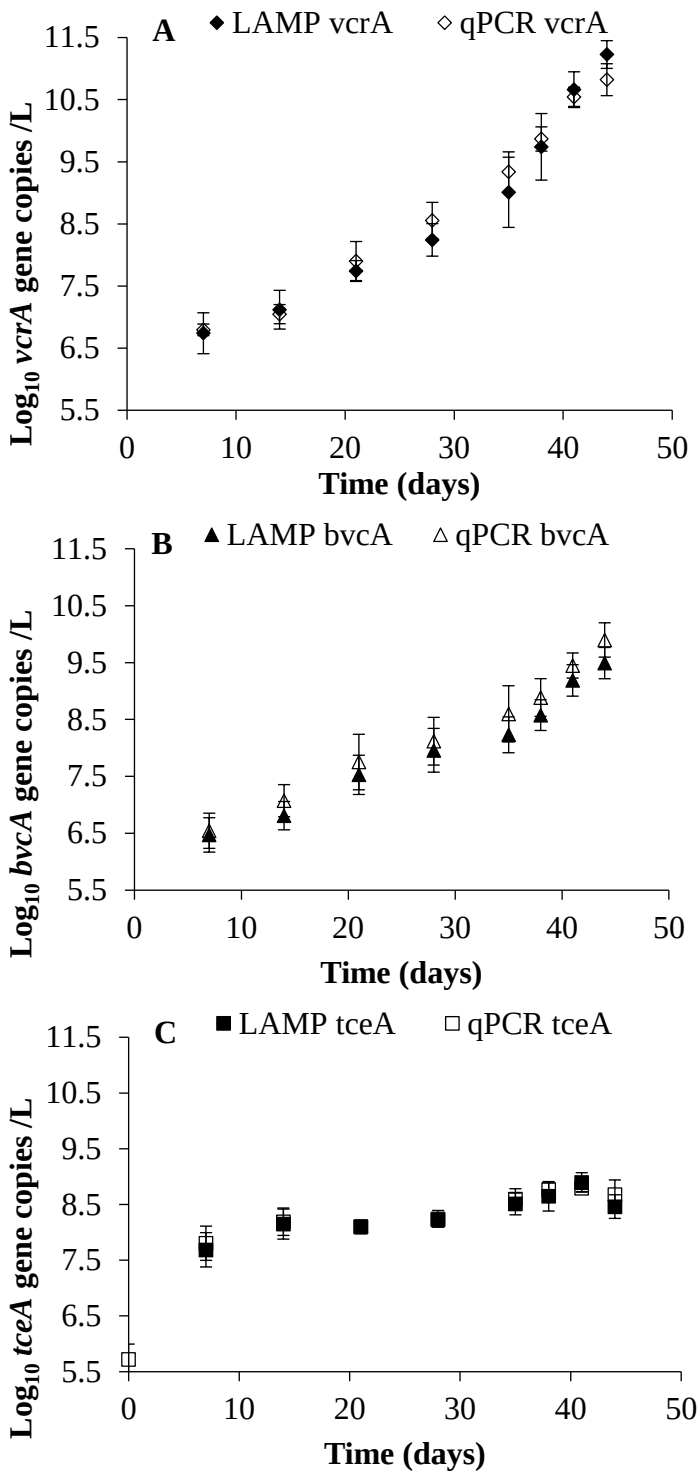


Figure 3.2. Gene copies of *vcrA* (A), *bvcA* (B), and *tceA* (C) per L in triplicate cultures of KB-1 while growing on TCE. LAMP vcrA set C was used to target *vcrA*.

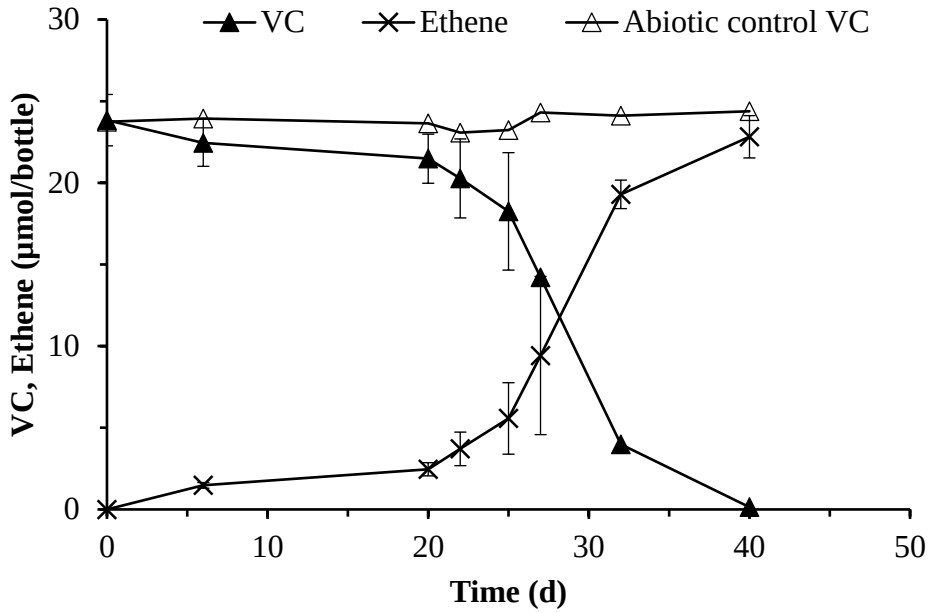


Figure 3.3. Mean mass of VC and ETH in triplicate SDC-9 cultures and an abiotic control. The bars represent standard deviation from the mean values.

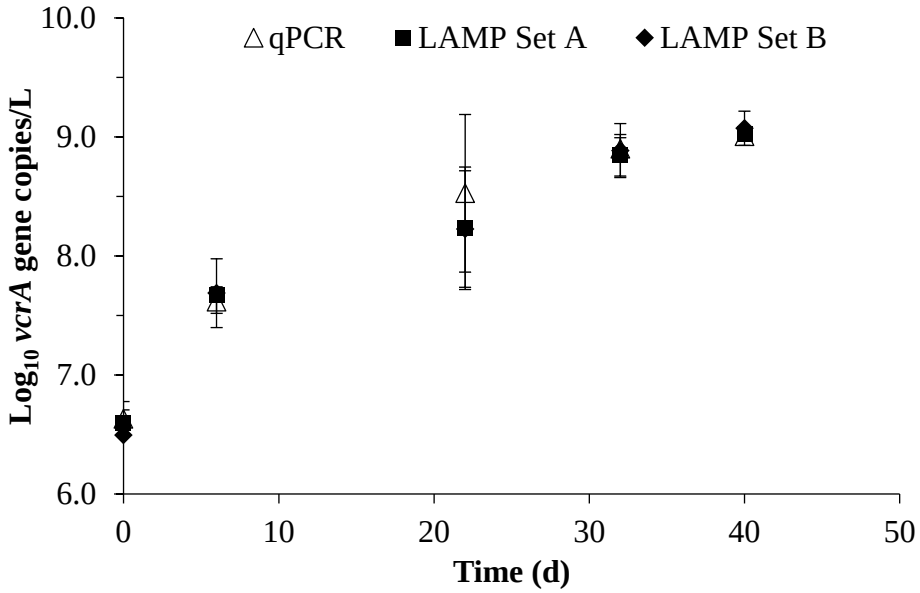


Figure 3.4. *vcrA* gene copies per liter measured via qPCR and two LAMP assays (vcrA set A and vcrA set B) in triplicate cultures of SDC-9 (A, B, C) during growth on VC.

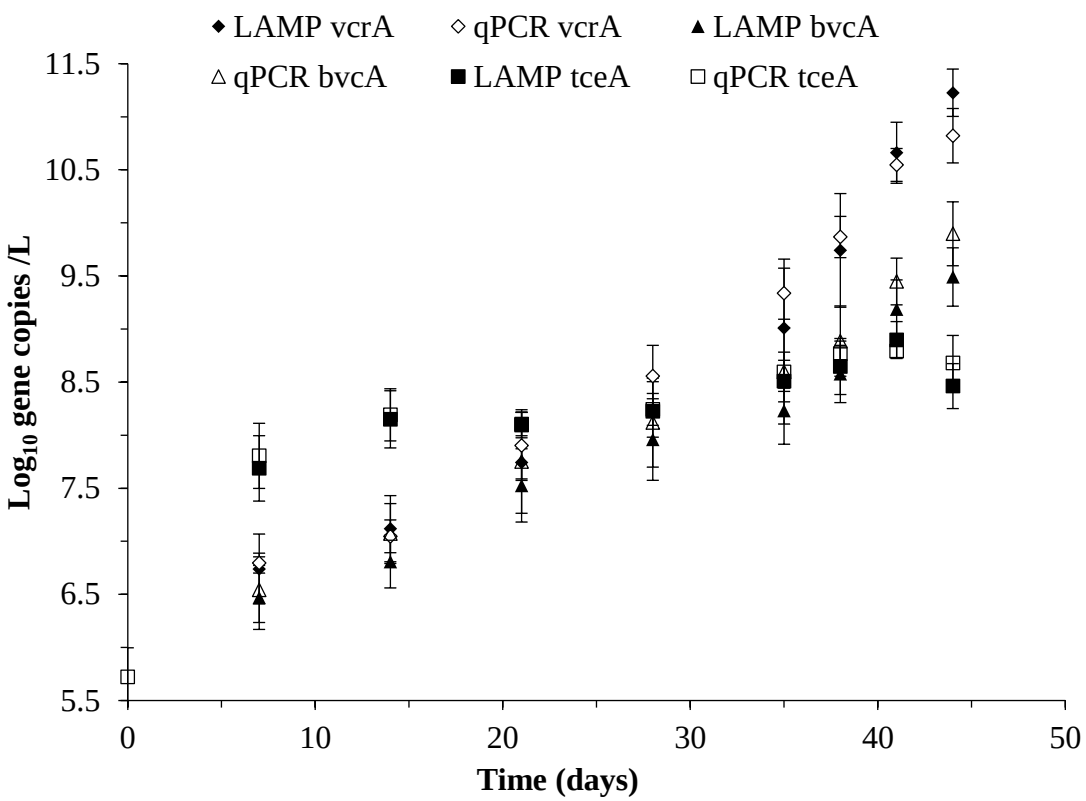
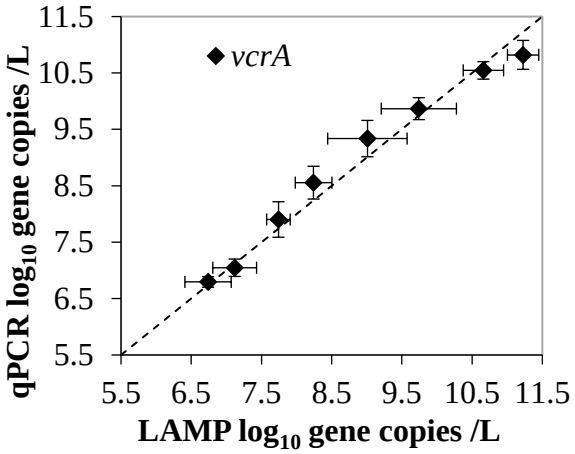
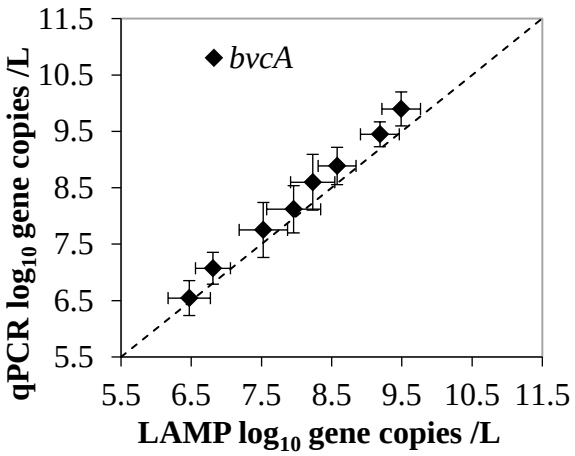


Figure 3.5. Mean gene copies of *vcrA* (A), *bvcA* (B), and *tceA* (C) per L in triplicate cultures of KB-1 while growing on TCE. The bars represent standard deviations from the mean values. Note: the y-axis is a log scale and does not start at zero.





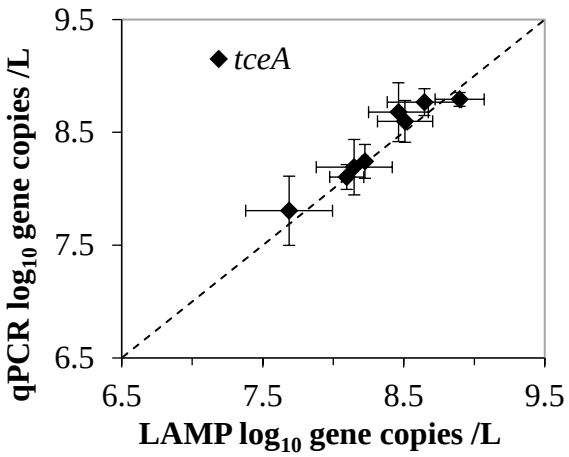


Figure 3.6. Comparison of *vcrA* (A), *bvcA* (B), *tceA* (C) mean gene copies (per L) in triplicate cultures of KB-1 while growing on TCE. The bars represent standard deviations from the mean values. The dashed line represents 1:1 comparison.

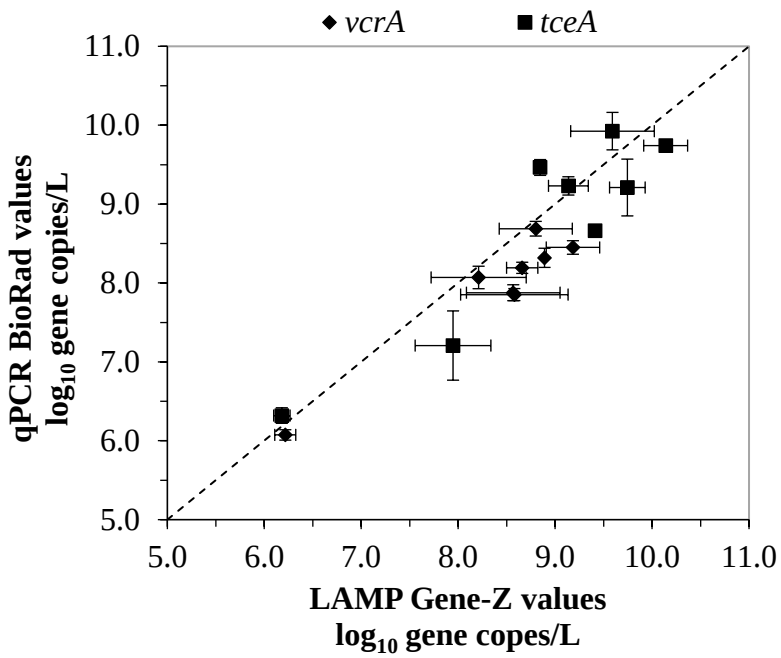


Figure 3.7. Comparison of *vcrA* and *tceA* mean gene copies (per L) in triplicates or eight different groundwater DNA templates observed using qPCR on real time thermal cycler and the Gene-Z. The dashed line represents 1:1 comparison.

Table 3.1. LAMP primers used in this study.

Target Gene	Primer set	Primer	Sequence (5'→3')	Target region (bp)
vcrA	vcrA set A	F3	GTAAGTTTTACGCGAGATGG	*Accesssion# NC_013552.1, region 1187298-1188857* (857-1072)
		B3	GTCATCGGCTGAGCTTTC	
		FIP	ACCCTCCCATTTTGGTACGCTTGTA TGGTCCGCCACAT	
		BIP	AAGACAATTTTCTAATGCTGAGGGC ATTTGGGATCTGCCAGGT	
		LF	CATCAGGTGGCGCTGAATC	
		LB	AGCTGCAAAATATTTTGGTGCTGG	
	vcrA set B [25]	F3	ACTAATATATAAGAAAGCTCAGCC	*Accesssion# NC_013552.1, region 1187298-1188857* (652-886)
		B3	TCTTATTGAGTTCTTGTGGTTG	
		FIP	GGTCAGGAACCTTGGGATAAATTTT GATGACTCTAGGAAAAGGAACA	
		BIP	AACTTTAAGGAAGCGGATTATAGC TATGGATTCACACTTTGTTGG	
		LF	CCTGGTCCACCTAATTCACTGTA	
		LB	ACTACAATGATGCAGAGTGGGTTA	
	vcrA set C	F3	GTAAGTTTTACGCGAGATGG	*Accesssion# NC_013552.1, region 1187298-1188857* (857-1072)
		B3	GTCATCGGCTGAGCTTTC	
		FIP	ACCCTCCCATTTTGGTACGCTTGTA TGGTCCGCCACAT	
		BIP	AAGACAATTTTCTAATGCTGAGGGC ATTTGGGATCTGCCAGGT	
		LF	CCATCAGGTGGCGCTGAA	
		LB	TGGTGCTGGTGGCGTT	
bvcA	bvcA Set A	F3	ACAATGCCTTTACCAGAAGA	*Accession# NC_009455.1 region 834959 to 836509* (895-1139)
		B3	ACCGTATTTGGGGCTGAT	
		FIP	TCGGCCTCCATTAAAAGCCATTCTC TAGGGTGGTCATGT	
		BIP	ATCAAGGACTTGGTGGCGACCTTGT TCGGAAAGACACTCA	
		LF	AGGCAATCATACTTGAAGCGTC	
		LB	TGTGGGGACCTGGTGGT	
tceA	tceA Set A	F3	GCCGTTTATTCCATTCATGG	Accession# AY165309.1 (882-1156)
		B3	GCATAGACTGGATGAAGGAA	
		FIP	ACATAATTGCTGGGAGAACCCG-TCGCATAGAGAGATAAGGCC	
		BIP	GCCATTCGTGGCGGCATATAT-CAGATTATGACCCTGGTGAA	
		LF	CTTTATGGACGCTATGAAGGTTCTA	
		LB	TCTTCCCTGCGGTCGCCATA	

Table 3.2. qPCR primers used in this study.

Target Gene	Primer	Sequence	Reference
vcrA	vcrA1022F	CGGGCGGATGCACTATTTT	[17]
	vcrA1093R	GAATAGTCCGTGCCCTTCCTC	[17]
	vcrA1042Probe	FAM-CGCAGTAACTCAACCATTTCCT GGTAGTGG-TAMRA	[17]
bvcA	bvcA925F	AAAAGCACTTGGCTATCAAGGAC	[17]
	bvcA1017R	CCAAAAGCACCACCAGGTC	[17]
	bvcA977Probe	FAM-TGGTGGCGACGTGGCTATGTGG-TAMRA	[17]
tceA	tceA1270F	ATCCAGATTATGACCCTGGTGAA	[26, 27]
	tceA1336R	GCGGCATATATTAGGGCATCTT	[26, 27]

Chapter 4

Stedtfeld, R. D., T. M. Stedtfeld, F. Samhan, Y. H. Kanitkar, P. B. Hatzinger, A. M. Cupples, and S. A. Hashsham. 2016. Direct loop mediated isothermal amplification on filters for quantification of *Dehalobacter* in groundwater. Journal of Microbiological Methods. 131: 61-67.

1. Abstract

Nucleic acid amplification of biomarkers is increasingly used to monitor microbial activity and assess remedial performance in contaminated aquifers. Previous studies described the use of filtration, elution, and direct isothermal amplification (i.e. no DNA extraction and purification) as a field-able means to quantify *Dehalococcoides* spp. in *groundwater*. This study expands previous work with direct loop mediated isothermal amplification (LAMP) for the detection and quantification of *Dehalobacter* spp. in groundwater. Experiments tested amplification of DNA with and without crude lysis and varying concentrations of humic acid. Three separate field-able methods of biomass concentration with eight aquifer samples was also tested, comparing direct LAMP with traditional DNA extraction and quantitative PCR (qPCR). A new technique was developed where filters were amplified directly within disposable Gene-Z chips. The direct filter amplification (DFA) method eliminated an elution step and provided a detection limit of 10^2 *Dehalobacter* cells per 100 mL. LAMP with crudely lysed *Dehalobacter* had a negligible effect on threshold time and sensitivity compared to lysed samples. The LAMP assay was more resilient than traditional qPCR to humic acid in sample, amplifying with up to 100 mg per L of humic acid per reaction compared to 1 mg per L for qPCR. Of the tested field-able concentrations methods, DFA had the lowest coefficient of variation among *Dehalobacter* spiked groundwater samples and lowest threshold time indicating high capture efficiency and low inhibition. While demonstrated with *Dehalobacter*, the DFA method can potentially be used for a number of applications requiring field-able, rapid (<60 min) and highly sensitive quantification of microorganisms in environmental water samples.

2. Introduction

The widespread contamination of groundwater aquifers with chlorinated solvents throughout the US and abroad has resulted in a number of treatment strategies [1]. Metabolic reductive dechlorination by *Dehalobacter restrictus* (*Dehalobacter* spp.) uniquely position its use for bioremediation of groundwater co-contaminated with tetrachloroethene, trichloroethene and

dichloromethane [2, 3]. The assessment of remedial performance increasingly relies on enumeration via nucleic acid amplification. Quantitative PCR (qPCR) is routinely used to quantify genes associated with *reductive dehalogenation, however, this method* can be inaccurate due to inhibition in environmental samples [4-6] or target loss during DNA extraction [7] and sample transport. Studies have also described an underestimation of reductive dehalogenase genes measured with qPCR compared to other methods [8].

As previously reviewed [9], isothermal amplification techniques such as loop mediated isothermal amplification (LAMP) have many field-able and low cost [10] advantages, including being less susceptible to environmental inhibition compared to qPCR [4]. In addition, LAMP analysis is faster than qPCR because amplification can be accomplished without DNA extraction or using a crude lysate [11, 12]. Previous studies demonstrated LAMP assays for quantification of *Dehalococcoides spp. in groundwater [13]*. Our studies also examined a field-able means to concentrate biomass from groundwater via Sterivex cartridges and direct amplification from filtrate elution on a field-ready Gene-Z device [14]. While a detection limit of 10 to 20 copies per reaction was observed for the *Dehalococcoides* LAMP assay, sensitivity in water samples varied with volume of concentrated water. For example, a detection limit of 10^5 cells per L was obtained with filtration of 4 L using the filter elution method and 10^8 cells per L was observed with no filtration. The lack of sensitivity, while suitable for some applications, was due to an elution step to remove concentrated biomass from filters, and the inability of the eluted sample to constitute over 10% of the amplification reaction volume.

For instances requiring higher sensitivity, a previously undescribed method was explored in which filters used to concentrate biomass were placed into reaction wells of disposable Gene-Z chips for direct filter amplification (DFA). By avoiding the elution step, this technique reduced time to results by 10 min and increased sensitivity over 100 fold. LAMP assays targeting the 16S rRNA gene specific to *Dehalobacter* spp. and the previously identified putative 1,2-DCA reductive dehalogenase gene (*rdhA* gene) [15] were tested. Experiments were performed to compare direct LAMP with qPCR including: i) spiking reactions with various concentrations of humic acid, ii) spiking multiple groundwater samples with *Dehalobacter* (from a commercially available mixed culture currently used for bioaugmentation, TCA-20) following a concentration step, and iii) comparing methods for field-able target enrichment followed by direct isothermal amplification or DFA.

3. Methods

3.1. Design of LAMP Primers

Three primer sets were initially designed targeting both the 16S rRNA gene (U84497.2) and *Dehalobacter* reductive dehalogenase genes. Sequences available in public databases were downloaded, aligned, and LAMP assays were designed from consensus sequences using Primer Explorer V4 software. Loop primers were also designed to decrease time to positive amplification (T_t). BLAST analysis was performed to determine coverage of each primer set. Degenerate bases were added to primer sets targeting the *rdhA* gene, which totaled 16 base variations (Supplementary Table 4.1.). LAMP primers were also designed from the luciferase (*luc*) gene to serve as a microbial internal positive control [16]. Previously described qPCR assays targeting the *rdhA* gene [15] and *luc* gene were compared with the LAMP assays.

qPCR and LAMP experiments

LAMP experiments were tested under isothermal conditions in conventional vials in the real-time cycler (Chromo4, BioRad) or in the Gene-Z device [17] with disposable chips. LAMP reactions consisted of 1X isothermal amplification buffer (New England Biolabs), 1.4 mM each dNTP (Invitrogen), 0.8 M Betaine solution (Sigma Aldrich), 6 mM $MgSO_4$ (New England Biolabs), 8 U Bst Polymerase 2.0 WarmStart (New England Biolabs), 200 μM SYTO82 Orange Fluorescent Nucleic Acid Stain (ThermoFisher Scientific), template that constitutes 10% of the reaction volume (DNA, crudely lysed, or direct cells), and PCR grade water. All DNA extractions were performed using the PowerWater DNA Isolation Kit (12888-100, MoBio Laboratories, Inc). For DFA experiments, cells were captured by the filter and thus template was not added to the reaction. All experiments were performed with an isothermal incubation at 63°C for 60 min with plate reads at one minute intervals in the real-time thermal cycler, and every 16 sec in the Gene-Z device. LAMP in the real-time cycler and Gene-Z device had 10 μL and 25 μL reaction volumes, respectively.

QPCR was performed in 25 μL volumes with the following constitutes: 500 nM forward and reverse primers, specified mass of gDNA, and reagents from the Power SYBR Green PCR Master Mix (Life Technologies). Real-time reactions were run using the real-time cycler, which included a 10-min enzyme activation at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. All experiments included a no-template control and triplicate reaction vials.

Experiments to test sensitivity were performed with dilutions of targeted *rdhA* gene amplicon and gDNA extracted from TCA-20, a mixed dehalogenating culture that contains *Dehalobacter* spp. among other organisms (ENV-TCA20™, CB&I). The quantity of *Dehalobacter* cells in TCA-20 was provided by CB&I and verified in house by qPCR of extracted DNA. Amplicons were generated by PCR of the F3 and B3 primers. Triplicate PCR reactions were pooled and purified using the Qiagen PCR purification kit. The mass of amplicon after purification was quantified using Qubit. Amplicons were diluted and tested with their respective primer sets. Triplicate reactions were performed for each experiment with positive and no template controls.

The *rdhA* gene and 16S rRNA gene assays used for subsequent experiments were selected based on initial sensitivity and specificity tests. Dilutions of gDNA extracted from TCA-20 and purified amplicons were tested with six designed LAMP assays. Experiments were performed in the real time thermal cycler. Primers selected for further analysis (Supplementary Table 4.1.) amplified down to 20 copies tested with purified amplicon and 100 genomic copies tested with gDNA extracted from TCA-20. Direct LAMP with the *luc* gene assay amplified in all dilutions down to 5 CFU per reaction. Lower dilutions did not amplify.

Specificity tests on the LAMP assays were performed with gDNA (5 ng) from 12 type strains. The 16S rRNA gene of selected type strains had varying levels of sequence similarity to *Dehalobacter* (Supplementary Table 4.2.). Specificity experiments were run in the real-time cycler. For the *rdhA* gene, gDNA from the non-targeted organisms did not amplify within the 60 minute LAMP reaction. However, the selected 16S rRNA gene assay amplified with gDNA from *Syntrophobotulus glycolicus* (DSM 8271). Thus, the 16S rRNA gene LAMP assay for *Dehalobacter* should only be used in parallel with *rdhA* gene primer as a secondary or redundant verification of presence.

3.2. Direct LAMP and qPCR in Groundwater

For the experiments comparing heat lysed versus non-heat lysed *Dehalobacter* template, 1 mL of TCA-20 ($3x10^7$ cells per mL) was spiked into 99 mL of phosphate buffered saline (PBS) and six dilutions were prepared yielding 3×10^5 to 3 cells per mL. Samples were passed through Sterivex filters (SVGPL10RC, Millipore) to remove extracellular DNA, eluted from filters using 0.9 mL of elution buffer as previously described (Stedtfeld et al. 2014). Briefly, samples were eluted from Sterivex filters by adding an elution buffer, vortexing Sterivex cartridges at

minimum speed for 10 min, and collecting elution buffer with enriched biomass using a syringe. Following elution each dilution was separated into two vials and one of each vial was heat treated at 95^{o}C for 5 min to crudely lyse cells. Amplification reactions were performed in the real time cycler using the selected *rdhA* gene LAMP primer. Reactions were performed in triplicate with positive and no template controls. Using 1 μL of sample eluted from filters, and assuming 100% capture efficiency using Sterivex cartridges, LAMP reactions were run with dilutions of 3 x 10^4 to 0.3 cells per reaction.

Experiments included testing various concentrations of humic acid spiked into LAMP and qPCR assays with 500,000 copies of *rdhA* gene amplicon. In detail, humic acid (53680-10G, Aldrich) was spiked with concentrations of 0, 0.1, 0.3, 1, 3, 10, 30, 100, 300 mg per L. Based on results, subsequent experiments included testing six dilutions of *rdhA* gene amplicons (5 x 10^5 to 5 copies per reaction) with and without 30 mg per L of humic acid. Reactions were performed with isothermal conditions in a real time cycler in triplicate with positive and no template controls.

Experiments also examined the influence of seven enriched groundwater samples (from chlorinated solvent impacted sites) on direct LAMP and qPCR. In summary, 250 mL of sample was passed through a Sterivex filters (SVGPL10RC, Millipore) for testing direct amplification, and another 250 mL was concentrated via vacuum through a 47 mm diameter 0.22 μm filter (GSWG047S6, Millipore) for testing with DNA. Genomic DNA was extracted from vacuum filtered samples, and Sterivex filters were eluted as previously described [14]. Direct LAMP and qPCR reactions were spiked with 1 μL of TCA-20 (3 x 10^7 *Dehalobacter* cells per mL) to yield 3 x 10^5 cells per reaction; and 0.4 μL of concentrated groundwater. For experiments with DNA, 5 ng of genomic DNA from TCA-20 was spiked into 2 ng of genomic DNA extracted from groundwater samples.

To test sensitivity of the DFA method, prepared dilutions of TCA-20 were spiked into 100 mL of sterile water yielding 10^4 to 0.1 cells per mL. Three replicate dilutions were passed through individual filters and all three filters along with a no template control were all run on a single Gene-Z chip (described below). The DFA method was performed with polycarbonate 13 mm diameter Isopore hydrophilic membrane filters with 0.45 μm pore size, track-etched black screen filter (HTBP01300, Merck Millipore Ltd). The filters were placed into autoclaved semi-clear polycarbonate reusable holders with silicone gaskets (EW-29550-40, Cole-Parmer).

Holders were attached to autoclaved 140 mL syringes with luer lock connections. Filter and syringe setup were sealed to a Büchner flask with parafilm to create a vacuum that pulled water samples through filters. After all water had passed, filters were removed from the reusable holders and placed into disposable Gene-Z chips. Reaction wells were sealed with clear optical film (MicroAmp, Applied Biosystems). LAMP reaction constitutes were dispensed into chips (300 μL per reaction well) via pipettes and run immediately following filtration. All DFA experiments were run with the LAMP assay targeting the *rdhA* gene.

For experiments testing various filtration methods, eight additional groundwater samples were collected from remediation sites. TCA-20 (1.25 mL) was spiked into each of five separate 200-mL groundwater samples to yield 2 x 10^5 *Dehalobacter* cells per mL. Samples were also spiked with a microbial internal amplification control (MIAC) [16] to yield 2 x 10^6 CFU MIAC per mL. Cultures of *Escherichia coli* clones with the luciferase gene (*luc* gene), used for the MIAC, were provided by Dr. Frank Löffler (University of Tennessee, Knoxville). Cultures of MIAC were grown overnight at 37 ºC in LB medium amended with 50 mg per mL ampicillin, and counted by dilution plating. Spiked groundwater samples were concentrated using one of five methods including i) no enrichment (Direct), (ii) Sterivex filtration and elution as previously described (Ster.), iii) a two tiered filter train that included a 5 micron filter (SLSV025LS, Millipore) followed by Sterivex filtration and elution as previously described (5+Ster.), iv) vacuum filtration and DNA extraction from filters (gDNA), and v) direct filter amplification after pre-filtration through 5 micron filter units (5+DFA). Following enrichment, samples were tested for qPCR in a real time cycler or LAMP on the Gene-Z device. Extracted gDNA was diluted to 5 ng per μL concentrations, and all qPCR and LAMP experiments with non lysed samples or DNA were run with sample constituting 10% of amplification reaction volume.

Gene-Z device and chips

The previously described Gene-Z device [17] was used for real-time LAMP of *Dehalobacter* spiked into groundwater samples. Disposable chips were laser etched (3rd Gen 40W laser, Full Spectrum Laser) into 1/16 inch black acrylic sheets (24112-07, Inventables), cleaned with distilled water, soaked in 70% ethanol for 5 min, dried with compressed air, and enclosed on one side using clear optical film (MicroAmp, Applied Biosystems). Primers were dispensed using a pipette, dried at 70 ºC for 10 min, enclosed on the top-side using MicroAmp tape, and chips were stored as previously described [18]. The chip configuration consisted of eight

reaction wells per sample lane, and four sample lanes per chip (i.e. 32 reaction wells per chip, each with 20 μL reaction volume), as previously described [13]. Primers targeting *rdhA* gene and 16S rRNA gene were each dispensed into three separate reaction wells per sample lane, and primers targeting the MIAC were dispensed into two reaction wells.

For DFA experiments, 1/16 inch black acrylic plastic was cut to have 4 reaction wells per chip. Wells were cut with 18 mm diameter for placement of 13 mm diameter DFA filters. The size of each well permitted six sensors (LEDs/optical fibers) within the Gene-Z device to monitor reactions in real-time (Figure 4.1.). Reaction wells had a tear-drop shape on both the entrance and exit for loading with minimal bubble formation (Figure 4.1C). DFA chips were designed so that three replicates and one negative control could be tested per chip for a single primer set. Each reaction well had a loading port and air vent that were sealed with clear optical film (MicroAmp, Applied Biosystems) after reaction mixes were dispensed.

Data analysis was performed as previously described [18] to calculate the threshold time (T_t) of LAMP, akin to threshold cycle (C_t). The signal to noise ratio (SNR) was calculated as the signal at a given time minus the median signal at the start of the reaction, divided by the standard deviation of the signal at the start of the reaction. For DFA experiments, the average T_t of all six sensors monitoring a single reaction well were used for analysis. A student T-test was used to test for significant differences between tested methods ($p < 0.05$).

4. Results and Discussion

4.1. Direct Amplification of *Dehalobacter*

Heat treatment of *Dehalobacter* prior to LAMP had no observable influence in T_t or sensitivity (Figure 4.2A) compared to direct amplification without lysis. Regardless of lysis, amplification was only observed in the highest tested dilutions (3×10^4 to 3×10^2 cells per reaction) and no amplification was observed in lower dilutions. Results showed no significant difference ($p < 0.05$) in sensitivity or T_t between crudely heat lysed and non-lysed dilutions of *Dehalobacter*.

The LAMP assays were less susceptible to inhibition by humic acid than qPCR, amplifying with up to 100 mg per L of humic acid per LAMP reaction compared to only 1 mg per L for qPCR (Figure 4.2B). Furthermore, the LAMP assay did not show a significant change in T_t for humic acid concentrations below 100 mg per L in the reaction. A dilution series of *rdhA* gene amplicon was also spiked with and without 30 mg per L of humic acid to test for inhibition

with lower target concentrations. Results showed that there was a slight shift in T_t, but only with the lowest number of copies showing amplification (50 copies per reaction). All other dilutions had no significant difference between reactions with and without humic acid (Figure 4.2C). Dilutions below 50 copies per reaction did not amplify with or without humic acid.

The influence of humic acid on qPCR and LAMP assays targeting the amoA gene was previously reported [19]. Samples were tested with 0-8 mg per L of humic acid, which had a concentration dependent influence on T_t with turbidity based LAMP. qPCR reactions tested in Tani's study failed to amplify with humic acid concentrations above 4 mg per L. Experiments described here were to examine inhibition with reagents selected/optimized for genetic testing of *Dehalobacter* with the Gene-Z device.

4.2. qPCR and LAMP in groundwater samples spiked into filtered samples

Direct LAMP of *Dehalobacter* assays was less influenced by inhibition in groundwater samples (collected from remediation sites) compared to qPCR. In detail, TCA-20 culture was added to seven groundwater samples following elution from Sterivex cartridges , yielding 3×10^5 *Dehalobacter* cells per reaction. Of note, amplification of the *rdhA* gene or *Dehalobacter* specific 16S rRNA gene was not observed in qPCR or LAMP reactions in non-spiked groundwater samples (genomic DNA, or direct cells). Four of the groundwater samples spiked with TCA-20 inhibited qPCR while none of the samples inhibited LAMP to the extent that amplification did not occur (Figure 4.3A). Six out of seven spiked groundwater samples had a T_t similar to the LAMP reaction run without groundwater. Sample MI315, which contained the highest concentration of suspended solids (Supplementary Figure 4.2.) had a shifted T_t, compared to the other six groundwater samples tested with direct LAMP. Excluding the MI315 sample, the T_t coefficient of variation (CV) was 9% among the seven groundwater samples tested with LAMP *rdhA* gene and 16S rRNA gene assays. The CV among qPCR-based C_t was also 9% between samples that amplified with qPCR. Subsequent experiments (described in next section) tested the use of a 5 micron filter prior to enrichment and DFA to reduce suspended solids in groundwater samples (e.g. MW315).

All groundwater samples spiked with gDNA extracted from TCA-20 amplified with qPCR and LAMP assays. Inhibition was not observed, as the C_t and T_t CV between all seven samples was 2%, 3%, and 6% for qPCR (*rdhA* gene), LAMP (*rdhA* gene), and LAMP (16S rRNA gene) assays, respectively (Supplementary Figure 4.1.).

4.3. Direct filter amplification (DFA) with Gene-Z

For DFA experiments, 100 μL of serially diluted TCA-20 was spiked into 100 mL of sterile distilled water, filtered, and analyzed on the Gene-Z device for the *rdhA* gene. Amplification was observed down to 10^2 *Dehalobacter* cells spiked into 100 mL. Amplification was not observed in controls run without TCA-20 spikes or lower tested dilutions (Figure 4.3B). At lower dilutions (e.g. 10^2 cells spiked into 100 mL), increased fluorescence due to an amplification event was initially observed on one side of the filter, and this fluorescence diffused across the reaction well temporally, varying the T_t among the six sensors used to monitor each reaction well (Figure 4.3C). As such, the mean T_t among the six sensing spots was reported for all DFA chips (Figure 4.3B, 4.4A, 4.4D).While not transparent, filters were translucent allowing a mean value of 13 $\pm$ 5% of green LED light to pass through the reaction (captured by the Gene-Z photodiode). Lower fluorescent signals caused by the filters did not influence T_t. More translucent filters that allow for greater transmission of light are currently being explored.

The DFA method was also tested to enrich for low target concentrations in environmental waters. During our previous study with Sterivex filters, a minimum detection limit of 10^5 *Dehalococcoides* cells per L [14] was observed with filtration of 4 L of water. Demonstrated with filtration of 100 mL in this current study, the lowest sensitivity observed with DFA exceeds this 100 fold. While tested with different microbial targets (e.g. *Dehalococcoides* versus *Dehalobacter*), LAMP assays for both targets had similar sensitivity (10 to 100 gene copies per reaction depending on the assay tested). Placement of filters directly onto Gene-Z chips also reduced the time required for an elution step (10-15 min), which required an elution buffer stored at 4 ^{o}C and a means to vortex the Sterivex cartridges. Thus, DFA may provide a field-able alternative for biomass enrichment of water for genetic diagnostics.

4.4. Sample Concentration Methods

Five methods of biomass concentration prior to LAMP on the Gene-Z device or qPCR in a conventional real-time cycler were tested in eight groundwater samples spiked with 2 x 10^6 CFU per mL of MIAC and 2 x 10^5 *Dehalobacter* per mL from TCA-20. Since the same amount of bacterial target was added to all eight groundwater samples, the ideal method of sample processing will have a minimum Tt and Ct CV among samples. Overall, the 5+DFA method tested on the Gene-Z device and gDNA tested with qPCR both had the lowest T_t and C_t CV among the eight different groundwater samples. A CV of 5.6% was measured for DFA and 3.8%

for qPCR of gDNA, both tested with the *rdhA* gene assay (Figure 4.4B-F). However, *Dehalobacter* genomic copies measured with qPCR of gDNA was underestimated in all but one of the groundwater samples (24 $\pm$ 40% estimated to actual *rdhA* gene copies). The DFA method appeared to be less influenced by inhibition since all groundwater had been removed, providing estimates that were closer to the actual amount of spiked *Dehalobacter* cells. Overestimation was observed in groundwater sample MW9 run with DFA. Compared to all other methods, the DFA method tested with *rdhA* gene assay provided the lowest T_t, higher estimated yield compared to qPCR, and lowest CV among the eight groundwater samples.

Sterivex with LAMP assays also had lower T_t, however, the higher CV compared to DFA method indicates more variability in biomass loss. In all three LAMP assays, pre-filtration with the 5 micron filter slightly increased the average T_t (18.5 $\pm$ 2.3 min for the *rdhA* gene) compared to sole filtration with Sterivex (17.7 $\pm$ 0.9 min for the *rdhA* gene). This indicates a slight loss of *Dehalobacter* cells using the 5 micron filters. Interestingly, C_t values were lower for qPCR experiments run with the 5 micron pore filter, which we surmise is due to higher level of inhibition in samples without pre-filtration. The level of suspended solids was lower when the 5 micron filtration step was implemented (Supplementary Figure 4.2B, C). Both C_t and T_t values were highest in non-enriched (direct) samples as biomass was not concentrated.

To our knowledge, no previous study has described a method similar to DFA with a field-able device such as the Gene-Z for remediation applications. A manuscript is also in preparation by our group describing real-time DFA for detection of *Legionella pneumophila* in water samples, monitored in real-time using a camera and the Gene-Z device [20]. A handful of studies have described lab on chip devices in which samples are automatically processed and crude or purified lysate is amplified isothermally (see reviews by [21, 22]. However, the DFA method is better suited for 1) genetic diagnostics applications that require greater sensitivity when organism/gene concentrations are low, and 2) potential use outside of the laboratory via direct amplification without DNA extraction and purification. The DFA method could also potentially be used in other field-able devices, in which filters are placed directly into amplification vials.

Limitations of the DFA method include the inability to test multiple assays in parallel, which would require a setup for enriching and handling of numerous smaller filters. Used with Gene-Z chips, the current configuration also requires 300 μL per reaction well; however, chips etched into thinner pieces of acrylic would reduce volumes. The smaller 13 mm diameter filter

may also have reduced filtration capacity compared to Sterivex and 47 mm filters, thus requiring the prefiltration step with 5 micron syringe filters. For some organisms and targets, direct LAMP amplification without DNA lyses may cause reduction in quantitative capacity or sensitivity. For example, previous studies with *Staphylococcus aureus* observed a 1 min difference between crudely lysed and non lysed cells (Kostic et al., 2015). Perhaps one of the greatest limitations of LAMP is increased possibility of contamination to subsequent reactions, due to the large concentration of generated amplicons. As such, proper handling of chips and vials is critical to ensure vials or chips remained unopened following an amplification event.

Genetic techniques such as qPCR have proven extremely useful in accessing remedial performance in contaminated sites. The development of LAMP assay targeting *Dehalobacter* will also allow for specific detection of samples from bioaugmented sites. Minimal sample processing associated with the DFA method can potentially reduce costs and time for detection, and compliments the use of the field-able real-time tools such as the Gene-Z device. The DFA technique facilitates detection of *Dehalobacter* far below the accepted limit for natural attenuation (10^7 gene copies per L).

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5. Tables and Figures

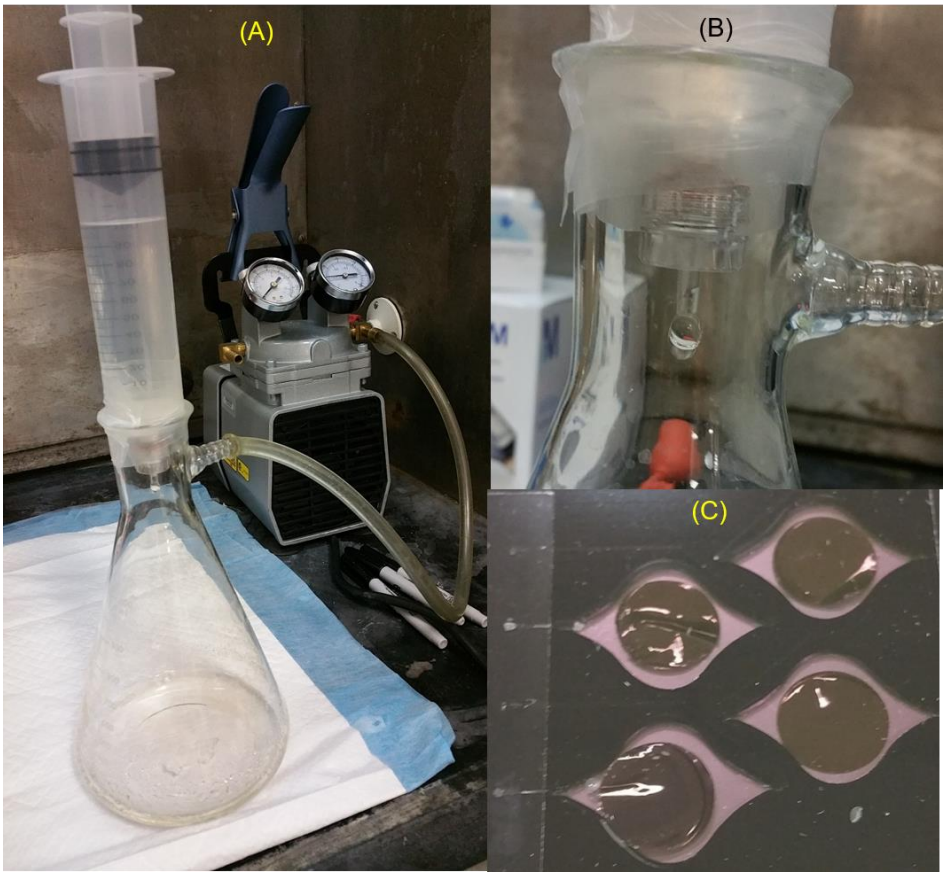

Figure 4.1. Pictures of filter apparatus used for DFA: A) placement of 140 mL syringe into Büchner flask, B) close up picture shows reusable filter holder with vacuum pressure retained with parafilm wrapped between the syringe and Büchner flask, and C) disposable Gene-Z chips with 13 mm filters placed into four individual reaction wells, enclosed with optical adhesive, and loaded with LAMP reagents.

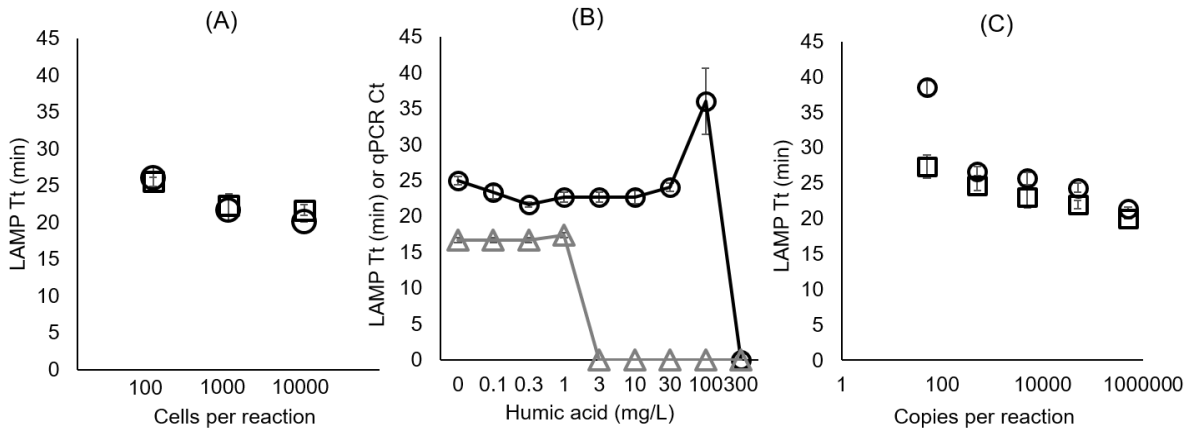

Figure 4.2. Testing direct amplification and inhibition with *rdhA* gene LAMP assay. A) T_t for dilution series of TCA-20 tested with heat lysis at 95 ºC for 5 minutes (open circles) and without lysis (open squares). X-axis is theoretical number of cells per reaction after filtering 100 mL of dilutions through Sterivex cartridges (to eliminate extracellular gDNA) and 0.9 mL of elution. B) 500,000 copies of *rdhA* gene amplicons with LAMP (circles, T_t) and qPCR (triangles, C_t) assays spiked with varying concentrations of humic acid. Points at 0 indicate less than two of three technical replicates amplified with the specified concentration. C) Six dilutions of *rdhA* gene amplicon with (circles) and without (squares) humic acid spiked at 30 mg per L in the LAMP reaction. Dilutions lower than 50 *rdhA* gene copies per reaction did not amplify with or without humic acid. Error bars represent standard error of three reaction vials.

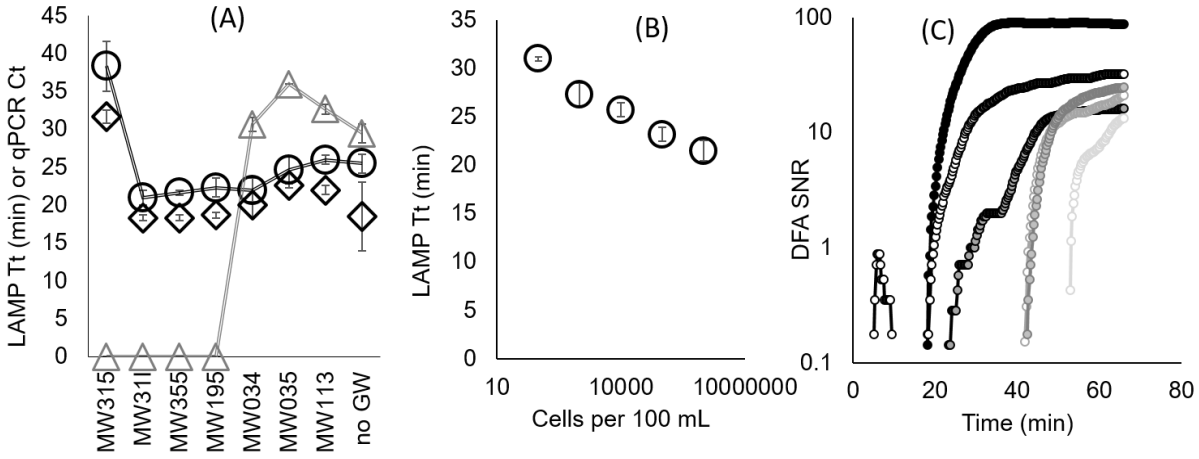

Figure 4.3. Direct LAMP in groundwater samples and testing DFA method. A) Sterivex enriched groundwater samples spiked with 3 x 10^5 *Dehalobacter* cells per reaction from TCA-20 following elution from cartridges; and tested for LAMP assays targeting *rdhA* gene *(*circles), 16S rRNA gene (diamond), and qPCR assay targeting *rdhA* gene (triangles). Points at 0 indicate less than two of three technical replicates amplified with the specified sample. B) Dilutions of TCA-20 spiked into 100 mL of sterile water and tested using the DFA method with the Gene-Z device. C) Signal to noise ratio (SNR) observed throughout a DFA reaction for all six sensors monitoring a single reaction well loaded with a filter used to concentrate 10^2 cells per 100 mL. This figure demonstrates how amplified product diffuses across the surface of the filter during the reaction. Error bars in (A-B) represent standard error of three technical replicates.

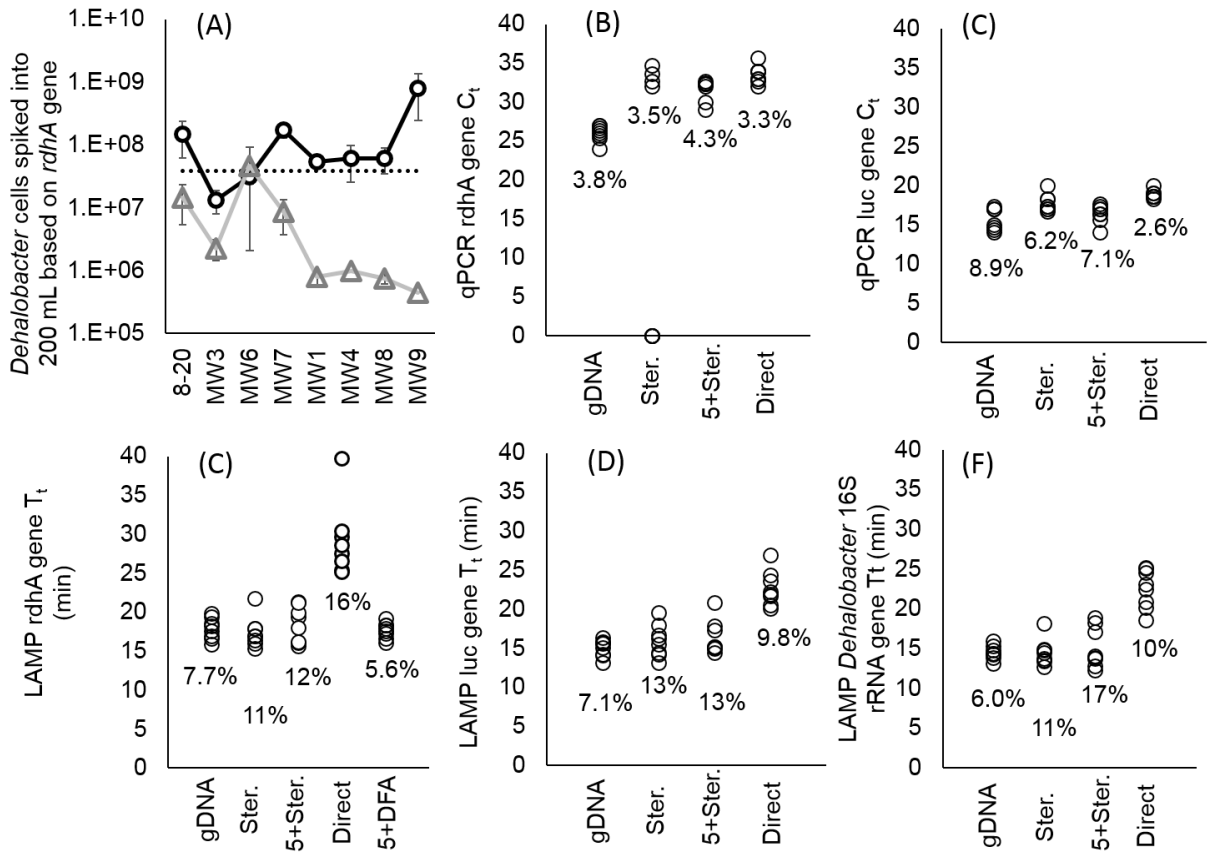

Figure 4.4. Comparing methods of biomass concentration followed by LAMP on Gene-Z or qPCR in real-time cycler with TCA-20 spiked into 200 mL of eight separate groundwater samples. A) Estimated quantity of *Dehalobacter* cells based on calibration curves and Tt and Ct of DFA (circle) and qPCR of gDNA (triangle), respectively. The dotted line represents total number of cells spiked into 200 mL of groundwater samples prior to sample processing. Error bars represent standard error of T_t between three replicates. B-F) Graphs showing T_t and C_t of different assays measured with MIAC and TCA-20 spiked into 200 mL of eight separate groundwater samples prior to sample processing including filtration with Sterivex and direct LAMP (Ster.), a filter train with 5 micron filter prior to Sterivex (5+Ster.), no filtration prior to amplification (Direct), conventional sample preparation using vacuum filtration followed by DNA extraction (gDNA), and direct filter amplification (DFA). Percentage indicate the T_t or C_t coefficient of variation (CV) among the eight groundwater samples.

Chapter 5

Kanitkar, Y. H., Stedtfeld, R. D., Hatzinger. P. B., Hashsham, S. A. and A. M. Cupples. 2017. Most probable number with visual based LAMP for the quantification of reductive dehalogenase genes in groundwater samples. Journal of Microbiological Methods. 143:44-49.

1. Abstract

The remediation of chlorinated solvent contaminated sites frequently involves bioaugmentation with mixed cultures containing *Dehalococcoides mccartyi*. Their activity is then examined by quantifying reductive dehalogenase (RDase) genes. Recently, we described a rapid, low cost approach, based on loop mediated isothermal amplification (LAMP), which allowed for the visual detection of RDase genes from groundwater. In that study, samples were concentrated (without DNA extraction), incubated in a water bath (avoiding the use of a thermal cycler) and amplification was visualized by the addition of SYBR green (post incubation). Despite having a detection limit less than the threshold recommended for effective remediation, the application of the assay was limited because of the semi-quantitative nature of the data. Moreover, the assay was prone to false positives due to the aerosolization of amplicons.

In this study, deoxyuridine triphosphate (dUTP) and uracil DNA glycosylase (UNG) were incorporated into the assay to reduce the probability of false positives. Optimization experiments revealed a UNG concentration of 0.2 units per reaction was adequate for degrading trace levels of AUGC based contamination (~1.4×10^4 gene copies/reaction) without significant changes to the detection limit (~100 gene copies/reaction). Additionally, the optimized assay was used with the most probable number (MPN) method to quantify RDase genes (*vcrA* and *tceA*) in multiple groundwater samples from a chlorinated solvent contaminated site. Using this approach, gene concentrations were significantly correlated to concentrations obtained using traditional methods (qPCR and DNA templates). Although the assay underestimated RDase genes concentrations, a strong correlation (R^2 = 0.78 and 0.94) was observed between the two data sets. The regression equations obtained will be valuable to determine gene copies in groundwater using the newly developed, low cost and time saving method.

2. Introduction

The chlorinated solvents tetrachloroethene (PCE) and trichloroethene (TCE) and their degradation products (dichloroethene and vinyl chloride) have contaminated many aquifers.

These sites are often remediated by biostimulation and bioaugmentation, which involve the injection of carbon sources and mixed dehalogenating cultures, respectively. Such practices facilitate the biological reductive dechlorination of the chlorinated chemicals by a process known as organohalide respiration [1]. The primary objective is to increase the population of *Dehalococcoides mccartyi* (DHC) cells in the subsurface, as this is the key species for the complete reduction of PCE and TCE to ethene [2].

It has become standard practice to monitor the concentration of DHC both before and during the remediation process using quantitative polymerase chain reaction (qPCR), because higher DHC concentrations (>10^6 16S rRNA gene copies/L) are often associated with greater dechlorination rates and ethene generation [3]. Consequently, qPCR primers and probes specific to the biomarker reductive dehalogenase (RDase) genes, *vcrA*, *bvcA*, and *tceA*, are now widely used for monitoring the *in situ* activity and growth of DHC [3, 4]. Many remediation professionals either perform qPCR in-house or use the service of a commercial laboratory with expertise in qPCR. Both approaches involve significant costs, such as the requirement for a real time thermal cycler (~$20K) for in-house analysis or the fee associated with having samples analyzed by a commercial laboratory (typically >$250 per sample). Given these economic constraints, there remains a need for a more cost-effective approach for quantifying RDase genes.

Loop mediated isothermal amplification (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 [5-7]. LAMP assays for the quantification of DHC 16S rRNA and *vcrA* genes on a proprietary microfluidic platform, the Gene-Z, have also been developed [8, 9]. In addition, LAMP was tested on two commercially available bioaugmentation cultures (SDC-9 and KB-1) containing DHC [10]. This research also involved comparing RDase gene concentrations using LAMP on the Gene-Z to qPCR on a real time thermal cycler. 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 [10]. Recently, we developed a visual based SYBR green LAMP assays for *vcrA* and *tceA* genes and tested the methods with multiple groundwater samples from different chlorinated solvent sites. The approach involves sample concentration (without DNA extraction) and requires only a bench top centrifuge and a water bath (no Gene-Z or thermal cycler) for RDase detection [11]. However, two limitations were associated with the assay. The approach was prone to false positives due to the

aerosolization and contamination of amplicons, which has been previously described for other targets [12, 13]. In addition, the assay provided only threshold data (samples turned green above a certain gene concentration) and was therefore only semi-quantitative. The objective of the current research was to address these two limitations.

Here, the previously developed SYBR green LAMP assay was optimized, while maintaining detection levels, to incorporate deoxyuridine triphosphate (dUTP) and uracil DNA glycosylase (UNG) to prevent false positives due to carry over contamination. Replacing deoxythymidine triphosphate (dTTP) with dUTP produces LAMP amplicons containing uracil. Then, before initiating LAMP, the reaction mixture is treated with UNG to destroy carry over contamination (containing uracil) from any previous amplification serving as a source of contamination. The second limitation was addressed by incorporating a most probable number (MPN) approach into the assay to enable quantification. This approach is well known to water microbiologists for culture-based techniques. It was recently used on a microfluidic chip to create a MPN-LAMP technique for the quantification of gram negative and gram positive water borne pathogens [14]. The final assay was used on multiple groundwater samples from a chlorinated solvent contaminated site and compared to data obtained using conventional approaches (qPCR on extracted DNA). The final assay offers significant potential advantages over previous approaches, including reduced cost and time for sample preparation, as well as the potential for on-site, real-time application.

3. Methods

3.1. Integration of dNTP-UNG into the SYBR Green LAMP Assay

SYBR green LAMP reactions contained a final volume of 25µl with 22µl master mix and 3µl template. The master mix for each reaction comprised of 2X reaction mix (2X isothermal amplification buffer, 2.3 mM dUTP-dNTPs mix, 1.6 mM betaine, 12.0 mM $MgSO_4$ and balance water), 32 units (2 µl) of BST 2.0 WarmStart enzyme, 1.0 µl pluronic, 2.5 µl bovine serum albumin, 2.5 µl 10X primer mix as described previously [10, 11] as 2.0 µM F3 and B3, 16.0 µM Forward Inner Primer (FIP) and Backward Inner Primer (BIP), 8.0 µM LF and LB.

The first goal was to determine the amount of UNG required for degrading trace levels of carry over contamination of *vcrA* gene, while preserving the amplification of *vcrA*. For this, trace

contamination (each tube received 1.44 x 10^4 gene copies of AUGC based *vcrA*), was purposefully introduced into the reaction mix. Following this, a four-fold 10X dilution series of a plasmid standard containing *vcrA* (ATGC based) was amplified, along with negative controls (LAMP mixture, water, 1.44 x 10^4 of *vcrA* AUGC contaminant, but no *vcrA* ATGC based template). The reaction mix recipe previously described was used as a basis for LAMP experiments [11]. 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 µl to make up a reaction mix volume of 22 µl per reaction. To determine the 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 incubated for 1.5 min at room temperature after adding UNG. Finally, 3.0 µl templates were dispensed to each tube. After dispensing the templates, PCR tubes were capped and placed in a polypropylene 96 well PCR tube rack. The rack was incubated in the water bath for 1 hour after which it was removed, dried, and allowed to cool to room temperature (~5.0 min.). Following this, 2.0 µl 0.1 X SYBR green 1 (Molecular Probes, Catalog #S7563) was added to each tube.

The second goal was to determine the effect of UNG concentrations on RDase gene copy detection limits using centrifuged cell templates. The detection limits were tested with seven fold 10X dilution series of cell templates prepared from the SDC-9 culture spiked into groundwater and 0.4, 0.3 or 0.2 units of UNG. For this, UNG was added to the master mix, such that each reaction received 0.2, 0.3 or 0.4 units (1 unit/µl) to make up a final volume of 22.0 µl. The following experimental procedure was the same as that described above. The negative controls consisted of the LAMP mixture, water, with no cell templates.

3.2. MPN Approach using Centrifuged Cell Templates of Groundwater Samples

Centrifuged cell templates were prepared from groundwater samples (Supplementary Table 5.1.) using a protocol previously described [11]. The groundwater samples were from a chlorinated solvent contaminated site (in San Antonio, Texas) previously bioaugmented with the DHC-containing SDC-9 mixed culture. Groundwater samples were collected using traditional low-

flow sampling [15], the water was pumped into sterile amber bottles (1 l), which were placed on ice and shipped overnight to Michigan State University. In the laboratory, a 5 µm pore-size nylon membrane filter (to remove turbidity) (Nalgene, Rochester, NY) and a 0.22 µm pore-size Sterivex filter (EMD Millipore Corp., Billerica, MA) were placed in series to form a groundwater filtration module. Groundwater samples (250 ml) were filtered through the filtration module using a sterile 160 mL syringe. DHC cells are 0.3- 1.0 µm [2] and are therefore retained on the 0.22 µm pore-size 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 Sterivex filter and the filter was capped at 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 (13,000 g x 15 min) to obtain a biomass pellet by decanting the liquid content from the tube. The pellet was resuspended in 100 µl of 1X phosphate buffer to obtain centrifuged cells. Using these centrifuged cells, a dilution series consisting of seven consecutive 10-fold dilutions of the cell suspension was created for each sample. Six such replicate dilution series were created for each groundwater sample and used as templates for SYBR green LAMP amplification. Negative controls consisted of the LAMP mixture, water and no template.

3.3. Quantification with MPN LAMP on Groundwater and qPCR on DNA

Photographs 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. Representative data recorded for MPN analysis of *vcrA* and *tceA* genes in groundwater sample MW100 using SYBR green LAMP method are shown (Supplementary Tables 5.2. and 5.3.). Concentrations of DHC cells in centrifuged cell templates were determined using the statistical method of result rejection (Equation 1) developed by Hurley and Roscoe [16]. Six replicates of each dilution were used for the MPN calculations.

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 (using Microsoft Excel 2016) was used to iterate the value of x.

Example calculations used in the iterative evaluation of *vcrA* and *tceA* gene concentrations (i.e., x) in groundwater sample MW100 are shown (Supplementary Tables 5.4. and S5.5.). We assumed that the DHC cells were colloidal and had 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, the calculation was performed using the most dilute samples. On the other hand, if dilutions provided all negative results, MPN values were calculated using the most concentrated dilutions. These assumptions are similar to those made previously for MPN-LAMP [14].

The approach previously described for qPCR on extracted DNA [11] was used to quantify *tceA* and *vcrA* in the same groundwater samples as those used for MPN-LAMP. The process (DNA extraction and qPCR) was repeated three times to generate triplicate data points for each groundwater sample.

4. Results and Discussion

The modifications to our previous assay for the visual detection of DHC RDase genes in groundwater samples [11] are illustrated in Figure 5.1. First, the reaction volume was reduced from 50 µl to 25 µl (the original protocol used 5 µl template and 45 µl master mix, the current protocol adds 3 µl template to 22 µl master mix). Second, the reaction mix was altered to include the 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 would be 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. Also, greater volumes of groundwater were filtered in order to avoid false negatives, which might result from the addition of UNG. Additionally, the optimized SYBR green LAMP assay was coupled to the MPN technique to determine *vcrA* and *tceA* gene copy numbers in groundwater samples using centrifuged cell templates prepared from each sample.

4.1. Optimization of SYBR Green LAMP Assay with dUTP-UNG (using DNA templates)

The use of dUTP and UNG to control carry over contamination in qPCR is common [17-20] and several commercial master mixes for qPCR with varying concentrations of UNG are available (e.g., Applied Biosystems SYBR Green PCR Master Mix, Catalog #4309155 or QuantiTect SYBR Green PCR, Catalog # 204141). However, the application of dUTP-UNG system to LAMP is relatively new [21-24]. In this study, the dUTP-UNG system was tested with the visual based SYBR green LAMP assay previously developed in our laboratory.

One initial goal was to determine the amount of UNG required to address AUGC contamination, while preserving detection limits from ATGC templates (10^2 gene copies per reaction). A summary of the results is shown with the endpoint visual color of the reaction being denoted on the y-axis (Figure 5.2.). On the y-axis, a positive detection is denoted by green and a negative detection is shown as orange. The gene copies of *vcrA* (ATGC based plasmid standard, four 10X dilution) per reaction is plotted on the left x-axis while the amount of UNG in the LAMP master-mix is plotted on the right x-axis. At high levels of UNG (1.0 and 0.8 units per reaction) amplification in all templates was inhibited. At 0.6 units of UNG, false negatives were generated in tubes containing 1×10^3 and 1×10^2 gene copies of the *vcrA* plasmid standard template. Similarly, 0.4 units of UNG produced a false negative at 1×10^2 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. At the two lowest levels (0.1 and 0 units) of UNG, contamination persisted and the negative controls fluoresced green. These data suggest that 0.2 units of UNG was the appropriate addition under these experimental conditions. The results indicate the inclusion of dUTP and UNG into the SYBR green LAMP successfully prevented false positives due to carry over contamination, but that the dose is very important. Further studies may be warranted to evaluate whether the optimal dose of UNG varies under different environmental conditions.

4.2. Detection Limit of SYBR Green LAMP Assay with dUTP-UNG on Cell Templates

The detection limit of the SYBR green LAMP assay was tested using a dilution series of cell templates prepared from the SDC-9 culture and 0.4, 0.3 and 0.2 units of UNG (Figure 5.3.). At the highest UNG level (0.4 unit), the SYBR green LAMP assay failed to produce any amplification below 8.4 x 10^6 gene copies and at this level only one replicate fluoresced green. With 0.3 units of UNG, the detection limit was reduced to 8.4 x 10^3 gene copies. In this case, two replicates each of dilutions containing 8.4 x 10^3 and 8.4 x 10^2 gene copies fluoresced green. In contrast, at 0.2 units of UNG, the detection limit was closer to ~84 gene copies, similar to the detection limit with DNA templates. This detection limit translates to a hypothetical *vcrA* gene concentration of 1.1 x 10^4 gene copies per l groundwater. This detection limit is acceptable from an applied perspective, as it is lower than the reported gene concentration required for monitored natural attenuation [3, 25].

4.3. Quantification of *vcrA* and *tceA* Genes with SYBR Green LAMP using MPN

In this study, we explored for the first time using MPN with LAMP for the quantification of *vcrA* and *tceA* genes in groundwater from several different wells at a SDC-9 bioaugmented chlorinated solvent site. An example image illustrating the endpoint colors for a dilution series prepared from a single centrifuged cell template of groundwater sample MW100 for the *vcrA* gene is shown in Figure 5.4. (image A shows the first set of triplicates, and image B shows the second set). No template controls for each replicate are on the right. The data set for each dilution series from this well (6 replicates) for *vcrA* and *tceA* are provided in the Supplementary Information (Supplementary Tables 5.2. and 5.3., respectively).

As discussed above, the MPN method was used to convert visual data results from all seven groundwater samples into *vcrA* and *tceA* gene copies/l. Example data sets for MPN are shown for MW100 for *vcrA* and *tceA* (Supplementary Tables 5.4. and 5.5.). The data sets were then compared to data generated using qPCR with DNA templates from the same samples (Figure 5.5.). Overall, gene concentrations from qPCR were greater than concentrations obtained using SYBR green LAMP (Figure 5.5. and Supplementary Table 5.6.). Between different groundwater samples, the maximum concentration of *vcrA* gene obtained using qPCR and DNA templates was 6.1 x 10^7 gene copies/l while that for *tceA* gene was 8.9 x 10^7 gene copies/l. The minimum concentration of *vcrA* gene obtained using qPCR and DNA templates in these samples was 1.4 x 10^6 gene copies/l while that for *tceA* gene was 2.4 x 10^6 gene copies/l. The maximum concentration of *vcrA* gene obtained using SYBR Green LAMP and centrifuged cell templates

was 3.2 x 10^7 gene copies/l while that for *tceA* gene was 2.9 x 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 x 10^5 gene copies/l while that for *tceA* gene was 5.0 x 10^5 gene copies/l.

When the two data sets (qPCR and LAMP) were plotted against each other a strong positive correlation was observed between the two methods (Figure 5.6.). For the *vcrA* gene, a Spearman's coefficient (ρ) of 0.952 and a p-value of 0.0329 was calculated while for the *tceA* gene these values were 0.994 and 0.0484 respectively. In Figure 5.6A, the slope and the intercept of the trend line for *vcrA* gene were 0.95 and 1.02 (R^2 = 0.78) while for the *tceA* gene, these values were 0.94 and 1.07 (R^2 0.93), respectively. In Figure 5.6B, for a trend line passing through the origin, the slope and the exponent values for *vcrA* gene were 10.39 and 0.95. Similarly, these values for the *tceA* gene were 11.87 and 0.94. The strong correlation between the techniques suggests that the newly developed LAMP-MPN method may represent a useful technique for estimating DHC numbers in groundwater, potentially saving both time and money compared to the current qPCR approach.

5. Conclusions

A visual based SYBR green LAMP- MPN approach was developed, offering a low cost and user-friendly alternative to qPCR for quantifying DHC RDase genes in groundwater samples. It offers three key advantages compared to existing methods: time, cost and the potential *in situ* application. The use of centrifuged cells, instead of DNA, reduces the time and cost required for sample preparation (no DNA extraction). Also, compared to qPCR, the LAMP assay has a shorter run time and the visualization of amplification products is immediate. The assay requires only basic laboratory equipment (benchtop centrifuge and water bath), does not require an expensive real time thermal cycler. With additional development and validation, it is possible that the method could be applied in the field. Additionally, the dUTP-UNG system reduces the probability of false positives due to carry over contamination and increases the overall robustness of visual detection with SYBR green LAMP. The regression equations generated for SYBR green LAMP assay with MPN technique can be used to calibrate the assay to relate the data to traditional qPCR data.

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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. Copyright 2018. American Society of Microbiology.

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6. Tables and Figures

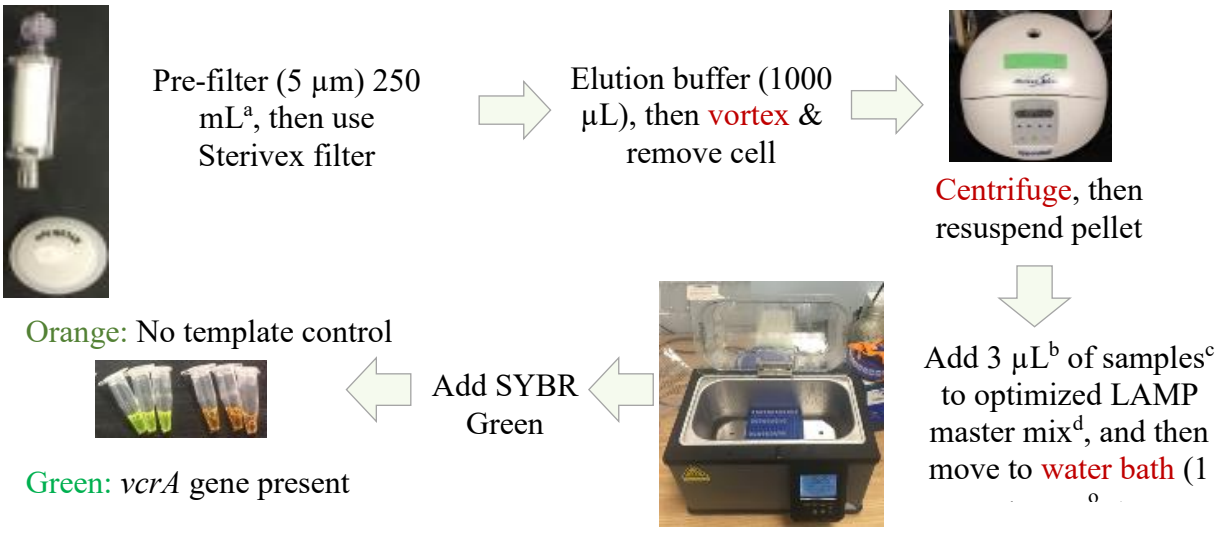

Figure 5.1. SYBR green LAMP method for detection of RDase genes from groundwater samples. Changes made in this study:

[a]Greater volumes of groundwater samples were filtered, [b]change in reaction volume (50 µL to 25 µL), [c]replicate dilution series included to enable MPN approach and [d]LAMP master mix (included dUTP instead of dTTP) was incubated with UNG before addition of templates.

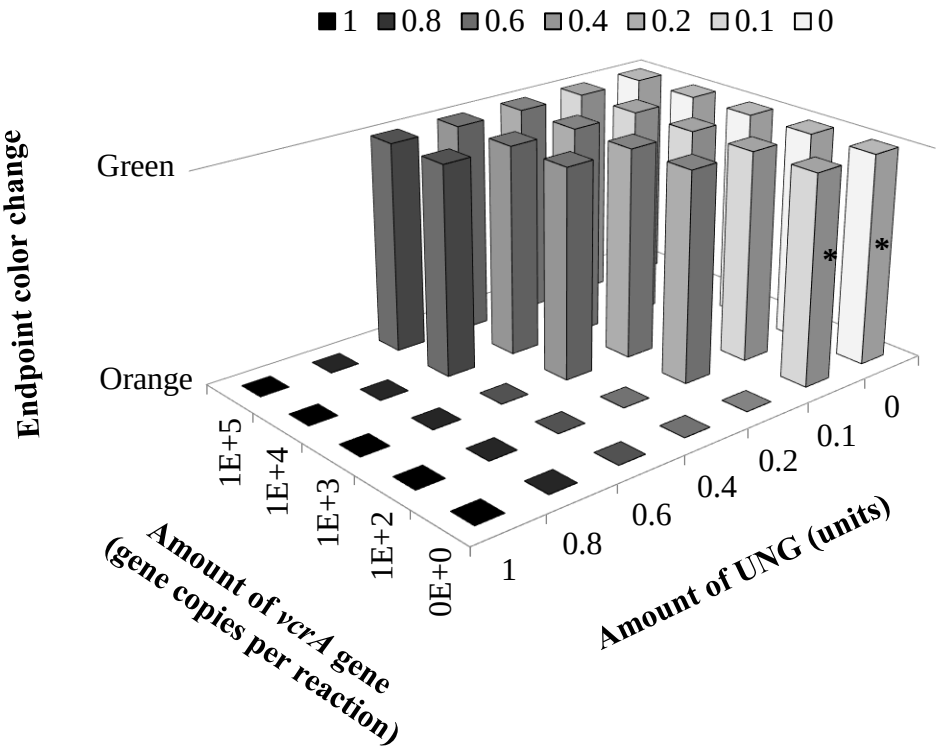

Figure 5.2. The effect of a range of UNG on the amplification of *vcrA* (ATGC, plasmid standard, $n = 1$) in the presence of added contamination (*vcrA* gene AUGC contamination of ~1.4 x 10^4 gene copies per reaction). Green is a positive detection and orange is no detection.

*Amplification was observed in the negative controls (LAMP mixture, water, ~1.4 x 10^4 of *vcrA* AUGC contaminant, no *vcrA* ATGC based template).

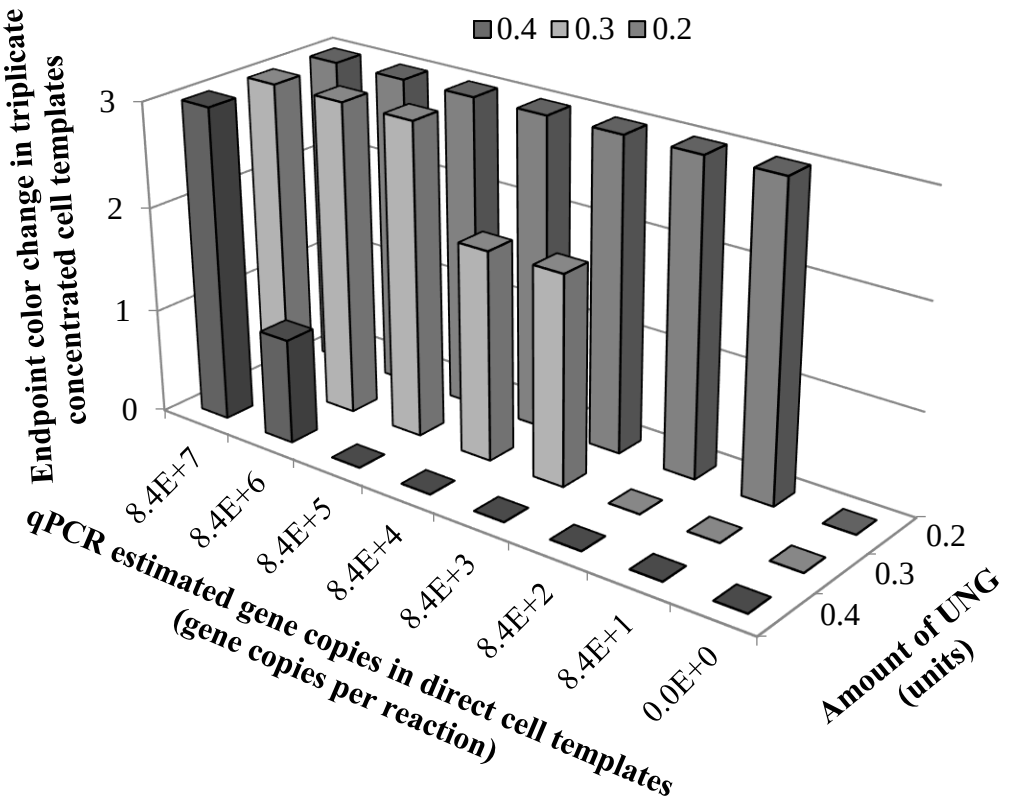

Figure 5.3. Determination of the detection limit over a range of UNG units and concentrated cell templates (n = 3) prepared from groundwater spiked with known quantities of *Dehalococcoides* cells. To score each reaction, a value of one was given to each triplicate that turned green and zero was given to each that remained orange. As each dilution had triplicates, the maximum and the minimum values on y-axis can be three and zero respectively. On the left x-axis, *vcrA* gene copies from groundwater spiked with known quantities (determined using qPCR) of *Dehalococcoides* cells is shown. On the right x-axis, the amount of UNG in the LAMP master-mix is shown. The negative controls consisted of the LAMP mixture, water, with no cell templates.

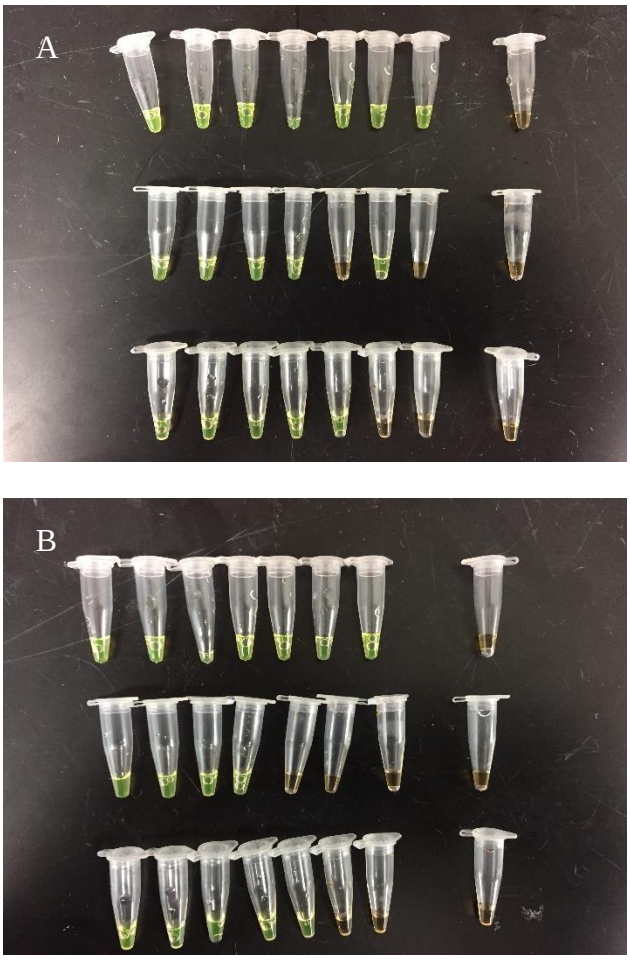

Figure 5.4. Endpoint color observed in a six replicate (*n*=6) seven fold 10X dilution series prepared from a single centrifuged cell template of groundwater sample MW100 for *vcrA* gene.

Note: Image A has the first set of triplicates, and image B has the second set. Negative controls (LAMP mixture, water, no template) for each replicate are on the right. If the endpoint color change was green, it was denoted with a value of one while that of orange was denoted with zero.

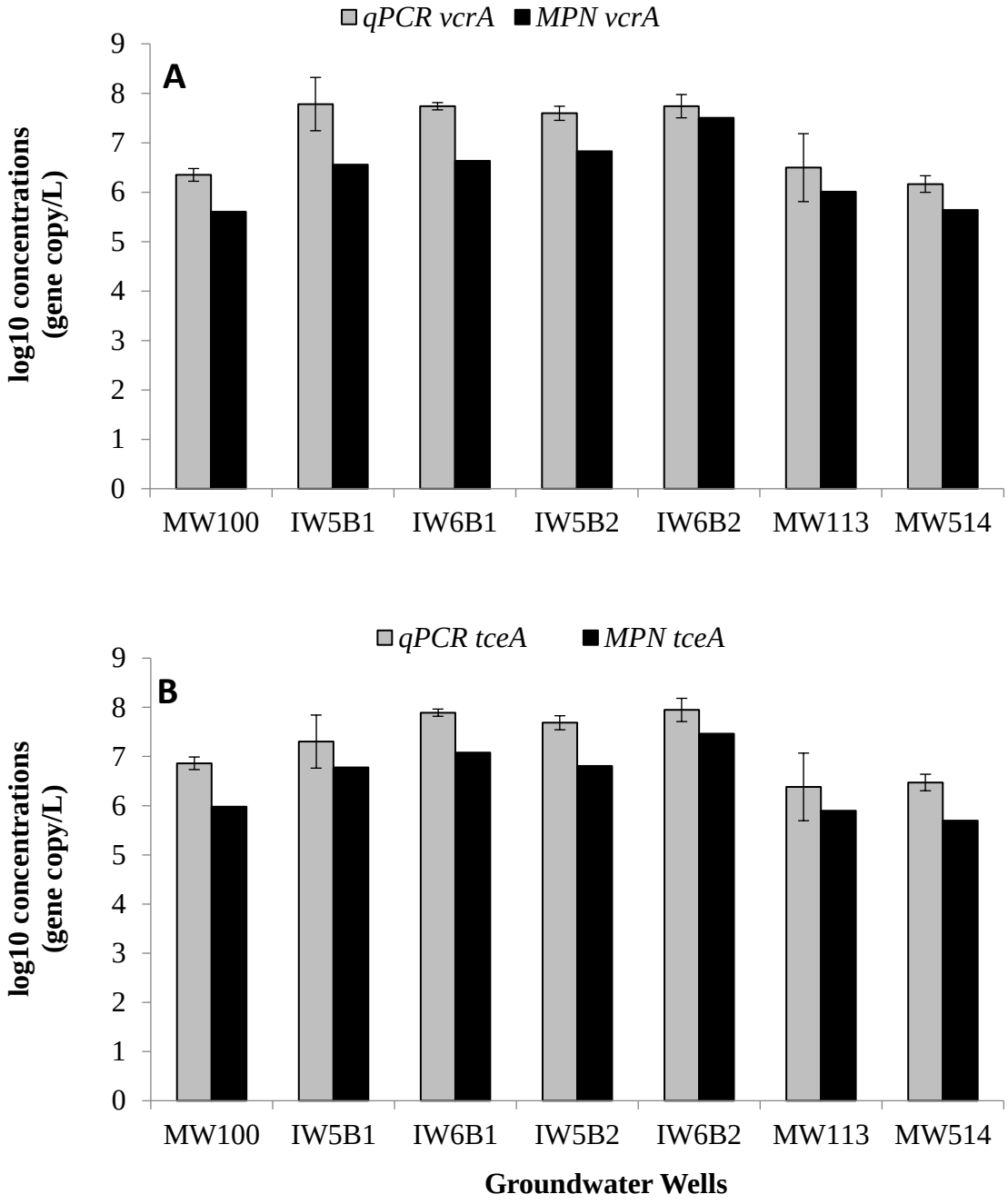

Figure 5.5. Gene concentrations (log10 gene copy/L) determined using qPCR with DNA templates (grey) and MPN coupled to SYBR green LAMP with centrifuged cell templates (black) for *vcrA* (A) and *tceA* (B) genes. The bars for the qPCR data represent standard deviations from triplicate DNA extracts (n = 3).

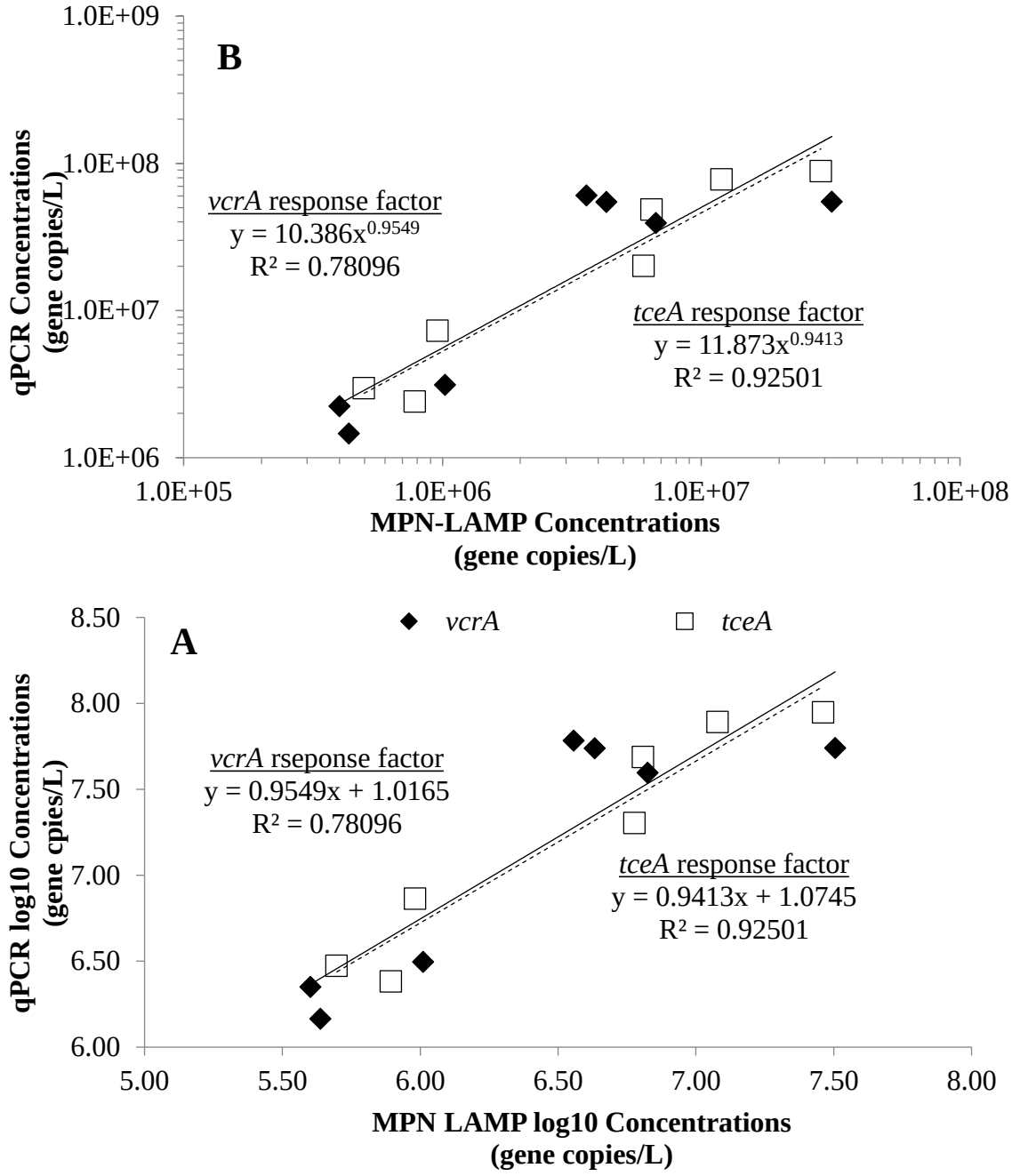

Figure 5.6. Correlation between concentrations of *vcrA* and *tceA* genes obtained using qPCR with DNA templates and MPN coupled to SYBR Green LAMP with centrifuged cell templates on linear scaled axes with log10 values (A) and log scaled axes (B). The values are averages of triplicate measurements (n = 3).

Chapter 6

Kanitkar, Y. H., Stedtfeld, R. D., Hatzinger. P. B., Hashsham, S. A. and A. M. Cupples. 2017. Development and application of a rapid, user-friendly and inexpensive method to detect *Dehalococcoides* sp. reductive dehalogenase genes from groundwater. Applied Microbiology and Biotechnology. 101: 4827–4835.

1. Abstract

TaqMan probe based quantitative polymerase chain reaction (qPCR) specific to the biomarker reductive dehalogenase (RDase) genes is a widely accepted molecular biological tool (MBT) for determining the abundance of *Dehalococcoides* sp. 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 X 10^5 gene copies per L for *vcrA* and 1.3 X 10^5 gene copies per L for *tceA*. Both values are below the threshold recommended for effective *in situ* dechlorination.

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, a process known as organohalide respiration [1]. 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 these tasks [2-6]. Other methods for detecting *Dehalococcoides* cells include catalyzed reporter deposition-fluorescent in situ hybridization (CARD-FISH) and

microarray based platforms [7-9]. 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.

The current research builds on previous work in our laboratories concerning the use of loop mediated isothermal amplification (LAMP) to detect *Dehalococcoides* RDase genes. LAMP is a single step amplification reaction that amplifies a target DNA sequence using four to six primers. The Bst large fragment DNA polymerase has strand displacement activity and helicase like activity allowing it to unwind and amplify DNA strands in the 60-65 ºC temperature range [10]. Because LAMP is sensitive, specific and occurs isothermally, it is often used as an alternative to point-of-care diagnostic methods which rely on PCR for a wide variety of applications [11-14]. For example, many LAMP assays have been developed for testing food borne bacterial pathogens and fungal contaminants [15, 16]. 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 [17, 18]. 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 [19]. 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 [19]. 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 in either 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. Methods

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[20]. The water 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[21-23] 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 (Supplementary Table 6.1.).

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)[24]. 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 ºC for future use. The entire process was repeated three times to generate triplicates of each groundwater sample.

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[25] 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 filters were cut open and the suspension was poured into a 1.5 mL tube. The resuspensed 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.

3.4. 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[19], 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.

3.5. Amplification for 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 [17, 19] . Each 20 µL 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 µL SYTO 82 orange fluorescent dye (Life Technologies, Inc., Grand Island, NY), 0.8 µL Pluronic (Life Technologies, Inc., Grand Island, NY), 0.8 µL Bovine Serum Albumin, 0.25 µM 10X Primer Mix and balance water to make up 18 µL. The reactions were incubated at 63 ºC for 60 min for amplification.

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 °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. Templates and standards were added to each LAMP and qPCR reaction as 2 µL aliquots. All qPCR primers and probes (Supplementary Table 6.2.) and LAMP primers (Supplementary Table 6.3.) are listed.

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 ºC. The reactions were performed using 0.2 mL PCR tubes or a 96 well plate. Each 50 µL reaction contained 45µL 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 [17, 19] 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.

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 [17]. Recently, new LAMP primers were developed for *tceA* and *vcrA* genes and these were used to quantify the growth of *Dehalococcoides* sp. in the bioaugmentation cultures, SDC-9 and KB-1 [17, 19]. 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.

4.1. Comparison of qPCR and LAMP for DNA Extracts from Groundwater

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 6.1.). 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 6.2.). These results suggest that quantification with LAMP will be comparable to quantification with qPCR at different sites.

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[17]. 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 6.3.).

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 63ºC. A visual comparison of the two cell based methods (direct and centrifuged cells, Figure 6.3.) 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, centrifuged cell templates produced threshold times which were quantifiable. In many samples (indicated with an asterisk, Figure 6.3.), 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 6.4.). 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 that 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 6.4.) could be used on data generated from the centrifuged cells method to determine the concentration expected using DNA extracts.

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 [26-28].

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 6.5.). 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.

4.4. Detection Limits for Visual Detection

Triplicate samples of centrifuged cell templates were created from groundwater from six wells (IW5, IW7, MW38, MW40, MW41, 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 6.6. and Supplementary Table 6.4.). 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 (Supplementary Figure 6.1.). The five reaction tubes in each row represent amplicons created with templates produced by making the five-fold 10X dilution series of that triplicate (Supplementary Figure 6.1.). 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 uncertainly associated with these ranges, we suggest that conclusions should only been made if all three triplicates produce the same result. The dilution data were examined to

determine the lowest concentration were all three triplicates turned green for *vcrA* and *tceA* and guidance tables were generated to estimate the gene concentrations (Tables 6.1. and 6.2.). 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 probable number (MPN) technique to enable the method to be used in a quantitative manner.

The accuracy of the detection thresholds were tested using the *vcrA* assay and groundwater from additional sites (Figure 6.7.). As predicted, in samples containing > 1.8×10^5 *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.

5. Discussion

The correlation between the in *situ* dechlorination activity and the observed quantity of *Dehalococcoides* sp. in groundwater has been documented [4, 29]. In groundwater samples where *Dehalococcoides* sp. 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* sp. counts ($>10^6$ 16S rRNA gene copies/L) are often associated with high dechlorination rates and ethene generation [4]. 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* sp. counts. Further, if the sample produces three green vials following one X10 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* sp. 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 (Supplementary Table 6.5.). Also, 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 (Supplementary Table 6.6.). A 50 µL LAMP reaction with centrifuged cells is slightly cheaper (~$ 0.30) than a 20 µL qPCR reaction when consumables and reagents are considered. However, commercially available master mixes are used for qPCR, whereas reagents are mixed manually for LAMP. When commercial master mixes for LAMP become available, this will further decrease the time and cost associated with LAMP. More importantly, qPCR requires DNA extraction, which adds approximately $9 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 in a dilution series, then the gene copies can be estimated, providing the concentration is above ~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. Also, 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).

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6. Tables and Figures

Table 6.1. *vcrA* 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	***vcrA***	**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
X 10	*3 replicates = green*	*> 1.8 $X10^6$ gene copies per L*	*Threshold for effective dechlorination*
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 $X10^5$)
[b] Derived from the highest copy number when all remained orange (10 fold dilution of 1.1 X 10^6)
These values were determined using DNA extraction and LAMP quantification with plasmid standards.

Table 6.2. *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	***tceA***	**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
X 10	*3 replicates = green*	*> 1.3 $X10^6$ gene copies per L*	*Threshold for effective dechlorination*
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 $X10^5$)
[b] Derived from the highest copy number when all remained orange (10 fold dilution of 3.19 X 10^5)
These values were determined using DNA extraction and LAMP quantification with plasmid standards.

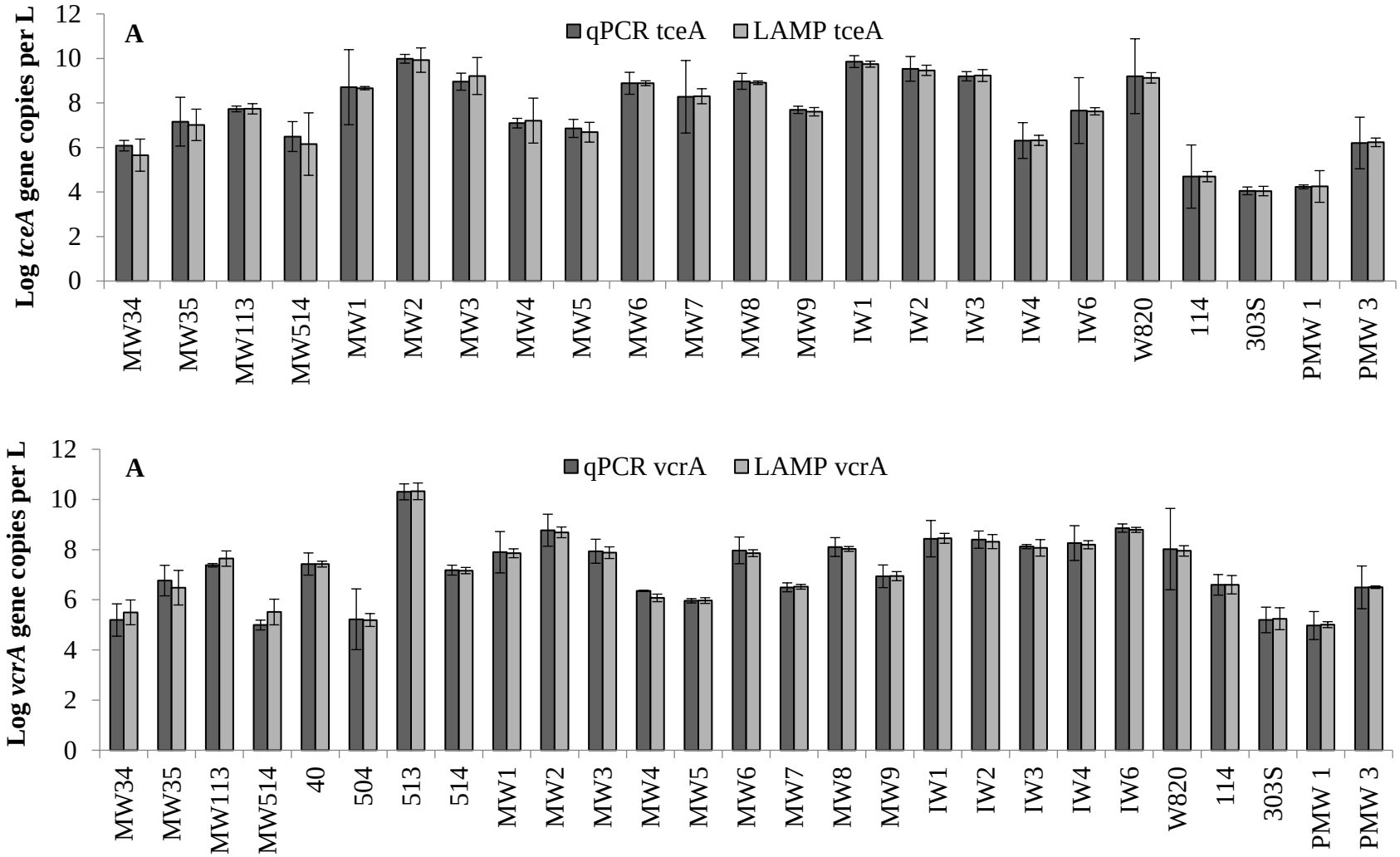

Figure 6.1. A comparison of qPCR and LAMP to quantify *tceA* (A) and *vcrA* (B) gene copies in DNA extracted from groundwater from different chlorinated solvent sites. The values represent means from triplicate DNA extracts and the error bars represent one standard deviation.

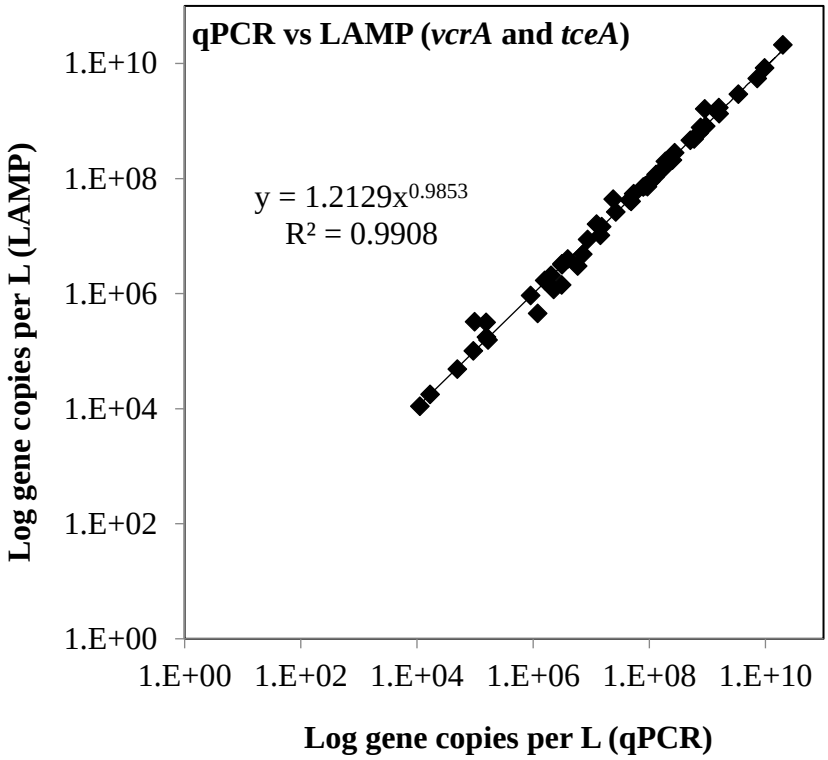

Figure 6.2. The correlation between gene concentrations (*tceA* and *vcrA*) determined via qPCR and LAMP using DNA extracted from numerous groundwater samples.

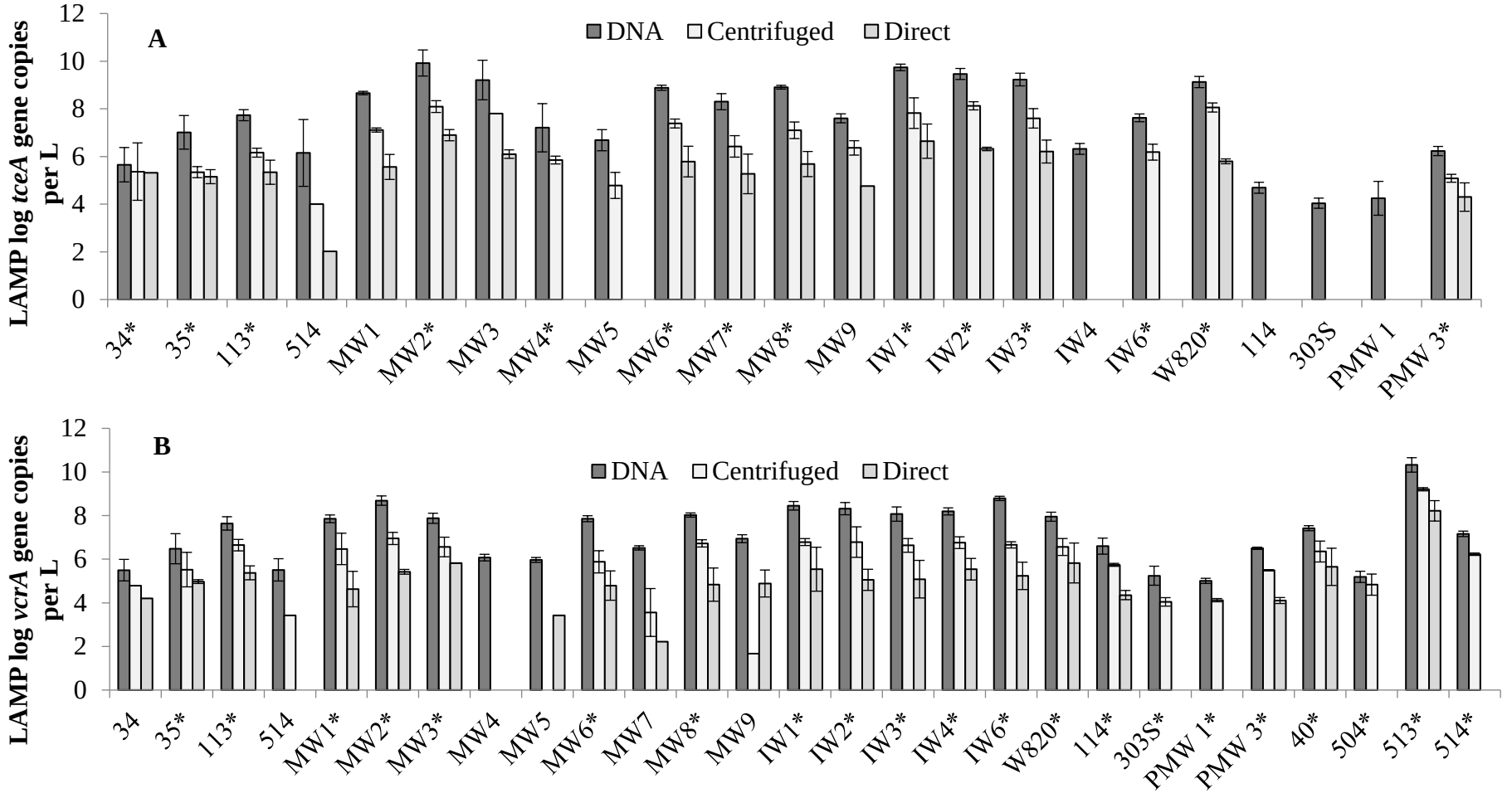

Figure 6.3. A comparison of *tceA* (A) and *vcrA* (B) gene concentrations (log gene copies per L) determined using DNA extracts, direct cells or centrifuged cells as templates. The values represent means of triplicate groundwater samples and the bars represent one standard deviation. *Templates were quantification was possible in all three replicates for centrifuged cells.

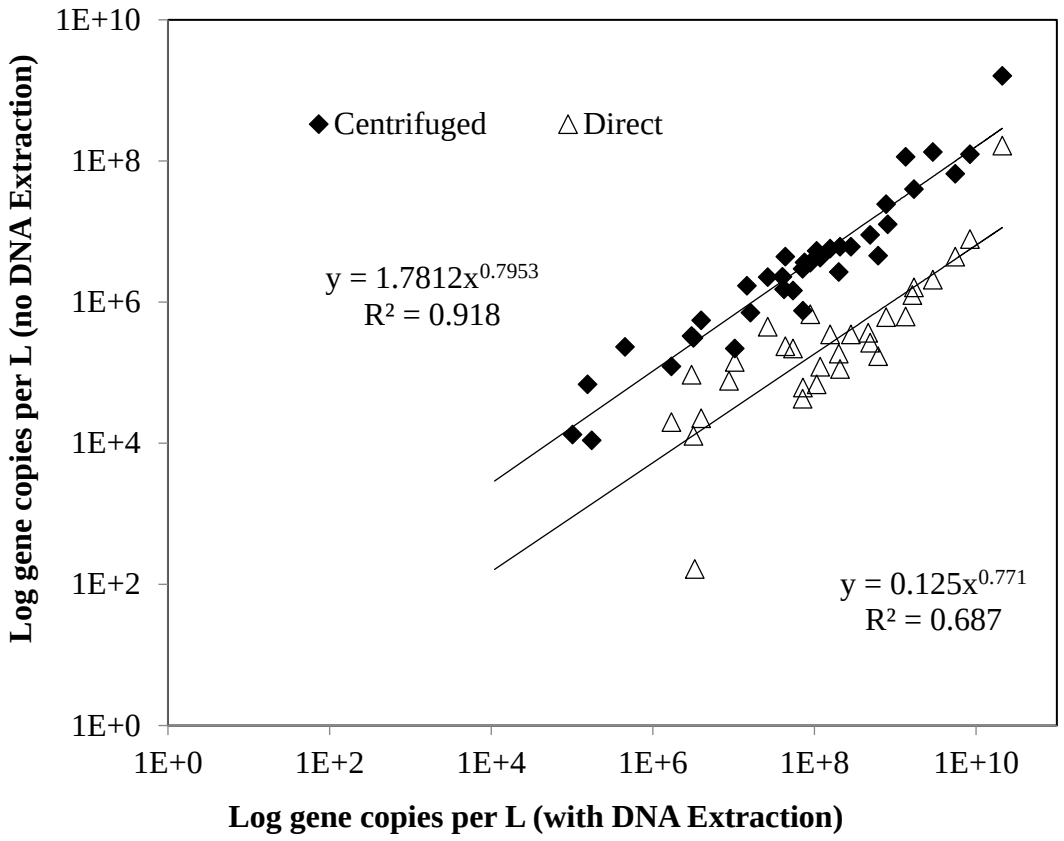

Figure 6.4. A comparison of the LAMP generated gene concentrations (vcrA and tceA) determined using DNA as a template to those values obtained using cells as a template (direct and centrifuged cells).

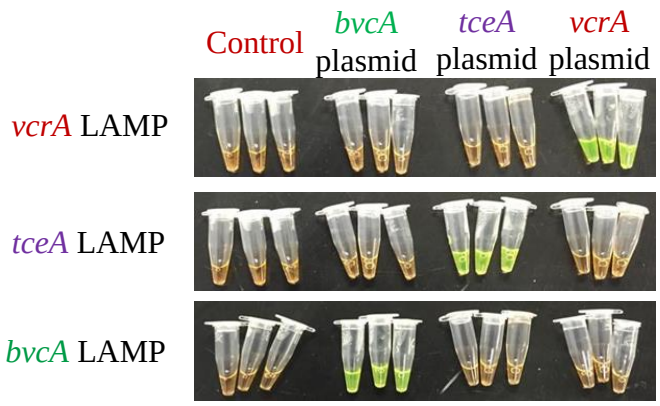

Figure 6.5. Specificity of LAMP/SYBR green assays with triplicates of plasmid standards (10^6 gene copies/reaction) containing RDase genes.

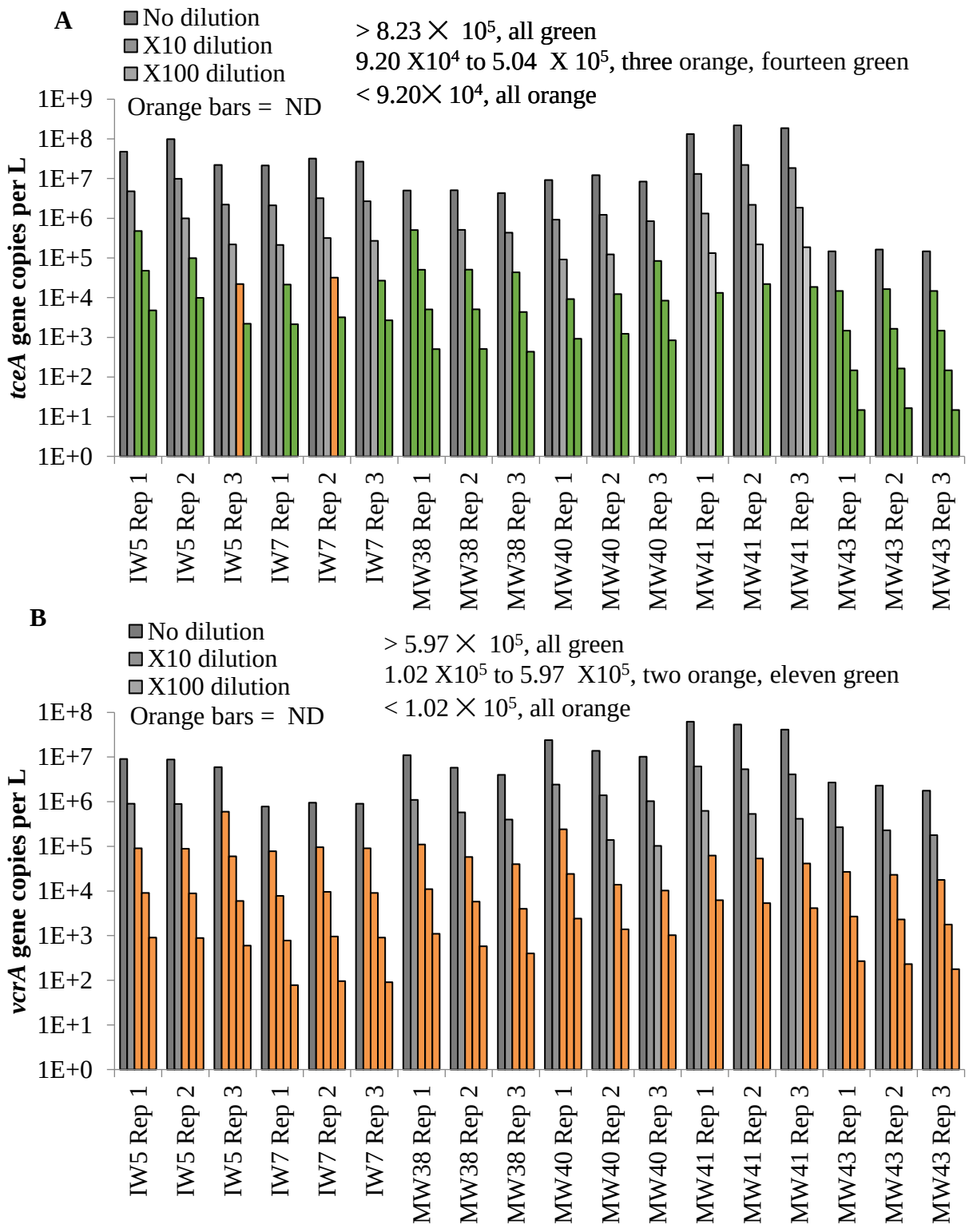

Figure 6.6. Amplification results using a 10X dilution series of centrifuged cell templates from groundwater for *tceA*(A) and *vcrA* (B). The highest value in each dilution series was measured (DNA extraction and LAMP) and the resulting dilutions values are estimated from this. The green bars indicate a positive gene detection

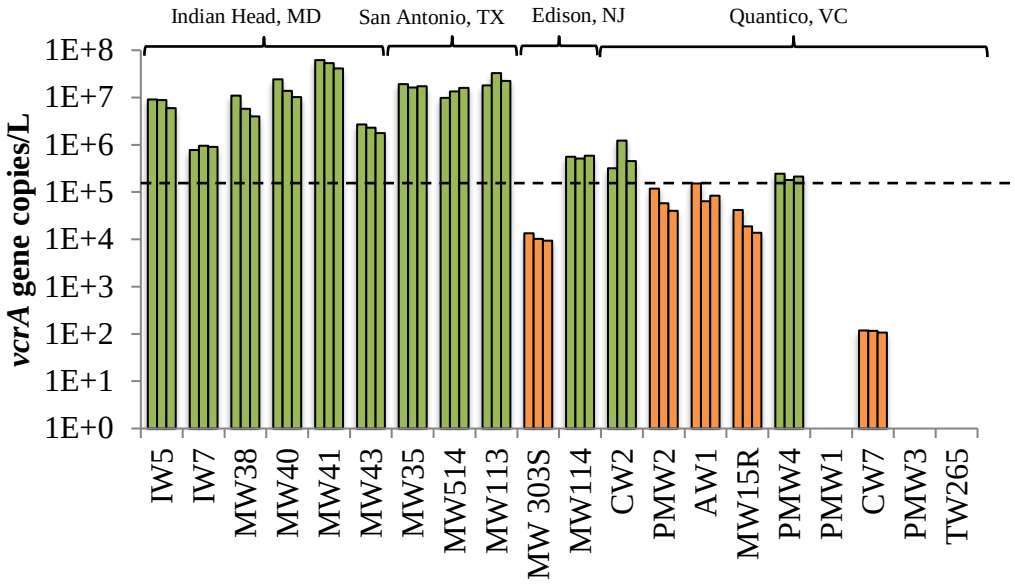

Figure 6.7. Testing of detection guidance values using groundwater from different sites. The dashed line represents the determined threshold for *vcrA* gene detection. Green and orange bars represent samples with the *vcrA* gene above and below the detection threshold, respectively. Three samples (PMW1, PMW3 and TW265) contained *vcrA* genes below the detection limit

Chapter 7

Dang, H., Kanitkar, Y. H., Stedtfeld, R. D., Hatzinger, P. B., Hashsham, S. A. and A. M. Cupples. *In press.* Abundance of chlorinated solvent and 1,4-dioxane degrading microorganisms at five chlorinated solvent contaminated sites determined via shotgun sequencing, Environmental Science and Technology.

1. Abstract

Shotgun sequencing was used for the quantification of taxonomic and functional biomarkers associated with chlorinated solvent bioremediation in twenty groundwater samples (five sites), following bioaugmentation with SDC-9. The analysis determined the abundance of 1) genera associated with chlorinated solvent degradation, 2) reductive dehalogenase (RDases) genes, 3) genes associated with 1,4-dioxane removal, 4) genes associated with aerobic chlorinated solvent degradation and 5) *D. mccartyi* genes associated with hydrogen and corrinoid metabolism. The taxonomic analysis revealed numerous genera previously linked to chlorinated solvent degradation, including *Dehalococcoides, Desulfitobacterium* and *Dehalogenimonas*. The functional gene analysis indicated *vcrA* and *tceA* from *D. mccartyi* were the RDases with the highest relative abundance. Reads aligning with both aerobic and anaerobic biomarkers were observed across all sites. Aerobic solvent degradation genes, *etnC* or *etnE,* were detected in at least one sample from each site, as were *pmoA* and *mmoX*. The most abundant 1,4-dioxane biomarker detected was *Methylosinus trichosporium OB3b mmoX*. Reads aligning to *thmA* or *Pseudonocardia* were not found. The work illustrates the importance of shotgun sequencing to provide a more complete picture of the functional abilities of microbial communities. The approach is advantageous over current methods because an unlimited number of functional genes can be quantified.

2. Introduction

The chlorinated solvents tetrachloroethene (PCE) and trichloroethene (TCE) and their metabolites, dichloroethene (DCE) and vinyl chloride (VC), are persistent groundwater contaminants, requiring remediation because of their risks to human health. Remediation efforts have involved biostimulation, through the addition of carbon sources, or bioaugmentation, which involves the injection of mixed microbial cultures containing *Dehalococcoides mccartyi* [1]. *D. mccartyi* is a key microorganism for the complete transformation of these chemicals to the non-

hazardous end product, ethene [2, 3]. *D. mccartyi* strains reduce chlorinated compounds obtaining energy from the reduction process [4-6]. Examples of commercially available mixed cultures containing *D. mccartyi* for chlorinated solvent remediation include SDC-9 (from APTIM, formerly CB&I, also marketed under several different names) and KB-1 (from SiREM) [1]. It was estimated that several hundred sites in the US have been subject to bioaugmentation with cultures containing *D. mccartyi* [7]. With the expansion of this remedial practice over the last decade, the number of sites in the US now numbers well over 2,300, and bioaugmentation has been performed in at least 11 other countries (*P Hatzinger, pers comm*). Following bioaugmentation, remediation professionals commonly monitor *D. mccartyi* populations, typically targeting reductive dehalogenase (RDase) genes such as *vcrA* [8-10] using quantitative PCR (qPCR) on nucleic acids extracted from groundwater [11-13].

While qPCR has been successful for documenting the occurrence and dechlorinating activity of *D. mccartyi* [9, 12, 14, 15] most laboratories only have the instrumentation (bench-top real-time thermal cycler) to target a small number of functional genes. Next generation sequencing (NGS) is now becoming the tool of choice for environmental samples. For example, 16S rRNA gene amplicon NGS (16S rRNA-NGS) has been used to monitor microbial communities during chlorinated solvent natural attenuation [16-18], following biostimulation [9, 19 20-22], during zerovalent iron-based [22, 23] and thermal-based [24, 25] chlorinated solvent remediation.

In contrast to 16S rRNA-NGS, shotgun (or whole genome) sequencing offers the opportunity to investigate both the taxonomic and the potential functional characteristics of microbial communities. However, only a limited number of researchers have adopted this approach for describing chlorinated solvent groundwater microbial communities. Notably, these studies have primarily focused on taxonomic data, without specifically addressing RDases or other functional genes related to chlorinated solvent degradation [26, 27]. Others have examined dehalogenating genes in forest soils using shotgun sequencing [28]. To our knowledge, the current work represents the first study to target contaminant degrading functional genes in groundwater from chlorinated solvent contaminated sites using shotgun sequencing.

The samples included groundwater (from twenty injection or monitoring wells, post bioaugmentation with SDC-9) from five contaminated sites as well as the bioaugmentation culture, SDC-9. Although other researchers have used NGS to study *D. mccartyi* containing enrichment cultures (e.g. KB-1, D2, ANAS) [29, 30], limited data is available on SDC-9.

The overall objective was to develop the methodology to quantify chlorinated solvent and 1,4-dioxane degrading microorganisms in contaminated site groundwater using both taxonomic and functional analyses. We propose that this approach (or a derivative) will ultimately be the method of choice for predicting biodegradation potential at contaminated sites.

3. Methods

3.1. DNA Extraction from Groundwater and SDC-9

Groundwater samples from injection and monitoring wells were collected at five different chlorinated solvent sites (San Antonio TX, Tulsa OK, Edison NJ, Quantico VA, and Indian Head MD) through traditional low-flow sampling [31]. Only one of the five locations (Tulsa, OK) was known to be contaminated with 1,4-dioxane. The water was pumped into sterile amber bottles (1L), which were placed on ice and then shipped overnight to Michigan State University. All sites were previously bioaugmented with the commercially available reductive dechlorinating culture, SDC-9 [32, 33]. Details concerning groundwater sampling times and site characteristics have been summarized (Supplementary Table 7.1.). Additional site information, when available, has also been provided (e.g. plume maps, plot layouts, concentration data over time) for each site (Supplementary Figures 7.1.-15.). DNA was extracted (collection on a filter, bead-beating and chemical lysis) from groundwater and mixed culture (SDC-9) samples using the PowerWater DNA isolation kit (Mo Bio Laboratories, a Qiagen Company) and previously described methods [8, 34].

3.2. Sequencing and Taxonomic Analysis

DNA extracts from twenty groundwater samples and the culture SDC-9 were submitted for library generation and sequencing to the Research Technology Support Facility Genomics Core at Michigan State University (MSU). Details on the preparation of libraries, the sequencing platform (Illumina HiSeq 4000) and the taxonomic analysis (Meta Genome Rapid Annotation using Subsystem Technology (MG-RAST) [35] are provided in the Supplementary Section (Supplementary Table 7.2.).

3.3. Reference Sequences Collection, Functional Gene Analysis, qPCR

Two approaches were employed to analyze the functional gene data. First, protein sequences associated with RDases for published genomes were collected from the National Center for

Biotechnology Information (NCBI). The microorganisms and genome information utilized in this analysis has been summarized (Supplementary Tables 7.3. and 7.4.). Secondly, to enable a wider number of sequences to be examined, protein sequences were collected from additional sources e.g. Functional Gene Pipeline and Repository (FunGene) [36], NCBI BLAST. DIAMOND (double index alignment of next-generation sequencing data) [37] was used as the alignment tool for all functional genes. A stringent screening criteria approach (minimum sequence identity of 90% and alignment length of 49 amino acids) was adopted because of the similarity in many of the *D. mccartyi* genes (e.g. hydrogenases and corrinoid metabolism genes) between different strains. Detailed information on the collection of these sequences and the DIAMOND analysis has been provided (Supplementary Section). Quantitative PCR was performed to enumerate *vcrA* gene copies in each DNA extract using methods previously developed [34, 38] (see Supplementary Section).

4. Results

4.1. Sequencing and Taxonomic Analysis

From the twenty groundwater DNA extracts, the majority (seventeen) generated between ~4 and ~6 million sequences each, post quality control. Three samples (PMW2, MWAW1, IW7) produced lower sequence counts (157,000, 471,513 and 1,547,247). The average sequence length varied from 226 to 241 bp (standard deviations from 34 to 41 bp) (Supplementary Table 7.2.). The rarefaction curves plateaued indicating the analysis had captured the majority of the diversity within the samples (Supplementary Figure 7.16.).

Sequencing analysis of SDC-9 indicated the genera *Dehalococcoides* (31% of all sequences) and *Methanocorpusculum* (10%) were major components of the culture (Supplementary Figure 17). Other important microorganisms included those within the phyla *Bacteroidetes* (23%, primarily the genera *Parabacteroides* and *Bacteroides*), *Firmicutes* (19%, primarily *Desulfitobacterium, Desulfotomaculum, Clostridium* and *Bacillus*) and *Proteobacteria* (9%). For the groundwater, between two and five samples were studied for each of the five sites, with *Proteobacteria* and *Archaea* being dominant in many samples (Supplementary Figures 7.18.-7.22.)

4.2. Occurrence of Chlorinated Solvent Degrading Microorganisms in SDC-9 and In Situ

The sequencing data for each site was examined to determine the relative abundance of genera previously associated with chlorinated solvent degradation (Figure 7.1.). It is important to note that this analysis is only at the genus level and therefore, except for *Dehalococcoides*, may overestimate the abundance of possible degrading microorganisms. *Dehalobacter* and *Desulfomonile* were not detected in any of the culture or groundwater samples by MG-RAST and are not included in Figure 7.1.

The relative abundance of methanotrophs in the groundwater samples was also investigated (Figure 7.1). Methanotrophs are important because of their ability to use particulate and soluble methane monoxygenases (pMMO and sMMO) to cometabolically oxidize several chlorinated solvents [39-41].

Dehalococcoides, the key dechlorinating genera in SDC-9 (31% in SDC-9), was detected in every sample at every site (averages for each site ranging from 0.2 to 1.4%). The sites had been bioaugmented with SDC-9 from ~ 6.5 months (Quantico) to more than 6 years (Edison) prior to groundwater sample collection (Supplementary, Table 7.1.). The abundance of *Dehalococcoides* was greater in the injection wells (IW3, IW4, IW5, IW, CW2) compared to the monitoring wells (Figure 7.1B, C). *Dehalococcoides* relative abundance levels (0.14-0.26%) were lowest at the Edison site (Figure 7.1D) which had the longest time between bioaugmentation and sample collection (76 months). The lower *Dehalococcoides* levels at the Quantico site (0.15-0.19%, Figure 7.1C) are puzzling, since it had the shortest time between bioaugmentation and sampling (6.5 months), and may be related to the electron donor utilized (hydrogen compared to a fermentable substrate). At the Tulsa site, *Dehalococcoides* relative abundance levels were on the higher side (monitoring wells, 0.44 -0.96%, Figure 7.1B), perhaps as a result of higher TCE concentrations at the time of sampling (Supplementary Figure 7.12.). *Dehalococcoides* abundance levels were also higher at the Indian Head site (0.40-0.75%), possibly related to a shorter time between bioaugmentation and sampling (9 months).

Desulfitobacterium was detected at all five sites, although the relative abundance (average ranging from 0.1 to 0.4%) was typically less than that of *Dehalococcoides*. Except for *Dehalococcoides*, *Desulfitobacterium* was present at a higher relative abundance in SDC-9 (2.7%) compared to other dechlorinating microorganisms (<0.4%). At three sites, *Geobacter* was the most abundant genus in this group (Figure 7.1A, B and C) and at two sites, it was either the second or third most abundant (Figure 7.1D and E).

The five methanotrophs examined were present only at low levels in SDC-9 (averages ranging from 0.006-0.035%). In the groundwater samples, *Methylococcus* or *Methylosinus* were typically the most abundant, followed by *Methylobacterium* and *Methylocella*.

4.3. Functional Gene Analysis

The groundwater sequencing data were aligned to characterized RDases from *D. mccartyi* and three other genera (*Dehalogenimonas*, *Dehalobacter* and *Desulfitobacterium*) (Figure 7.2.). Not surprisingly, RDases from *D. mccartyi* were the most abundant (Figure 7.2A). Samples from Tulsa illustrated some of the highest values for *tceA* and *vcrA*, again a pattern perhaps caused by the higher chlorinated ethene concentrations at this site (Supplementary Table 7.1., Supplementary Figure 7.12.). Following Tulsa, the wells at Indian Head contained the second most abundant reads aligning to RDases from *D. mccartyi*. These results agree with the MG-RAST analysis, which illustrated the highest relative abundance of *Dehalococcoides* at Indian Head and Tulsa (Figure 7.1B and E).

The abundance of RDases from *Dehalogenimonas*, *Dehalobacter* and *Desulfitobacterium* were found in lower numbers and the results varied between sites (Figure 7.2B, C, D). The majority of reads aligning with *cerA* and *tdrA* from *Dehalogenimonas* were from Tulsa (MW2, MW3, MW4, IW3, IW4, IW6), followed by Indian Head (IW5, IW7, MW38, MW40) and Edison (MW303S) (Figure 7.2B). The average relative abundance values for *Dehalogenimonas* from the MG-RAST analysis indicated the highest values for San Antonio, Edison and Indian Head (Figure 7.1A, D, E). Reads aligning to RDases from *Dehalobacter* and *Desulfitobacterium* were less abundant but were found in at least one well from three of the five sites (except San Antonio and Edison) (Figure 7.2C, D). Although *Desulfitobacterium* was detected with the MG-RAST analysis, *Dehalobacter* was not.

Additional differences between the MG-RAST and the functional gene data sets included the presence of the genera *Anaeromyxobacter* and *Sulfurospirillum* with MG-RAST, but the absence of functional genes (associated with the removal of chlorinated chemicals) from these microorganisms. Also, *Geobacter* and *Polaromonas* were present at all sites, however, reads aligning to *pceA* of *Geobacter lovleyi* and cytochrome P450 of *Polaromonas JS666* were observed from only one sample each (MW40 and MW4, respectively, data not shown). These findings emphasize the importance of functional gene analysis to clearly define *in situ* potential biodegradation capabilities.

The majority of the RDases found in SDC-9 were from *D. mccartyi,* with *tceA* and *vcrA* being the most abundant (~two orders of magnitude higher than the RDases from other species) (inserts in Figure 7.2.). RDases from *Dehalogenimonas, Dehalobacter, Desulfitobacterium* were also present in SDC-9.

Reads aligning to the genes associated with the aerobic degradation of 1,4-dioxane [42] were also investigated (Figure 7.3). From the twelve genes examined, only six were identified in the groundwater samples (Figure 7.3A). These genes were detected in at least one sample from all five sites, despite the fact that only one of the sites (Tulsa) was known to be contaminated with 1,4-dioxane. Surprisingly, no genes associated with *Pseudonocardia* were detected. The MG-RAST taxonomic data were examined for the presence of the genera associated with these genes (Figure 7.3B). From this group, *Pseudomonas* was the most dominant genus, followed by *Burkholderia, Mycobacterium, Methylosinus* and *Rhodococcus*. Similar to the functional gene data, the genus *Pseudonocardia* was not detected in any groundwater sample.

The shotgun data sets were also queried against reference databases that contained both RDases from complete genomes as well as those from uncultured microorganisms (Figure 7.4A). The results were consistent with those found using sequences from complete genomes only (Figure 7.2A). Reads aligning with the genes associated with the aerobic degradation of the chlorinated ethenes (*pmoA, mmoX, etnC, etnE)* [40, 41, 43] were detected in the groundwater samples from a number of samples from Edison and Indian Head (Figure 7.4B, C). Additionally, *etnC* and *etnE* were also found at high levels in the monitoring wells from the Tulsa site, again perhaps as a result of higher cVOC concentrations at the time of sampling. Notably, the highest normalized relative abundance values for *etnC* and *etnE* were two orders of magnitude lower than *vcrA* or *tceA*.

The DIAMOND analysis included alignments to a gene encoding for a formate dehydrogenase-like protein (*fdhA*), hydrogenase genes (*hup*, *vhc*, *hym* and *ech*) and corrinoid metabolism genes (*btu, cbi* and *cob*) from *D. mccartyi*. In previous research, the formate dehydrogenase-like protein was found to be highly expressed and ubiquitous in *D. mccartyi*, representing a specific indictor for activity [44]. Hydrogenases are thought to oxidize H_2, the electron donor for *D. mccartyi* [45]. Corrinoid metabolism genes are relevant for up-taking and transforming of cobamides and cobinamide, which are critical for *D. mccartyi* RDases [45]. Samples containing the most abundant reads of *fdhA* were from Tulsa following by samples from

Indian Head (Supplementary Figure 7.23.). The abundance patterns for the hydrogenase and corrinoid metabolism genes across samples were similar to those for *vcrA, tceA* and *fdhA* (Supplementary Figures 7.24. and 7.25.). The *fdhA* abundance patterns across samples were similar to those observed for *tceA* and *vcrA* (Spearman's rank correlation coefficients 0.939 and 0.89 for *fdhA* vs. *vcrA* and *fdhA* vs. *tceA*, respectively, *p* values both < 0.0001, Supplementary Figure 7.26.), indicating this gene acts as an effective biomarker for *D. mccartyi*.

To investigate the accuracy of the shotgun sequencing data quantification method, the relative abundance of *vcrA* determined via shotgun sequencing was compared to *vcrA* gene copies determined via qPCR (Supplementary Figure 7.27.). In general, the abundance of *vcrA* determined using shotgun sequencing correlated well (Spearman's rank correlation coefficient 0.808, *p* value < 0.0001) with the qPCR data (3.9×10^4 to 7.0×10^9 *vcr* gene copies per L).

Principal component analyses were completed for the functional genes (Figure 7.5A) and genera (Figure 7.5B) associated with chlorinated solvent and 1,4-dioxane biodegradation. The genes *tdrA, vcrA* and *tceA* were positively correlated to *fhdA* as well as the hydrogenase and corrinoid metabolism genes, consistent with their similar abundance distribution in the wells. These genes correlated with injective wells from the Tulsa site, which would be expected considering the high relative abundance of *Dehalococcoides* in these samples. Genes relevant to aerobic chlorinated ethene degradation correlated with *mmoX* (from *M. trichosporium* OB3b) suggesting the genetic potential for degradation of these co-contaminants occurs at the same site. In this case, the genes correlated with MW114 from the Edison site. The remaining genes associated with 1,4-dioxane degradation correlated together (bottom left quadrant) perhaps indicating multiple functional genes will contribute to 1,4-dioxane degradation at the same site. RDases (*pceA*) from *Desulfitobacterium* and *Dehalobacter* also correlated together, along with MW3 (from Tulsa) which was previously found to contain RDases from these genera. For the taxonomic principal component analysis (Figure 7.5B), the anaerobic genera *Dehalococcoides, Desulfitobacterium and Desulfuromonas* correlated together along with the injection wells from the Tulsa site. For the methanotrophs, *Methylococcus* and *Methylobacterium* illustrated a positive correlation to each other and to the wells from several sites e.g. MW114, MW303, PMW4. The genera PCA is less meaningful because it is unknown if the majority of these microorganisms are truly associated with contaminant degradation.

5. Discussion

Biostimulation and bioaugmentation are becoming increasingly popular approaches for the remediation of groundwater contaminated with PCE, TCE and their daughter products. However, limited research has focused on groundwater microbial communities post bioaugmentation. This work is important because of the requirement for *Dehalococcoides* to co-exist with other "supporting" microorganisms and to survive over time. Further, it is also valuable to determine if other chlorinated solvent degrading microorganisms are present, and the extent to which these organisms persist following bioaugmentation with exogenous strains.

Not surprisingly, the genus *Dehalococcoides* was a major component of SDC-9. This was also reported for another common bioaugmentation culture, KB-1 [29]. Of additional interest is the presence (4% relative abundance) of *Desulfitobacterium* in SDC-9, as this genus has also been associated with dechlorination [46-50]. Similarly, others have reported *Desulfitobacterium* type RDase genes in *Dehalococcoides* enriched cultures [29]. Other genera linked to chlorinated solvent degradation were also detected in SDC-9 (as discussed above); however, their relative abundance in the culture was low compared to *Dehalococcoides* or *Desulfitobacterium.*

As in other *Dehalococcoides* enrichment cultures, SDC-9 contained methanogens (*Methanocorpusculum*), acetogens (*Clostridiaceae*) and *Geobacter* [9, 29]. *Geobacter* has previously been associated with interspecies corrinoid transfer with *Dehalococcoides* [51]. In addition, *Geobacter* has also been associated with dechlorination [52, 53]. The genera *Thermosinus* and *Selenomonas* within the family *Veillonellaceae* were detected in SDC-9 at low levels (3% and 0.4%, respectively). *Veillonellaceae* were previously found to be important corrinoid supplying microorganisms to *Dehalococcoides* in another enrichment culture [54]. SDC-9 contained *Desulfovibrio* (2.5%), which, in previous research, was linked to more robust dechlorination rates and growth when grown in co-culture with *Dehalococcoides* [55]. It was reported that *Desulfovibrio* can support *Dehalococcoides* by providing acetate, hydrogen and corrinoid cofactors [55].

Following *Dehalococcoides* and *Methanocorpusculum*, the third and fourth most abundant genera in SDC-9 were *Bacteroides* (5.4%) and *Parabacteroides* (10%) (within *Bacteroidetes*). Members of the *Bacteroidetes* phylum have also been reported as important bacteria in other dechlorinating mixed cultures [29] and in contaminated groundwater [9].

For the groundwater samples, *Geobacter* was more abundant at all sites compared to the SDC-9 culture and may therefore be important in playing a supportive role for *Dehalococcoides* at contaminated sites. In contrast, *Thermosinus* and *Selenomonas* were not detected in any groundwater samples. Other potentially supportive microorganisms, including *Desulfovibrio, Bacteroides* and *Parabacteroides*, were detected in the groundwater at all sites (ranging from 0.1- 4.2%) and therefore may also play a supportive role for *Dehalococcoides in situ*.

Similar to many previous studies examining microbial communities at chlorinated solvent sites (undergoing some kind of bioremediation), the genera *Dehalococcoides, Dehalogenimonas* and *Geobacter* were found in groundwater from all five sites [9, 19-27]. The current study identified *Desulfitobacterium* and *Anaeromyxobacter* in the majority of samples and these genera have also been frequently detected at contaminated sites [17, 20-22, 24]. In contrast, fewer previous studies have reported the presence of *Polaromonas* and *Nocardioides* [16, 17, 25]. Previous researchers have also detected methanotrophs *in situ* [16, 18, 24]. It was surprising that *Dehalobacter* was absent in the MG-RAST data, as this genus has been commonly reported in groundwater from chlorinated solvent contaminated sites [17, 19, 21, 22, 24]. However, *cprA* and *pceA* from *Dehalobacter* were found in the functional gene analysis, suggesting this genus could be present, but at levels undetectable by the MG-RAST analysis.

Although taxonomic data is important for characterizing microbial communities *in situ,* it is well recognized that certain limitations are associated with such data. A key limitation concerns an inability to classify to the species level when short sequences are analyzed. This issue is particularly relevant to bioremediation applications, as it impacts an identification of a known degrader, e.g. *Polaromonas* JS666 [56] or *Geobacter lovleyi* [57], over others in the same genus that are not capable of contaminant degradation. In the current study, relying on taxonomic data alone would have been misleading, because although the genera *Polaromonas* and *Geobacter* were present, the functional genes were largely absent (*P450* from *Polaromonas* JS666 and *pceA* from *Geobacter* were detected only once). Another related limitation concerns the inability of taxonomic data to provide in-depth information on function. This concern is important when considering *D. mccartyi*, as strains with similar 16S rRNA gene sequences may contain different

RDases. Clearly, to generate a full picture of the functional abilities of microorganisms to degrade contaminants *in situ*, both taxonomic and functional analyses are needed.

The taxonomic and functional analysis detected both aerobic and anaerobic biomarkers across the five sites. For example, both *vcrA* and *etnC* were found in MW2, MW3, MW4 from the Tulsa site (although the values for *vcrA* were higher). This trend has previously been noted for groundwater from other chlorinated solvent sites [13, 16, 58]. The genes *etnC* or *etnE* were detected in at least one groundwater sample from each site, with the normalized relative abundance values covering a wide range. Similarly, *pmoA* and *mmoX* were detected in at least one groundwater sample from each site and were particularly abundant at the Edison site. Given the occurrence of these genes in the current study, future research directions should include a consideration of both aerobic and anaerobic genes when accounting for chlorinated solvent removal rates.

To our knowledge, this study represents the first analysis of the genes associated with 1,4-dioxane degradation in groundwater using shotgun sequencing. Here, from the twelve sequences investigated, the most abundant number of reads (collectively, in all groundwater samples) aligned to *Methylosinus trichosporium OB3b mmoX,* followed by *Burkholderia cepacia G4 tomA3* and *Pseudomonas pickettii PKO1 tbuA1*. Notably, although *mmoX* from *M. trichosporium OB3b* has been associated with 1,4-dioxane degradation at high concentrations [59], at low, environmental relevant concentrations, no removal was observed [60]. Three others (*Pseudomonas mendocina KR1 tmoA, Rhodococcus jostii RHA1 prmA, Rhodococcus sp. RR1 prmA*) were detected at lower levels in at least one well from each site. In some cases, remarkably, the normalized relative abundance values were in the same range as those for *vcrA* and *tceA,* even though 1,4-dioxane was not previously reported at 4 of the 5 sites, and reducing conditions (i.e., negative oxidation-reduction potential; nORP) generally prevailed. Previously, others have observed *thmA* in samples from 1,4-dioxane contaminated sites using qPCR primers designed to *thmA* from *Pseudonocardia* [61-63]. However, reads aligning to *thmA* were not found in the current study. The taxonomic data confirmed this finding, as the genus *Pseudonocardia* was absent from the MG-RAST results. Reads aligning to *Mycobacterium* 1,4-dioxane degrading gene sequences (*prmA*) were also not detected in the current study, even though the taxonomic MG-RAST data indicated this genus was present. This discrepancy again illustrates the importance of functional gene data to corroborate taxonomic data and assumptions about

function. Further, the current work illustrates the importance of shotgun sequencing to provide a more complete picture of the potential of *in situ* microbial communities to degrade 1,4-dioxane compared to qPCR, which typically only targets a small number of genes.

Previous research indicated that transcripts of the proteins Fdh and Hup may be better indicators of cell respiration compared to RDases [64, 65]. In fact, it was concluded that HupL transcripts were the most robust activity biomarker across multiple *D. mccartyi* strains [66]. Given importance of Hup, the relative abundance of *fdhA* and other genes encoding for hydrogenases from *D. mccartyi* were investigated in the groundwater samples. Building on the approach developed in the current study, future research could include shotgun sequencing of transcripts to obtain an improved indicator of *D. mccartyi* cell respiration. These gene targets, as well as those involved in corrinoid metabolism, could be used as additional biomarkers for *D. mccartyi*.

To examine the quantitative robustness of the data generated, the normalized relative abundance values for *vcrA* were compared to those obtained via TaqMan qPCR. The correlation indicated the methods produced similar values across a range of concentrations for the five sites. Two important future research directions for using shotgun sequencing for bioremediation applications will be 1) to determine detection limits and 2) to generate more in depth comparisons to values determined with qPCR.

In summary, methods were developed to determine the abundance of genes associated with chlorinated solvent and 1,4-dioxane biodegradation in groundwater samples from multiple samples from multiple contaminated sites. The use of shotgun sequencing enabled a larger selection of genes to be targeted compared to traditional qPCR. In fact, the number of functional genes that can be analyzed is limitless. The method also does not require primer design or primer assay verification for each target (as is the case for qPCR). The most labor-intensive part of the approach involved the collection of reference fasta files for the DIAMOND alignment (following this, all remaining steps were not time consuming). The sequencing price is perhaps the largest limitation to the method. In the current study, for 22 samples, the cost was approximately $210 per sample. However, it is likely that sequencing costs will drop as the technology evolves, making the approach more attractive. The data indicated the presence of both aerobic and anaerobic biomarkers for chlorinated solvent degradation. Not surprisingly, the taxonomic data

alone was insufficient to determine the functional abilities of these communities. The relative abundance of hydrogenases and corrinoid metabolism genes suggest these may be appropriate additional biomarkers for *D. mccartyi.* The approach developed will enable researchers to investigate the abundance of any contaminant degrading gene in any sample, greatly expanding the analytical toolbox for natural attenuation, biostimulation or bioaugmentation.

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6. Tables and Figures

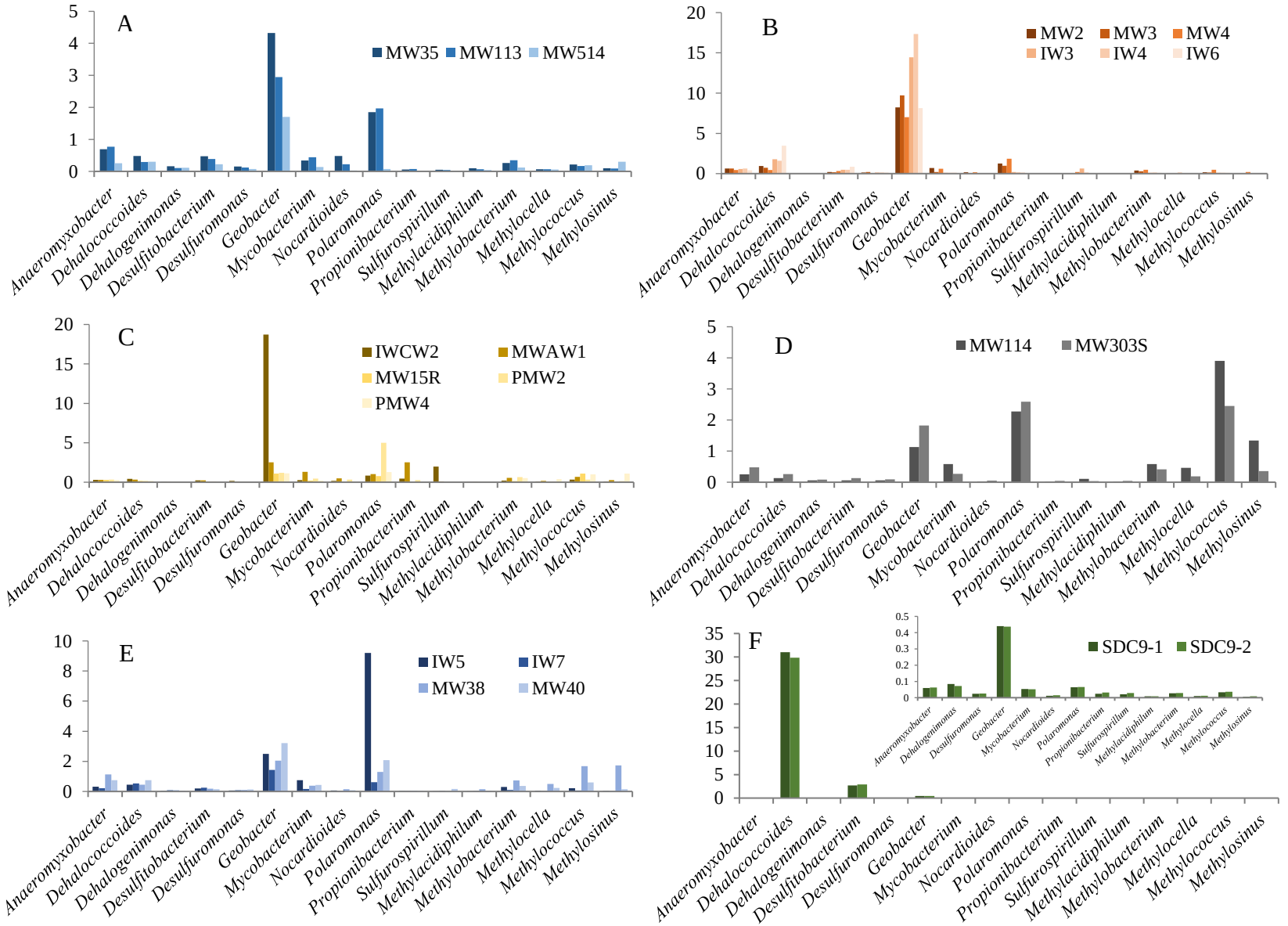


Figure 1. Relative abundance (%, as determined using MG-RAST) of methanotrophs and genera associated with chlorinated solvent biodegradation in groundwater from San Antonio (A), Tulsa (B), Quantico (C), Edison (D), Indian Head (E) and SDC-9 (F). The genus *Dehalococcoides* was present in all groundwater samples ranging from 0.1 – 3.5%. Note,"MW" in name refers to a monitoring well and "IW" in name refers to an injection well. The insert in F does not include *Dehalococcoides* or *Desulfitobacterium* to enable a y-axis with a different scale.

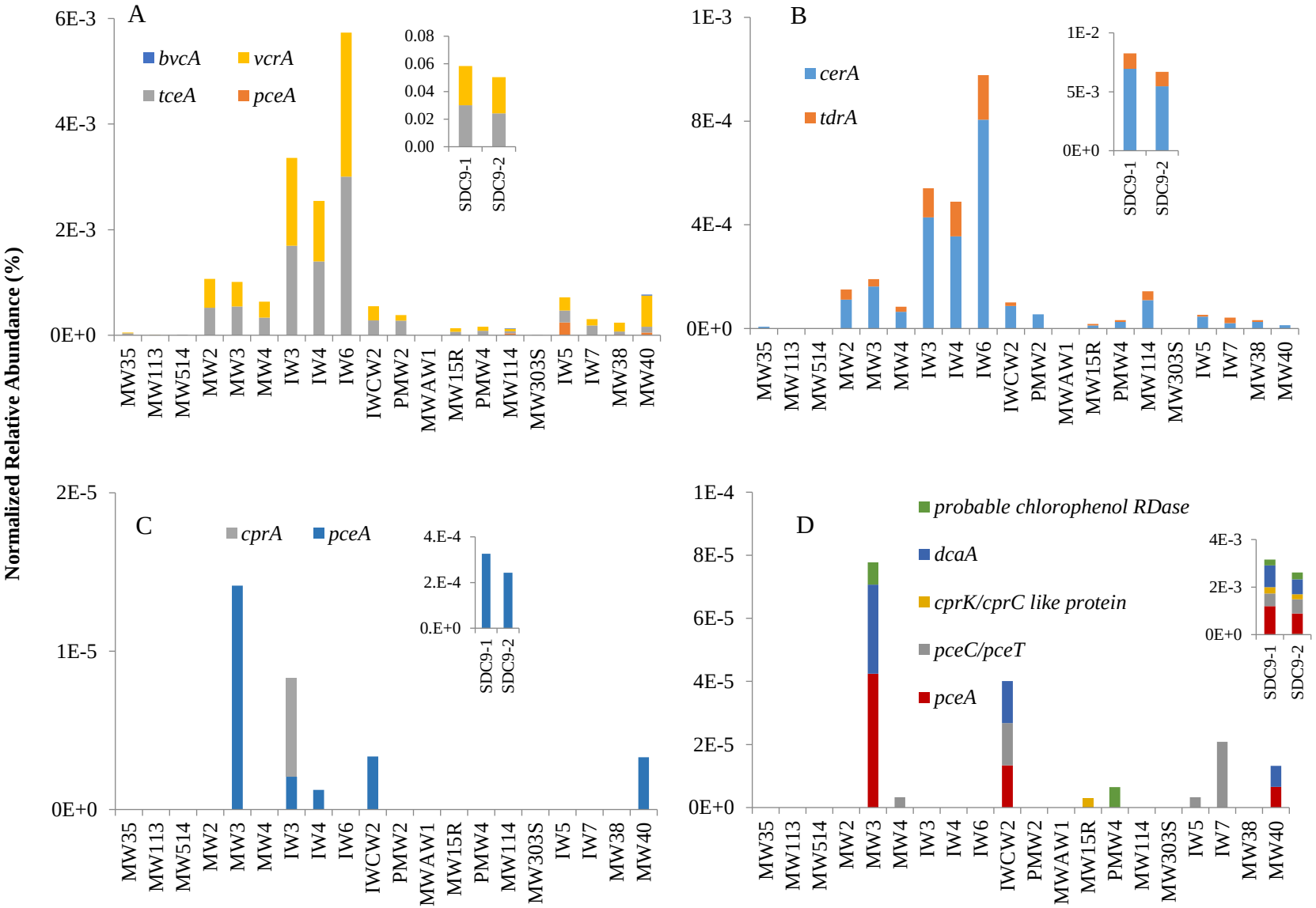


Figure 2. Normalized relative abundance (%, as determined by DIAMOND) of genes associated with reductive dechlorination in *Dehalococcoides mccartyi* (A), *Dehalogenimonas* spp. (B), *Dehalobacter* spp. (C) and *Desulfitobacterium* spp. (D) in SDC-9 (inserts) and in groundwater from the five chlorinated solvent sites. The highest abundance values are from *tceA* and *vcrA* from *Dehalococcoides*, followed by *cerA* from *Dehalogenimonas.*

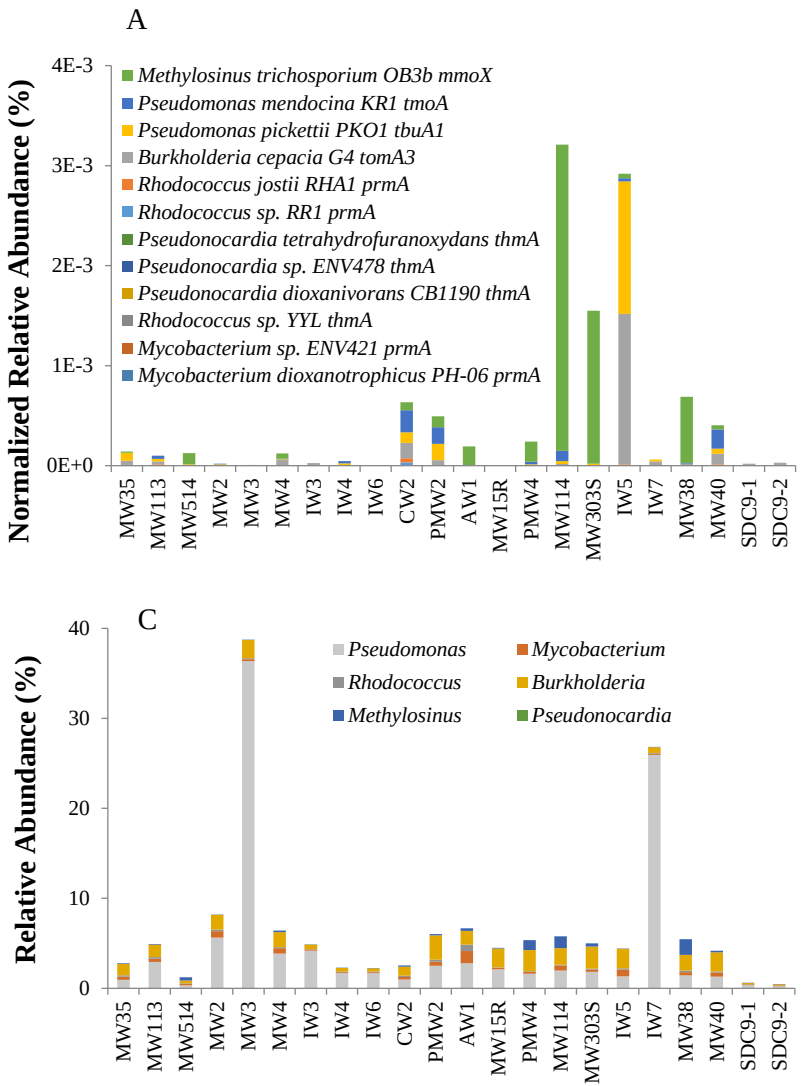


Figure 3. Normalized relative abundance (%, determined with DIAMOND) of genes (A) and relative abundance (%, determined with MG-RAST) of genera (B) previously associated with 1,4-dioxane degradation in all groundwater samples and in SDC-9. The relative abundance of *Pseudonocardia* was zero in all groundwater samples and in SDC-9. *Methylosinus trichosporium* OB3b *mmoX* was the dominant 1,4-dioxane degrading gene in the majority of the groundwater samples.

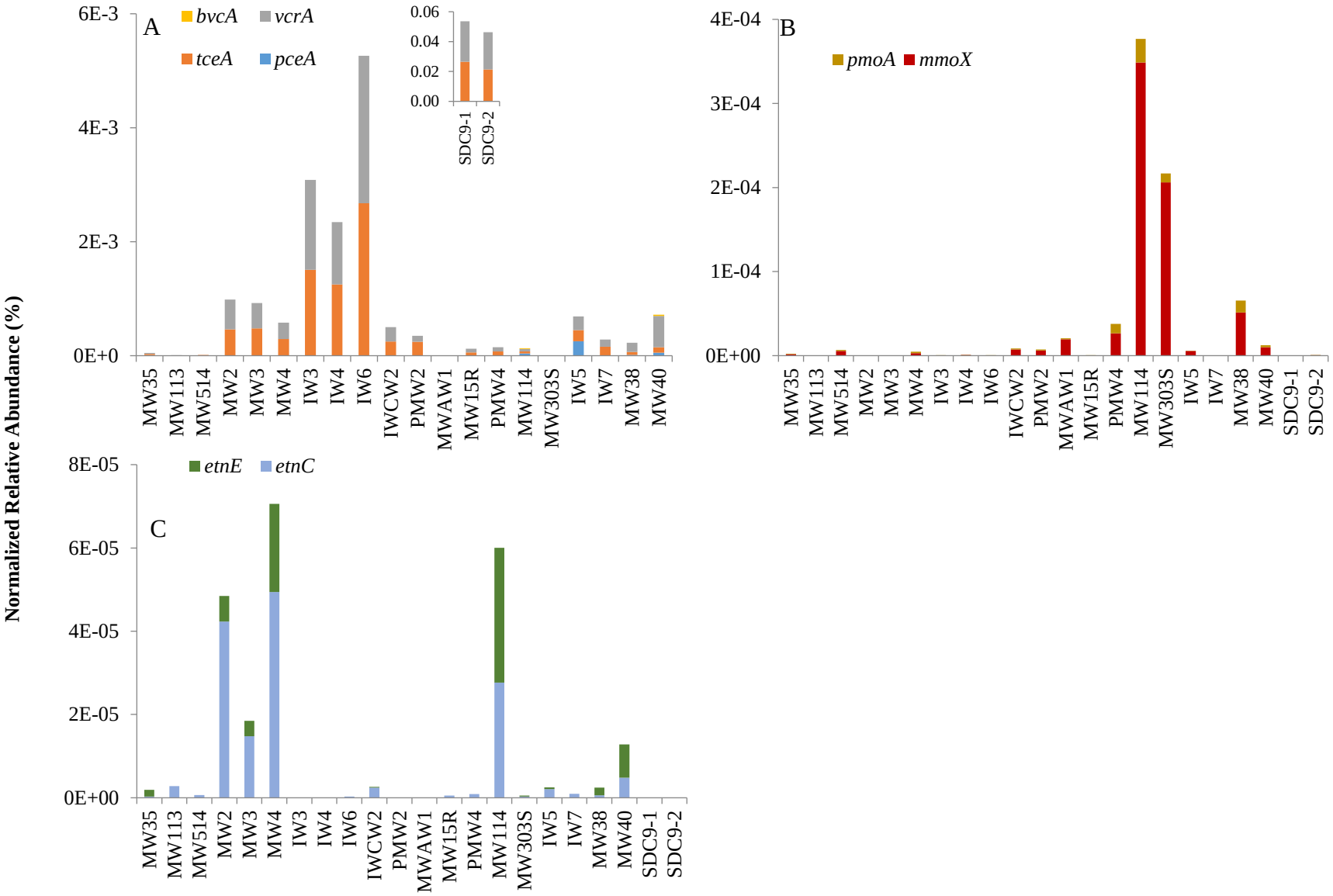


Figure 4. Normalized relative abundance (%, determined with DIAMOND) of genes associated with the chlorinated solvent reductive dechlorination (A) and the aerobic degradation of the chlorinated solvents (B, C) in SDC-9 (insert for A) and in groundwater from the five chlorinated solvent sites. The aerobic genes occurred at lower levels compared to the anaerobic genes. Note, the analysis approach differed from the approach used to generated figure 2, in that all sequences from the databases were compared to each dataset.

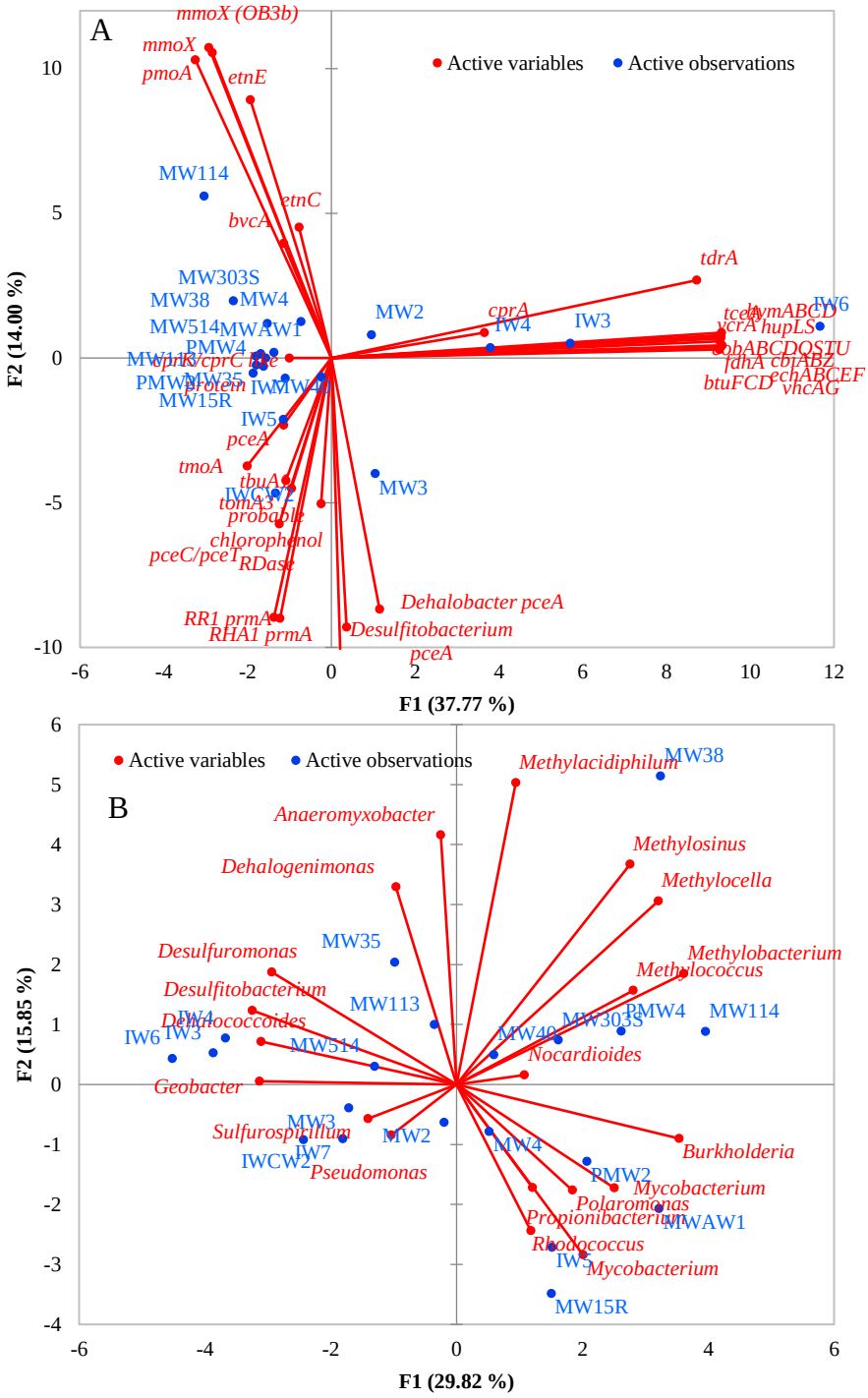


Figure 5. Principle component analyses of functional genes (A) and genera (B) associated with chlorinated solvent and 1,4-dioxane biodegradation in all groundwater samples.

Conclusions

The visual based SYBR green LAMP- MPN approach offers significant advantages compared to existing methods: time, cost and the potential *in situ* application. The use of centrifuged cells, instead of DNA, reduces the time and cost required for sample preparation (no DNA extraction). Also, compared to qPCR, the LAMP assay has a shorter run time and the visualization of amplification products is immediate. The assay requires only basic laboratory equipment (benchtop centrifuge and water bath), does not require an expensive real time thermal cycler. With additional development and validation, it is possible that the method could be applied in the field. Additionally, the dUTP-UNG system reduces the probability of false positives due to carry over contamination and increases the overall robustness of visual detection with SYBR green LAMP. The regression equations generated for SYBR green LAMP assay with MPN technique can be used to calibrate the assay to relate the data to traditional qPCR data.

In summary, LAMP would be beneficial at sites containing groundwater with higher humic acid contents, as LAMP amplification is less sensitive to inhibition, compared to qPCR. LAMP would be beneficial if funds for monitoring were limited, as the only equipment needed include an incubator and a waterbath. However, the individuals performing the assays would still need basic skills in microbiology/molecular methods. In comparison, qPCR requires an expensive thermal cycler. Further, LAMP can be performed without DNA extraction, which also reduces costs.

The work illustrates the importance of shotgun sequencing to provide a more complete picture of the functional abilities of microbial communities. The approach is advantageous over current methods because an unlimited number of functional genes can be quantified. Additional work should focus on RDase detection limits for shotgun sequencing data and comparisons to data generated with qPCR.

Appendices

A. Supplementary Material

Chapter 2

Supplementary Table 2.1. Direct amplification of *vcrA* gene with the microfluidic chip and Gene-Z™ device with varying amounts of filtration. The number of positive reaction wells over the number of replicate reaction wells is shown.

Concentration (cells L^{-1})	Range 1: no filtration	Range 2: 100 mL filtered	Range 3: 4 L filtered
10^4	NT	NT	NT
10^5	0/16	0/16	16/16
10^6	0/16	16/16	NT
10^7	0/16	16/16	NT
10^8	6/16	16/16	NT
10^9	16/16	16/16	NT
10^{10}	16/16	16/16	NT
10^{11}	16/16	NT	NT
10^{12}	16/16	NT	NT

NT: not tested

Supplementary Table 2.2. Geochemistry and contaminate concentration for both groundwater samples.

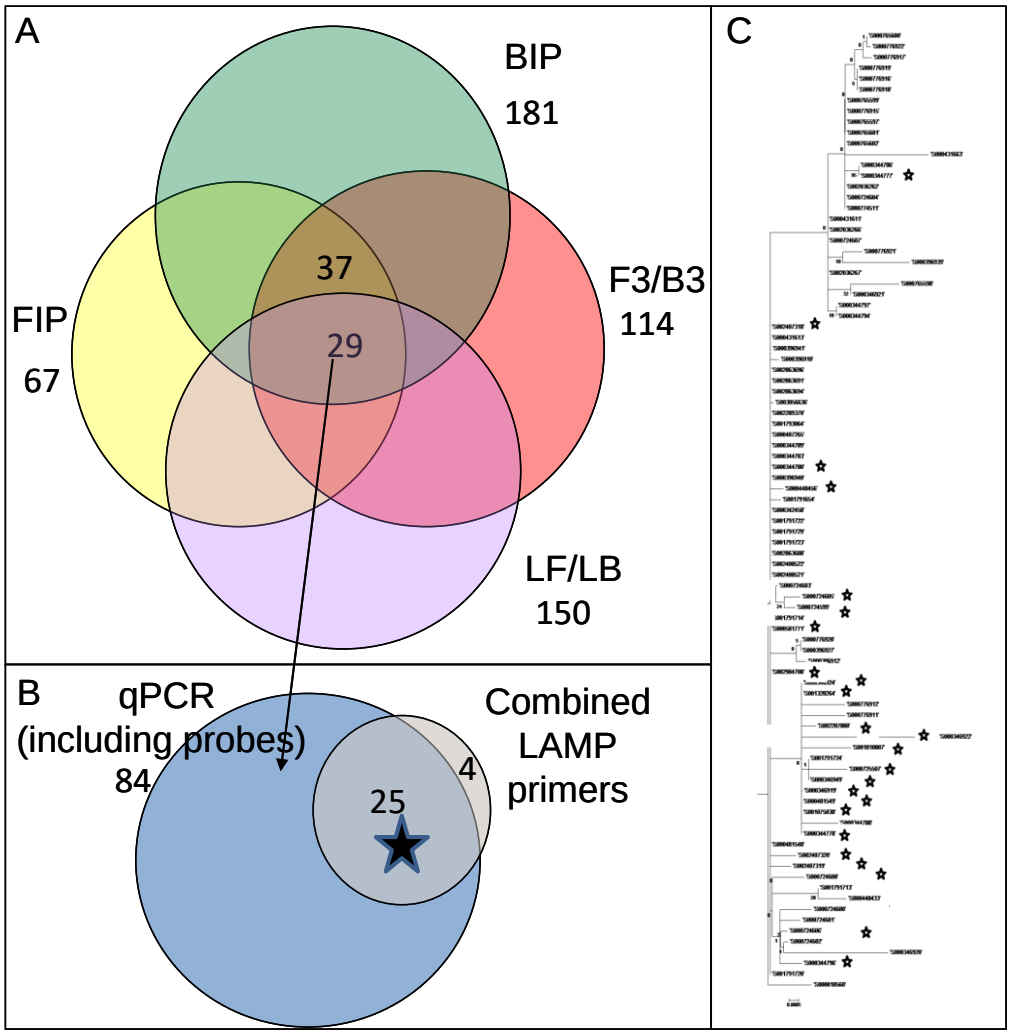
Parameter	HTOC groundwater	LTOC groundwater
Tetrachloroethene	140[c] μg L^{-1}	0.27[c] μg L^{-1}
Trichloroethene	140[c] μg L^{-1}	3.5 μg L^{-1}
cis-1,2-Dichloroethene	410,000 μg L^{-1}	450 μg L^{-1}
Vinyl chloride	90,000 μg L^{-1}	2.5 μg L^{-1}
Methane	2120[a] mg L^{-1}	0.250[b] mg L^{-1}
Ethene	2950[a] μg L^{-1}	0.500[b] μg L^{-1}
Ethane	105 μg L^{-1}	0.450[b] μg L^{-1}
Chloride	615 mg L^{-1}	40.6 mg L^{-1}
Nitrite	0.500[b] mg L^{-1}	0.500[b] mg L^{-1}
Sulfate	1.95 mg L^{-1}	97.5 mg L^{-1}
Nitrate	0.500[b] mg L^{-1}	6.27 mg L^{-1}
Lactic acid	1.00[b] mg L^{-1}	1.00[b] mg L^{-1}
Acetic acid	120 mg L^{-1}	1.00[b] mg L^{-1}
Propionic acid	34.4 mg L^{-1}	1.00[b] mg L^{-1}
Formic acid	0.648 mg L^{-1}	0.500[b] mg L^{-1}
Butyric acid	1.00[b] mg L^{-1}	1.00[b] mg L^{-1}
Pyruvic acid	1.00[b] mg L^{-1}	1.00[b] mg L^{-1}
Valeric acid	1.00[b] mg L^{-1}	1.00[b] mg L^{-1}

Temp (°C)	25.13	23.29
Electrical Conductivity (Mhos/cm)	3.742	1.223
pH	6.13	6.62
ORP (mV)	-100.9	113.9
Dissolved Oxygen (mg/L)	8.94	14.96
Turbidity (NTU)	-6.9	1.1

[a]Sample analyzed at dilution factor of 215.
[b]Compound not detected above method practical quantitation limit.
[c]Not detected. The analyte was analyzed for but not detected above the associated method detection limit

Supplementary Table 2.3. Assay used to test for inhibition in groundwater samples, LAMP primers targeting *cadA* gene of *L pneumophila*.

LAMP primers for *Legionella cadA* gene	
F3	GTCTTGCGATTCGAAGCT
B3	AAAAACCAAATCACCCACTT
FIP	CCAAGGTTTGCCATTGACCAT-ATGCAAATTGCCCCTGAG
BIP	GAGAGCGTATTCCCCTGGAT-ATTCACCAGTAATCGGCG
LF	CGTCCTGTTTCACTGAAGCC
LB	GGCGTGGTTATTTCAGGTCAGA



Supplementary Figure 2.1. Venn Diagram (unscaled) of DHC 16S rRNA sequences targeted by combined set of LAMP primers (A) versus combined set of qPCR primers (B), and (C) phylogenetic tree of alleles targeted by qPCR assay. Allele marked with a (☆) indicate sequences that are targeted by both qPCR and LAMP.

Chapter 4.

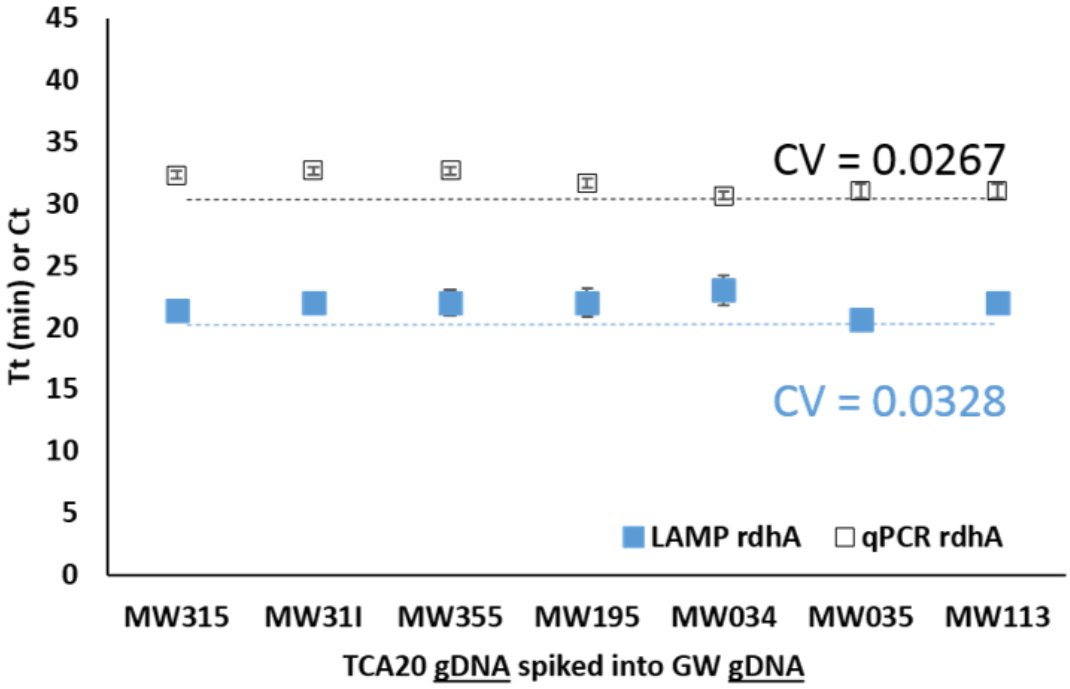
Supplementary Table 4.1. LAMP and qPCR primers used for experiments in this study. Degenerate bases are underlined.

Primer	Start Position	Sequence 5' - 3'
16S rRNA gene targeting *Dehalobacter*		
F3	182	GAGAAGAAAGCTGGCCTCTG
B3	394	GGCCTTCTTCATACACGCG
FIP	258-206	GATCGTCGCCTTGGTAGGCC-TGCTAGCGCTTAGGGATGG
BIP	302-363	GGCCACACTGGGACTGAGACA-TCAGACTTTCGTCCATTGCG
LF	228	CCAACTAGCTAATCAGACGCG
LB	342	AGGCAGCAGTGGGGAATCTTC
rdhA gene		
F3	1171	TTCGGTCCGAGAMWTCGC
B3	1340	TCGGMTACCTCAMMATCCT
FIP	1234-1192	ACTCGCGTACCCCGAATTTTYT-GCCAAAGTCTACACCGACC
BIP	1258-1321	TGCCGCCTGTGCAAAAAATGTG-CTGGCTGCAGAACCTTAGG
LF	1211	TGTCCGGAGCAAGTTCCA
LB	1295	CCCAGGCCATCTCCCAYGA
MIAC luc gene F3		
	1098	AGGACTCTGGTACAAAATCG
B3	1302	ACGTGAATTGCTCAACAGTA
FIP	1166-1120	ACGGATTACCAGGGATTTCAGTC-TTCATTAAAACCGGGAGGT
BIP	1235-1283	TGCACGTTCAAAATTTTTTGCAAC-GAACATTTCGCAGCCTAC
LF	1139	ACACGTTCGTCACATCTCATCT
LB	1259	CCCTTTTTGGAAACAAACACTACG
qPCR rdhA		
Forward		GCAGGAAGATTCTAAAACCTTG
Reverse		CACCGAGGTACTGGAAATGA
qPCR luc		
Forward		TACAACACCCCAACATCTTCGA
Reverse		GGAAGTTCACCGGCGTCAT

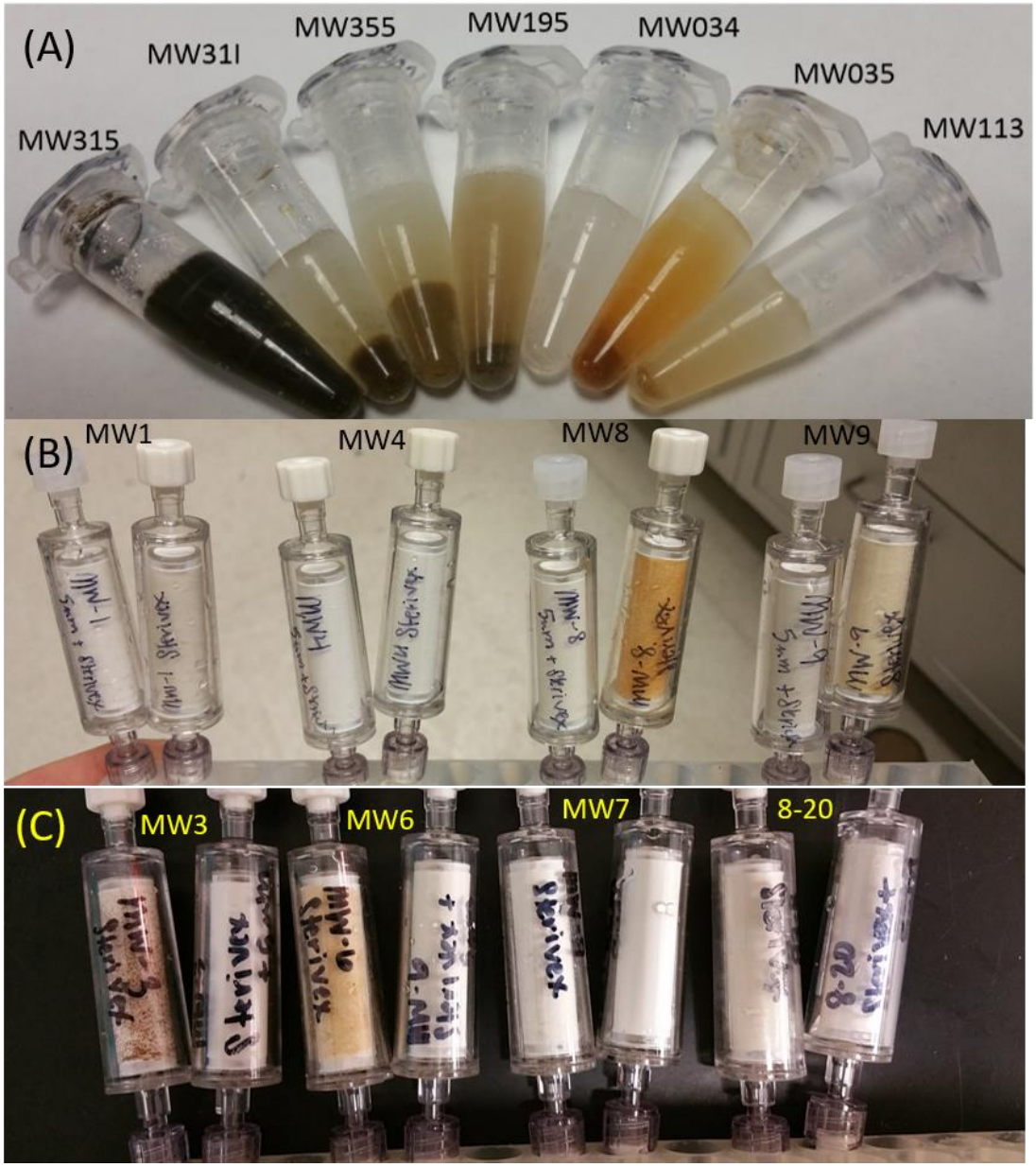
Supplementary Table 4.2. Testing selected LAMP assay specificity with gDNA from *Dehalobacter* and non-targeted organisms. Percent similarity is based on 16S rRNA gene of organisms or close relative if the 16S rRNA gene was not available in public databases.

16S rRNA gene classification	% Similarity to *Dehalobacter*	Organism	*rdhA* gene	16S rRNA gene

d: Bacteria, c: Clostridia, f: Peptococcaceae 1	-	*Dehalobacter* spp. (in CB&I TCA-20^{TM} culture)	+	+
d: Bacteria, c: Clostridia, f: Peptococcaceae 1	93.2%	*Syntrophobotulus glycolicus* (DSM 8271)	-	+
d: Bacteria, c: Deltaproteobacteria	79.3%	*Desulfacinum infernum* (DSM 9756)	-	-
d: Bacteria, c: Deltaproteobacteria	79.7%	*Desulfobacterium autotrophicum* (DSM 3382)	-	-
d: Bacteria, c: Deltaproteobacteria	82.8%	*Desulfomicrobium baculatum* (DSM 4028)	-	-
d: Bacteria, c: Deltaproteobacteria	85.1%	*Desulfonauticus submarinus* (DSM 15269)	-	-
d: Bacteria, c: Deltaproteobacteria	82.2%	*Syntrophobacter wolinii* (DSM 2805)	-	-
d: Bacteria, c: Thermodesulfobacteria	79.6%	*Thermodesulfobacterium commune* (DSM 2178)	-	-
d: Bacteria, c: Deltaproteobacteria	82.1%	*Thermodesulforhabdus norvegica* (DSM 9990)	-	-
d: Bacteria, c: Nitrospira	77.8%	*Thermodesulfovibrio yellowstonii* (DSM 11347)	-	-
d: Archaea	64.0%	*Methanococcus sp.*(DSM 8766)	-	-
d: Archaea	63.2%	*Methanosarcina sp.* (DSM 4659)	-	-



Supplementary Figure 4.1. C_t and T_t measured by spiking 5 ng of DNA extracted from TCA-20 into DNA extracted from the groundwater samples. CV indicates the coefficient of variation. Error bars represent standard error of three technical replicates. In some cases error bars are smaller than symbols.



Supplementary Figure 4.2. Picture of concentrated groundwater samples collected from remediation sites A) elution collected after concentration with Sterivex filters, B-C) Sterivex filters after passing 200 mL of eight groundwater samples with and without a 5 micron filter to remove suspended solids. In (B) Sterivex filters used after 5 micron filtration are on the left of each pair, in (C) Sterivex filters used after 5 micron filters are on the right side of each pair.

Chapter 5

Supplementary Table 5.1. Information on the 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

Supplementary Table 5.2. 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

Supplementary Table 5.3. 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

Supplementary Table 5.4. 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 x d_i x n_i	150	15	1.5	0.15	0.015	0.0015	0.00015
v_i x d_i x p_i	150	15	1.5	0.15	0.01	0.00075	0.000025
v_i x d_i	25	2.5	0.25	0.025	0.0025	0.00025	0.000025

Supplementary Table 5.5. 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 **x** d_i **x** n_i	150	15	1.5	0.15	0.015	0.0015	0.00015
v_i **x** d_i **x** p_i	150	15	1.5	0.15	0.0125	0.00125	0.000075
v_i **x** d_i	25	2.5	0.25	0.025	0.0025	0.00025	0.000025

Supplementary Table 5.6. 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 *vcrA*	**MPN *tceA***	**qPCR *vcrA***	**qPCR *tceA***	***vcrA***	***tceA***
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

Supplementary Table 6.1. The gene targets, assay type and template type used for each well and site.

Site	Well Name	Date	DNA Templates						Cells					
			qPCR DNA *tceA*	LAMP DNA *tceA*	qPCR DNA *vcrA*	LAMP DNA *vcrA*	qPCR DNA *bvcA*	LAMP DNA *bvcA*	Direct cells *tceA*	Direct cells *vcrA*	Direct cells *bvcA*	Centrifuged cells *tceA*	Centrifuged cells *vcrA*	Centrifuged cells *bvcA*
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
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 ND = template was not detected B1 = Batch 1
✓= Assay was performed X = Assay was not performed B2 = Batch 2

Supplementary Table 6.2. qPCR primer and probe sequences used for *vcrA*, *bvcA* and *tceA* in this study.

Target Gene	Primer	Sequence	Reference
vcrA	vcrA1022F	CGGGCGGATGCACTATTTT	[1]
	vcrA1093R	GAATAGTCCGTGCCCTTCCTC	[1]
	vcrA1042Probe	FAM-CGCAGTAACTCAACCATTTCCT GGTAGTGG-TAMRA	[1]
bvcA	bvcA925F	AAAAGCACTTGGCTATCAAGGAC	[1]
	bvcA1017R	CCAAAAGCACCACCAGGTC	[1]
	bvcA977Probe	FAM-TGGTGGCGACGTGGCTATGTGG-TAMRA	[1]
tceA	tceA1270F	ATCCAGATTATGACCCTGGTGAA	[2, 3]
	tceA1336R	GCGGCATATATTAGGGCATCTT	[2, 3]
	tceA1294Probe	FAM-TGGGCTATGGCGACCGCAGG-TAMRA	[23, 24]

Supplementary Table 6.3. LAMP primers for *vcrA*, *bvcA* and *tceA* used in this study.

Target Gene	Primer set	Primer	Sequence (5'→3')	Reference
vcrA	vcrA set C	F3	GTAAGTTTTACGCGAGATGG	[4]
		B3	GTCATCGGCTGAGCTTTC	
		FIP	ACCCTCCCATTTTGGTACGCTTGTA TGGTCCGCCACAT	
		BIP	AAGACAATTTTCTAATGCTGAGGGC ATTTGGGATCTGCCAGGT	
		LF	CATCAGGTGGCGCTGAA	
		LB	TGGTGCTGGTGGCGTT	
bvcA	bvcA Set A	F3	ACAATGCCTTTACCAGAAGA	[4]
		B3	ACCGTATTTGGGGCTGAT	
		FIP	TCGGCCTCCATTAAAAGCCATTCTC TAGGGTGGTCATGT	
		BIP	ATCAAGGACTTGGTGGCGACCTTGT TCGGAAAGACACTCA	
		LF	AGGCAATCATACTTGAAGCGTC	
		LB	TGTGGGGACCTGGTGGT	
tceA	tceA Set A	F3	GCCGTTTATTCCATTCATGG	[4]
		B3	GCATAGACTGGATGAAGGAA	
		FIP	ACATAATTGCTGGGAGAACCCG-TCGCATAGAGAGATAAGGCC	
		BIP	GCCATTCGTGGCGGCATATAT-CAGATTATGACCCTGGTGAA	
		LF	CTTTATGGACGCTATGAAGGTTCTA	
		LB	TCTTCCCTGCGGTCGCCATA	

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Supplementary Table 6.4. 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 #	*tceA* gene copies/L					*vcrA* 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

Supplementary Table 6.5. Time based comparison of qPCR and SYBR green LAMP assays

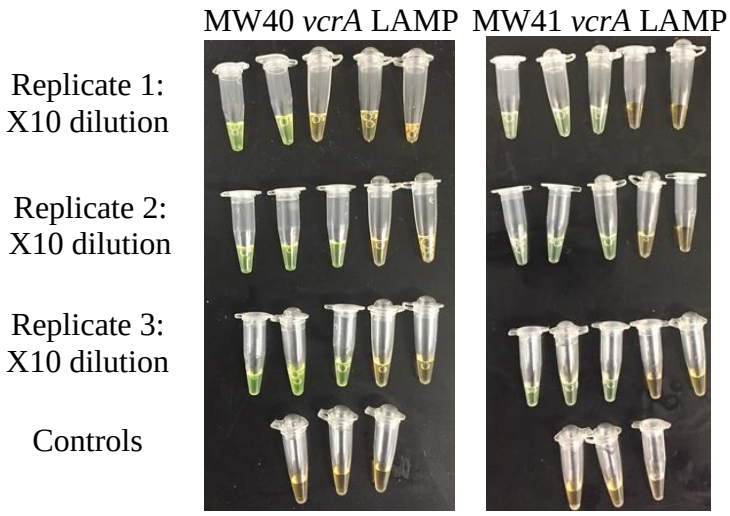
Process	**qPCR**	**SYBR Green LAMP**
Filtering	5-25 min. per sample	5-25 min. per sample
DNA extraction	35-45 min per sample	No DNA extraction
Centrifugation and template elution	No centrifugation or sample elution	<15 min per sample
Master Mix Preparation	<5 min. if commercial Master mix is used.	20-30 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)

Supplementary Table 6.6. 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)

[a]MO BIO PowerWater DNA Isolation Kit ($465 for 50 preps)



Supplementary Figure 1. Examples of SYBR green LAMP assays for *vcrA* with triplicates of X 10 dilutions of centrifuged cells from groundwater from the Indian Head site. The dilution levels increase to the right.

Chapter 7

Background on Functional Genes

The abundance of the genes associated with reductive dechlorination were examined from the genera *Dehalococcoides, Dehalogenimonas, Desulfitobacterium, Dehalobacter, Geobacter, Sulfurospirillum* and *Anaeromyxobacter*. The genes encoding for enzymes associated with aerobic VC degradation were also targeted (*etnC*/alkene monooxygenase and *etnE*/epoxyalkane:CoM transferase) [1-5]. Also, the gene encoding for cytochrome P450 from *Polaromonas* JS666 was investigated, as this initializes the degradation of *cis*-1,2-dichoroethene [6]. The genes encoding for the α subunits of soluble and particulate methane monooxygenases (*mmoX* and *pmoA*) were examined due to their role in chlorinated ethene degradation [7-9]. The genes encoding the enzymes associated with 1,4-dioxane biodegradation (as summarized in [10]) were also investigated. This chemical is a probable human carcinogen [11] and is frequently detected at sites where the chlorinated solvents are present [12-14]. Finally, the genes encoding for enzymes associated with hydrogen metabolism (*fdhA, hupSL, vhcAG, hymABCD* and *echABCEF*) and corrinoid metabolism (*btuFCD, cobA, cobB, cobC, cobD, cobQ, cobS, cobT, cobU, cbiA, cbiB* and *cbiZ*) in *D. mccartyi* were also quantified [15].

Collection of Sequences for Functional Genes

A primary source for RDases was the Functional Gene Pipeline and Repository (FunGene) [16] website using the link 'vcrA_ver2'. For this, the contents (e.g. score, protein and nucleotide accession numbers, microorganism name, length of the protein) of the topmost 3000 sequences from 'vcrA_ver2' were exported into excel. Then, accession number lists of RDases for each species were created by setting a filter in Excel. Each accession number was checked for accuracy using complete genome information (NCBI) (Supplementary Table 3). The protein sequence fasta files were also downloaded from FunGene (again by selecting the same 3000 sequences of 'vcrA_ver2'). The accession number RDase lists were used to collect RDases reference protein sequences from the protein sequence fasta files (from FunGene) using a tool (Readseq.jar) developed by Ribosomal Database Project (https://github.com/rdpstaff/RDPTools). The overall process produced individual RDase protein sequence files for each microorganism.

A number of microorganisms (including *Dehalococcoides mccartyi JNA, SG1, Sulfurospirillum strains, Anaeromyxobacter dehalogenans 2CP-C, Geobacter lovleyi SZ, Dehalobacter E1 and FTH1, Desulfitobacterium sp. PCE1, Desulfitobacterium hafniense TCP-A and PCP-1* and *Polaromonas sp. JS666*) did not have functional gene data on

FunGene, therefore their reference sequences were collected manually by downloading the fasta files from NCBI complete genomes (Supplementary Table 3).

The *vcrA* reference sequences (39 *vcrA* sequences, protein fasta files) were collected from the link 'vcrA' in FunGene by selecting those sequences with a score higher than 900 (Hidden Markov Model score alignment by FunGene). The *tceA* and *bvcA* reference sequences were collected using both NCBI and FunGene. Protein sequences (AAN85588, AAT64888) previously used for designing *tceA* and *bvcA* primers [17] were first used to collect sequences from the NCBI database using BLAST [18]. Sequences with a maximum score higher than 900 from the BLAST search were collected (31 *tceA* sequences, 13 *bvcA* sequences). In FunGene, using the 'Probe Match Search' function, primers for *tceA* (TceA1270F, TceA1336R and TceA1294Probe) and *bvcA* (Bvc925F, Bvc1017R and Bvc977Probe) [17] were used to search the first 3000 sequences of 'vcrA_ver2', producing 38 *tceA* sequences and 11 *bvcA* sequences. The *tceA* and *bvcA* sequences from the two sources were compared. The *tceA* sequences from NCBI (except ADV18463) were all present in the *tceA* sequences obtained from FunGene. Therefore, the final *tceA* reference list consisted of the sequences from FunGene along with ADV18463. A similar approach was used for generating the *bvcA* reference list.

Sequences for *pceA* (5 sequences) and *fdrA* (30 sequences) from *Dehalococcoides mccartyi* were collected by downloading the fasta files from the NCBI complete genomes. The sequence for the putative VC RDase (*cerA*) from *Dehalogenimonas* [19] was kindly provided by Dr. Frank Loeffler (Locus Tag JP09_004725, Protein ID PPD58423.1).

Reference sequences for *etnC* and *etnE* (31 *etnC* sequences, 95 *etnE* sequences) were collected from FunGene using scores higher than 700 and 500, respectively. Additionally, primers for *etnC* (RTC_F (*etnC*) and RTC_R (*etnC*)) and *etnE* (RTC_F (*etnE*) and RTC_R (*etnE*)) [20] were used with the 'Probe Match Search' function in FunGene to search for sequences in all pages of 'etnC' and 'etnE', resulting in 9 *etnC* sequences, 31 *etnE* sequences. Reference sequences for *etnC* and *etnE* (20 *etnC* sequences, 53 *etnE* sequences) were also collected from UniProt. The final *etnC* and *etnE* reference sequences were generated by combining all data sets discussed above.

mmoX and *pmoA* reference sequences (21 *mmoX* sequences, 30 *pmoA* sequences) were first collected using 'mmoX' and 'pmoA' links in FunGene (sequences with a score higher than 980 and 500, respectively). Additionally, all other sequences annotated as 'mmoX' or 'soluble methane monooxygenase' in all pages of *mmoX* in FunGene were also collected. For this, information, such as score, protein and nucleotide accession number,

name of the microorganism, length of the protein, was imported to excel. Then, a filter in excel was set for the name of the gene to create an accession number list for *mmoX*. The accession number list was used to collect reference protein sequences from protein sequences downloaded from FunGene using Readseq.jar (generating 580 sequences). Sequences annotated as '*pmoA*' or 'particulate methane monooxygenase' were also collected using methods similar to those described from *mmoX* (generating 8327 sequences).

A list of functional genes (12 sequences) associated with 1,4-dioxane metabolism or cometabolism was obtained from a recent publication [10]. The protein sequences of these genes were then collected from NCBI. The functional genes associated with hydrogen and corrinoid metabolism in *D. mccartyi* were also examined. Reference sequences for all hydrogenase (*hupLS*, *vhcAG*, *echABCEF* and *hymABCD*) and corrinoid (*btuFCD*, *cbiABZ* and *cobABCDQSTU*) metabolism genes were collected by using NCBI BLAST search. Additional information on the collection of sequences associated with hydrogen and corrinoid metabolism is provided in the supplementary section.

Sequences ACZ61293.1 and ACZ61294.1 from *D. mccartyi* VS were used for starting the BLAST search for *hupL* and *hupS*, separately. Then *hupL* and *hupS* reference sequences (13 *hupL* sequences, 6 *hupS* sequences) were collected with an identity > 95% and >94%, respectively. All identity values were selected because of the large identity decrease after the last selected reference sequences. *vhcA* and *vhcG* reference sequences (9 *vhcA* sequences, 8 *vhcG* sequences) were collected with an identity > 90%. The sequences used for the BLAST search were ACZ61705.1 and ACZ61704.1 from *D. mccartyi* VS. *hymA1* and *hymA2* reference sequences (4 *hymA1* sequences, 5 *hymA2* sequences) were collected with an identity > 98% and >96%, respectively. The sequences used for starting the BLAST search were ACZ61326.1 and ACZ61777.1 from *D. mccartyi* VS. *hymB1* and *hymB2* (3 *hymB1* sequences, 19 *hymB2* sequences) were collected with an identity > 98% and > 97%, respectively. The sequences used for starting the BLAST search were ACZ61327.1 and ACZ61778.1 from *D. mccartyi* VS. *hymC1* and *hymC2* (15 *hymC1* sequences, 15 *hymC2* sequences) were collected with an identity >96% and >87%, respectively. The sequences used to start the BLAST search were ACZ61328.1 and ACZ61779.1 from *D. mccartyi* VS. *hymD1* (11 *hymD1* sequences) was collected with an identity > 89%. The sequence used to start the BLAST search was ACZ61329.1 from *D. mccartyi* VS.

Additional *hymABC* genes were found in *D. mccartyi* 195 and following the similar nomenclature for the genes, they were named *hymA3, A4, B3* and *C3*. *hymA3* and *hymA4* (9 *hymA3* sequences, 13 *hymA4* sequences) were collected with identities > 98% and > 93%,

respectively. The sequences for the BLAST search were AAW39863.1 and AAW40249.1. *hymB3* (18 sequences) was collected with an identity > 94%. The sequence used for starting the BLAST search was AAW39862.1 *hymC3* (13 sequences) was collected with an identity > 90%. The sequence used for starting the BLAST search was AAW39861.1

The sequences used for starting BLAST search for *echABCEF* were from *D. mccartyi* CBDB1 with accession number of CAI82985.1, CAI82986.1, CAI82987.1, CAI82992.1 and CAI82993.1. *echABCEF* reference sequences (23 *echA* sequences, 16 *echB* sequences, 7 *echC* sequences, 10 *echE* sequences, 11 *echF* sequences) were collected with an identity > 92%, 94%, 94%, 96% and 84%, respectively. The sequences used for starting BLAST search for *btuFCD* were from *D. mccartyi* DCMB5 with accession number of AGG06280.1, AGG06281.1 and AGG06282.1. *btuFCD* reference sequences (17 *btuF* sequences, 14 *btuC* sequences, 6 *btuD* sequences) were collected with an identity > 89%, 93% and 93%, respectively.

The sequences used for starting BLAST search for *cbi* were from *D. mccartyi* VS. *cbiA* and *cbiB* reference sequences (16 *cbiA* sequences, 13 *cbiB* sequences) were collected both with an identity > 90%. The sequences used for starting the BLAST search were ACZ61308.1 and ACZ61741.1.

There were four *cbiZ* sequences from *D. mccartyi* VS (hereafter named *cbiZ1234*). The accession number of the sequences of *cbiZ1234* for the BLAST search were ACZ61242.1, ACZ61249.1, ACZ61740.1 and ACZ62455.1. *cbiZ1234* reference sequences (16 *cbiZ1* sequences, 11 *cbiZ2* sequences, 10 *cbiZ3* sequences, 46 *cbiZ4* sequences) were collected with an identity > 72%, 97%, 92% and 87%, respectively.

The majority of the sequences used for starting BLAST search for *cob* were from *D. mccartyi* VS, with one from *cobC* from *D. mccartyi* CBDB1. The accession number of sequences used for starting BLAST search for *cobA123* were with of AAW40449.1, AAW39561.1 and AAW39547.1. cobA123 reference sequences (8 *cobA1* sequences, 14 *cobA2* sequences, 14 *cobA3* sequences) were collected with an identity > 91%, 92% and 94%, respectively.

The accession number of sequences used for starting BLAST search for *cobBCQ* were with of AAW40541.1, CAI82815.1 and AAW39791.1. *cobBCQ* reference sequences (16 *cobB* sequences, 10 *cobC* sequences, 20 *cobQ* sequences) were collected with an identity > 89%, 90% and 92%, respectively. *cobD1* and *cobD4* reference sequences (11 *cobD1* sequences, 17 *cobD4* sequences) were collected with an identity > 84% and 80%, respectively. The sequences used for starting the BLAST search were AAW40448.1 and

AAW39562.1. *cobS1*, *cobT1* and *cobU1* reference sequences (14 *cobS1* sequences, 12 *cobT1* sequences, 14 *cobU1* sequences) were collected with an identity > 90%, 94% and 90%, respectively. The sequences used for starting the BLAST search were AAW40093.1, AAW40094.1 and AAW40091.1.

The BLAST search of *cobD2* and *cobD3* generated the same results as *cbiB*. Also, the BLAST results of *cobS2*, *cobT2* and *cobU2* were the same as those of *cobS1*, *cobT1* and *cobU1*, respectively. Therefore, the results of *cobD2*, *cobD3 cobS2*, *cobT2* and *cobU2* were not included in the analysis.

Library Preparation, Sequencing, MG-RAST and DIAMOND analysis

The Takara ThruPLEX low input DNA library preparation kit was used to generate libraries based on manufacturer's recommendations. Completed libraries were subject to quality control and quantification using a combination of Qubit dsDNA HS and Caliper LabChipGX HS DNA assays. All libraries were pooled in equimolar amounts to a maximum usable volume based on quantification obtained using the Kapa Biosystems Illumina Library Quantification qPCR kit. This pool was loaded on one lane of an Illumina HiSeq 4000 flow cell and sequenced in a 2x150 bp paired end format. Base calling was performed by Illumina Real Time Analysis (RTA) v2.7.6 and output of the RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.18.0.

The Meta Genome Rapid Annotation using Subsystem Technology (MG-RAST) [21] version 4.0.2. was used for the taxonomic analysis of the metagenomes. The processing pipeline included merging paired end reads, SolexaQA [22] to trim low-quality regions from FASTQ data and dereplication to remove artificial duplicate reads. Gene calling was completed using FragGeneScan [23]. For taxonomic profiles, the best hit classification at a maximum e-value of $1e^{-5}$, a minimum identity of 60% and a minimum alignment length of 15 against the ReqSeq database [24] were used. The MG-RAST plugin Krona was used to illustrate the taxonomic composition of each sample [25]. MG-RAST was used to generate rarefaction curves. MG-RAST ID numbers and pre- and post- QC sequencing data have been summarized (Supplementary Table 2) and the datasets are publicly available on MG-RAST. The following chlorinated solvent degrading genera were investigated in the MG-RAST data: *Anaeromyxobacter* [26], *Dehalococcoides* [27-32], *Polaromonas* [6, 33], *Nocardioides* [4, 34], *Desulfitobacterium* [35-38], *Geobacter* [39], *Sulfurospirillum* [40-42], *Dehalobacter* [43-45], *Desulfomonile* [46, 47], *Desulfuromonas* [48, 49], *Propionibacterium* [50], *Mycobacterium* [1, 51], *Dehalobacter* [44, 52], *Desulfomonile,* [53] and *Dehalogenimonas* [54-57].

DIAMOND (double index alignment of next-generation sequencing data) [58] was used as the alignment tool for all functional genes. The collected protein sequences from same species or with the same function were aligned to themselves for dereplication (removing sequences with 100% identity) and one representative sequence was left as the reference for that group. Then, low quality sequences and Illumina adapters sequences were removed using Trimmomatic [59]. The shotgun reads were then aligned to the dereplicated references for each groundwater sample and SDC-9 using DIAMOND. Only reads that exhibited an identity of ≥ 90% and an alignment length ≥ 49 amino acids to the reference sequences were counted as aligned reads to each sequence. For each, relative abundance values were calculated using the number of aligned reads divided by the total number of sequences for each sample. The relative abundance values were then normalized by (divided by) the number of dereplicated reference sequences for each gene to produce normalized relative abundance values. Details concerning qPCR targeted towards *vcrA* are provided in the Supplementary Section.

vcrA qPCR

The PCR tubes (20 µL reactions) contained 10 µL iTaq Universal Probe Supermix (Bio-Rad), 1.2 µL TaqMan probe [17, 60] and balance water to 18 µL. PCR amplifications were performed in three stages: 1) 95 °C for 15 min, 2) 40 cycle of 95 °C for 15 s, 58 °C for 1 min, 3) a slow ramp of 1% to 95 °C for 15 s and 58 °C for 15 s. DNA templates and plasmid standards (containing a partial *vcrA* gene in a GenScript plasmid) were added to each reaction as 2 µL aliquots. All qPCR experiments were performed in a bench top thermal cycler (C1000 Touch Thermal Cycler, Bio-Rad).References

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Supplementary Table 7.1. Groundwater and sampling data.

Site	Date Bioaugmented	Date Sampled	Months between Inoculation & Sampling	Basic Geochemistry	Carbon Source	cVOCs at time of sampling (µg/L)
Quantico, VA	12/2/2015	5/17/2016	6.5	pH 5-8 ORP: -100 to 0 Dis Fe: 0-140 mg/L	None, H_2 generated via proton reduction	VC – 0-60 Cis-DCE - 0-120
Tulsa, OK	8/2013	6/9/2015	22	pH 6.1-7.1 ORP: -64 to 248 Dis Fe: 0-110 mg/L	EOS	VC – 60-830 Cis-DCE – 110-1500 TCE – 200-9000 Trans-DCE – 3-17 1,1-DCE – 5-1400 1,2-DCA – 1-34 1,1-DCA – Trace levels (<5) 1,4-Dioxane – 78-220
Indian Head, MD	9/23/15	6/22/2016	9	pH 6.2-8.9 ORP: 37 to -326	lactate	VC – 0-29 Cis-DCE – 0-178 TCE – 0-40 Carbon Disulfide – 1-8 MEK – 0-8
San Antonio, TX	113 & 514: 10/7 & 10/8/2014 35: 10/17 & 10/30/2014	7/28/2016	20	pH 5.6-6.8 ORP: -32 to 237	EVO	VC – 2.5-8.4 Cis-DCE – 2.5-7.4 TCE – 0-2
Edison, NJ	7/8/2009	11/10/2015	76	Overall: pH 5-6.5 ORP: -100 to 3 Dis Fe: 0.7 – 13 mg/L MW-114: pH 6.0 ORP: -70 Dis Fe: 5.19 mg/L MW-303S: pH 6.1 ORP: -80 Dis Fe: 2.05 mg/L	Lactate, yeast extract, potassium bicarbonate	Overall: VC – 0-1170 Cis-DCE – 0-1190 TCE – 0-8 1,2,4-Trimethylbenzene – 0-8. Trace levels (<5) of benzene, MTBE, ethylbenzene, xylenes, isopropyl benzene, 1,3,5-trimethylbenzene, sec-butylbenzene, 1,1-DCE, tDCE MW-114: VC – 83 Cis-DCE – 70 TCE – 8 MW-303S: VC – 2J Cis-DCE – 4J

Supplementary Table 7.2. Groundwater and SDC-9 MG-RAST sequence analysis data.

Location	Monitoring Well	MG-RAST ID #	Pre QC Sequence Count	Post QC Sequence Count	Post QC Mean Length
SDC-9	Culture-1	mgm4795922.3	6,845,624	5,090,799	236 ± 37 bp
	Culture-2	mgm4795924.3	6,181,247	4,478,198	229 ± 39 bp
San Antonio, TX	MW35	mgm4795328.3	5,301,996	4,513,530	239 ± 35 bp
	MW113	mgm4795332.3	6,185,927	5,404,716	239 ± 35 bp
	MW514	mgm4795329.3	5,934,109	4,847,401	240 ± 35 bp
Tulsa, OK	MW2	mgm4795334.3	5,691,547	4,714,786	239 ± 35 bp
	MW3	mgm4795333.3	6,872,780	5,425,995	227 ± 38 bp
	MW4	mgm4795342.3	6,200,534	5,327,773	238 ± 35 bp
	IW3	mgm4795340.3	5,889,710	4,891,993	241 ± 34 bp
	IW4	mgm4795341.3	6,938,129	5,228,938	229 ± 38 bp
	IW6	mgm4795673.3	7,800,767	6,112,693	230 ± 38 bp
Quantico, VA	MWCW2	mgm4795675.3	5,171,923	3,998,002	241 ± 35 bp
	PMW2	mgm4795339.3	662,422	471,513	231 ± 39 bp
	MWAW1	mgm4795335.3	233,177	157,539	226 ± 38 bp
	MW 15R	mgm4795679.3	6,710,609	5,018,326	241 ± 35 bp
	PMW4	mgm4795678.3	5,429,417	4,433,348	241 ± 35 bp
Edison, NJ	MW114	mgm4795676.3	6,174,464	5,242,089	240 ± 35 bp
	MW303S	mgm4795677.3	5,824,346	5,008,287	239 ± 35 bp
Indian Head, MD	IW5	mgm4795927.3	5,674,151	4,837,429	241 ± 34 bp
	IW7	mgm4795847.3	2,036,212	1,547,247	219 ± 41 bp
	MW38	mgm4795845.3	5,832,647	5,071,187	240 ± 35 bp
	MW40	mgm4795846.3	5,265,951	4,480,623	241 ± 35 bp

Supplementary Table 7.3. Genomes used for collecting functional protein sequences.

Organism/Name	Strain	Size (Mb)	GC%	Replicons	WGS	Gene	Protein	# of RDase	Release Date
Dehalococcoides mccartyi	195	1.46972	48.9	chromosome:NC_002936.3/CP000027.1	-	1582	1497	19	10/3/2001
Dehalococcoides mccartyi	CG5	1.36215	47.2	chromosome:NZ_CP006951.1/CP006951.1	-	1459	1395	25	8/4/2014
Dehalococcoides mccartyi	CBDB1	1.3955	47	chromosome:NC_007356.1/AJ965256.1	-	1479	1412	32	8/19/2005
Dehalococcoides mccartyi	BAV1	1.34189	47.2	chromosome:NC_009455.1/CP000688.1	-	1444	1374	10	5/7/2007
Dehalococcoides mccartyi	VS	1.41346	47.3	chromosome:NC_013552.1/CP001827.1	-	1505	1432	37	12/3/2009
Dehalococcoides mccartyi	GT	1.36015	47.3	chromosome:NC_013890.1/CP001924.1	-	1468	1399	20	2/17/2010
Dehalococcoides mccartyi	DCMB5	1.4319	47.1	chromosome:NC_020386.1/CP004079.1	-	1524	1461	23	2/22/2013
Dehalococcoides mccartyi	BTF08	1.45233	47.3	chromosome:NC_020387.1/CP004080.1	-	1556	1485	20	2/22/2013
Dehalococcoides mccartyi	GY50	1.40742	47	chromosome:NC_022964.1/CP006730.1	-	1499	1427	26	11/26/2013
Dehalococcoides mccartyi	CG4	1.38231	48.7	chromosome:NZ_CP006950.1/CP006950.1	-	1470	1401	13	8/4/2014
Dehalococcoides mccartyi	CG1	1.48668	46.9	chromosome:NZ_CP006949.1/CP006949.1	-	1600	1527	32	8/4/2014
Dehalococcoides mccartyi	IBARAKI	1.45106	47	chromosome Unknown:NZ_AP014563.1/AP014563.1	-	1556	1471	28	9/9/2015
Dehalococcoides mccartyi	11a5	1.46791	46.87	chromosome:NZ_CP011127.1/CP011127.1 plasmid pDhc6:NZ_CP011128.1/CP011128.1	-	1587	1521	30	4/5/2016
Dehalococcoides mccartyi	CG3	1.52129	46.9	chromosome:NZ_CP013074.1/CP013074.1	-	1657	1589	20	12/6/2016
Dehalococcoides mccartyi	KBTCE2	1.3292	49.1	chromosome:NZ_CP019865.1/CP019865.1	-	1431	1364	4	2/28/2017
Dehalococcoides mccartyi	KBDCA1	1.42846	47.4	chromosome:NZ_CP019867.1/CP019867.1	-	1563	1483	6	2/28/2017
Dehalococcoides mccartyi	KBDCA2	1.39432	47.5	chromosome:NZ_CP019868.1/CP019868.1	-	1523	1443	6	2/28/2017
Dehalococcoides mccartyi	KBTCE3	1.2716	49.3	chromosome:NZ_CP019866.1/CP019866.1	-	1361	1295	4	2/28/2017
Dehalococcoides mccartyi	KBDCA3	1.33749	47.6	chromosome:NZ_CP019946.1/CP019946.1	-	1441	1372	7	3/6/2017
Dehalococcoides mccartyi	KBVC2	1.33773	47.2	chromosome:NZ_CP019969.1/CP019969.1	-	1440	1378	16	3/9/2017
Dehalococcoides mccartyi	KBVC1	1.3599	47.3	chromosome:NZ_CP019968.1/CP019968.1	-	1456	1393	21	3/9/2017
Dehalococcoides mccartyi	KBTCE1	1.38891	47.3	chromosome:NZ_CP019999.1/CP019999.1	-	1502	1441	16	3/15/2017
Dehalococcoides mccartyi	UCH-ATV1	1.38778	48.8	chromosome:NZ_AP017649.1/AP017649.1	-	1489	1408	15	7/8/2017
Dehalococcoides mccartyi	MB	1.57151	48.3	-	JGYD01	1711	1614	27	12/4/2015
Dehalococcoides mccartyi	11a	1.32452	47.2	-	JGVX01	1415	1339	8	12/4/2015
Dehalococcoides mccartyi	JNA	1.46251	47.1	-	JSWM01	1582	1515	26	1/15/2016
Dehalococcoides mccartyi	SG1	1.42874	47.1	-	JPRE01	1535	1467	28	8/18/2014
Dehalococcoides mccartyi	WBC-2	1.37458	47.4	chromosome:CP017572.1	-	1466	1386	15	10/12/2016
Dehalococcoides mccartyi	EV-VC	1.4716	46.8	-	LZFK01	1535	1475	28	7/1/2016
Dehalococcoides mccartyi	EV-TCE	1.3573	48.5	-	LZFJ01	1451	1369	11	7/1/2016
Dehalogenimonas lykanthroporepellens	BL-DC-9	1.68651	55.5	chromosome:NC_014314.1/CP002084.1	-	1732	1650	20	1/28/2014
Dehalogenimonas formicexedens	NSZ-14	2.092789	54	chromosome:NZ_CP018258.1/CP018258.1	-	2176	2091	25	10/17/2017
Dehalogenimonas alkenigignens	IP3-3	1.85	55.9	-	LFDV01	1940	1856	29	6/23/2016
Dehalogenimonas	WBC-2	1.72573	49.2	chromosome:CP011392.1	-	1800	1721	22	5/8/2015
Geobacter lovleyi	SZ	0.077113	52.97	chromosome:NC_010815.1/CP001090 plasmid pGLOV01: NC_010815.1/CP001090.1	-	3640	3552	2	6/26/2016
Sulfurospirillum	SL2-1	2.87654	38.7	chromosome:NZ_CP021416.1/CP021416.1	-	2943	2701	2	5/31/2017
Sulfurospirillum	JPD-1	2.81409	38.8	chromosome:NZ_CP023275.1/CP023275.1	-	2882	2793	2	9/18/2017
Sulfurospirillum	SL2-2	2.87661	38.7	chromosome:NZ_CP021979.1/CP021979.1	-	2924	2699	2	6/22/2017

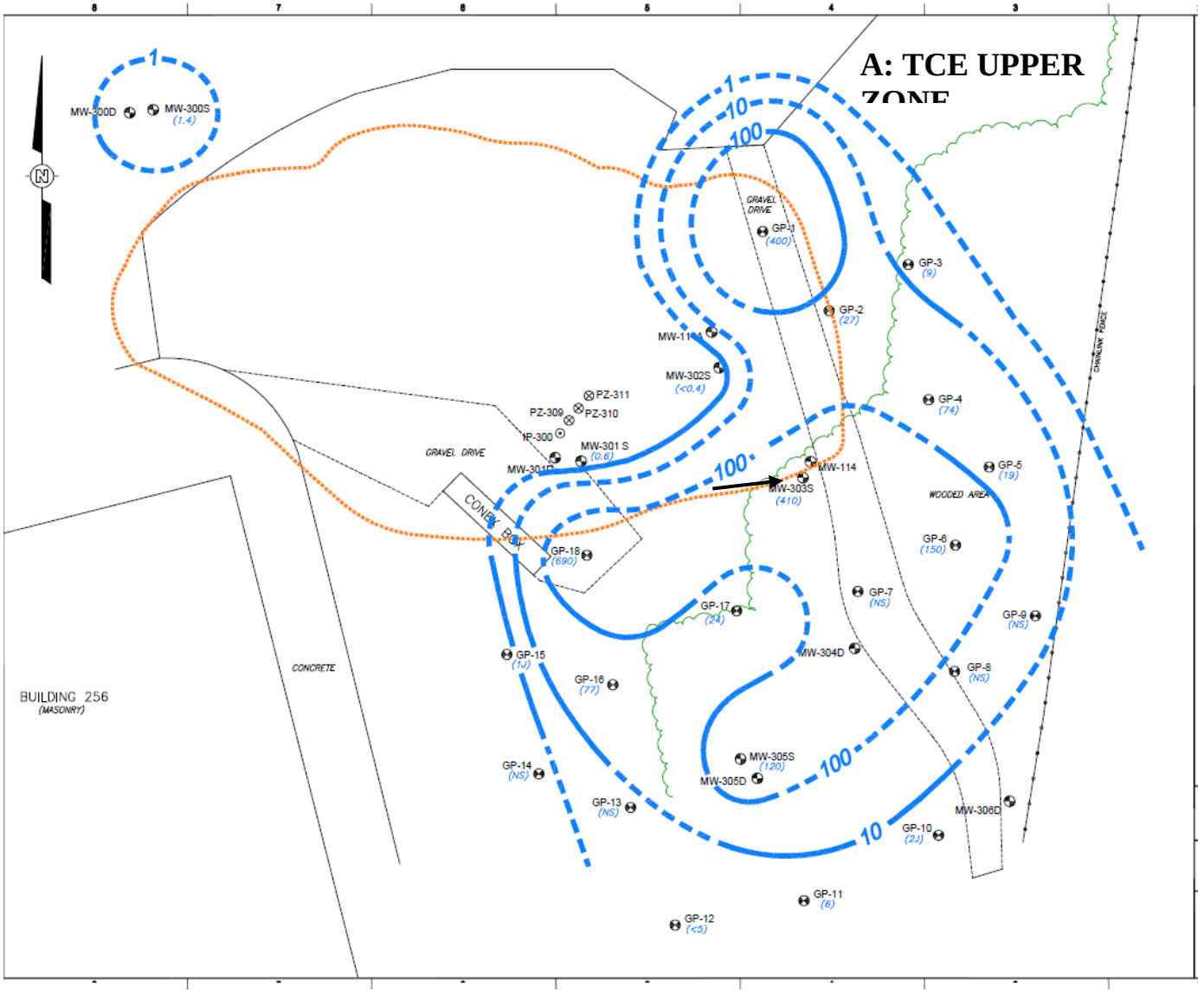
Sulfurospirillum halorespirans	DSM 13726	3.03	41.3	chromosome:NZ_CP017111.1/CP017111.1	-	3035	2967	2	9/8/2017
Sulfurospirillum multivorans	DSM 12446	3.18	40.9	chromosome:NZ_CP007201.1/CP007201.1	-	3288	3186	2	4/29/2015
Anaeromyxobacter dehalogenans	2CP-C	5.01348	74.9	chromosome:NC_007760.1/CP000251.1	-	4522	4416	2	1/27/2006
Dehalobacter restrictus	DSM 9455	2.94	44.6	chromosome:NZ_CP007033.1/CP007033.1	-	2848	2647	23	5/14/2014
Dehalobacter	DCA	3.06995	44.6	chromosome:NC_018866.1/CP003869.1	-	2974	2848	18	10/16/2012
Dehalobacter	CF	3.09205	44.3	chromosome:NC_018867.1/CP003870.1	-	2985	2882	18	10/16/2012
Dehalobacter	E1	2.95026	43.8	-	CANE01	2866	2719	7	9/19/2012
Dehalobacter	FTH1	6.32936	58.9	-	AQYY01	5934	5727	33	4/19/2013
Dehalobacter	UNSWDHB	3.20156	44.9	-	AUUR01	3105	2944	19	8/9/2013
Dehalobacter	TeCB1	3.13322	44	-	MCHF01	3106	2961	24	8/18/2016
Desulfitobacterium hafniense	DCB-2	5.27913	47.5	chromosome:NC_011830.1/CP001336.1	-	5038	4821	7	1/5/2009
Desulfitobacterium hafniense	Y51	5.72753	47.4	chromosome:NC_007907.1/AP008230.1	-	5484	5227	2	3/10/2006
Desulfitobacterium hafniense	TCP-A	4.96723	47.3	-	AQZD01	4839	4556	5	4/22/2013
Desulfitobacterium hafniense	PCP-1	5.56321	47.5	-	ARAZ01	5327	5095	7	4/22/2013
Desulfitobacterium hafniense	PCE-S	5.6667	47.3	-	-	5490	5417	6	-
Desulfitobacterium	PCE1	4.22	45	-	AQZF01	4070	3873	6	9/16/2013
Desulfitobacterium chlororespirans	DSM 11544	5.61	47.3	-	FRDN01	5367	5282	2	12/2/3016
Desulfitobacterium dichloroeliminans	LMG P-21439	3.62	44.2	chromosome:NC_019903.1/CP003344.1	-	3463	3300	1	6/17/2013
Desulfitobacterium dehalogenans	ATCC 51507	4.32	45	chromosome:NC_018017.1/CP003348.1	-	4212	3974	7	9/10/2015
Polaromonas JS666	JS666	5.89868	62.00	chromosome:NC_007948.1/CP000316.1 plasmid 1:NC_007949.1/CP000317.1 plasmid 2:NC_007950.1/CP000318.1	-	5660	5485	1[a]	2006/04/10

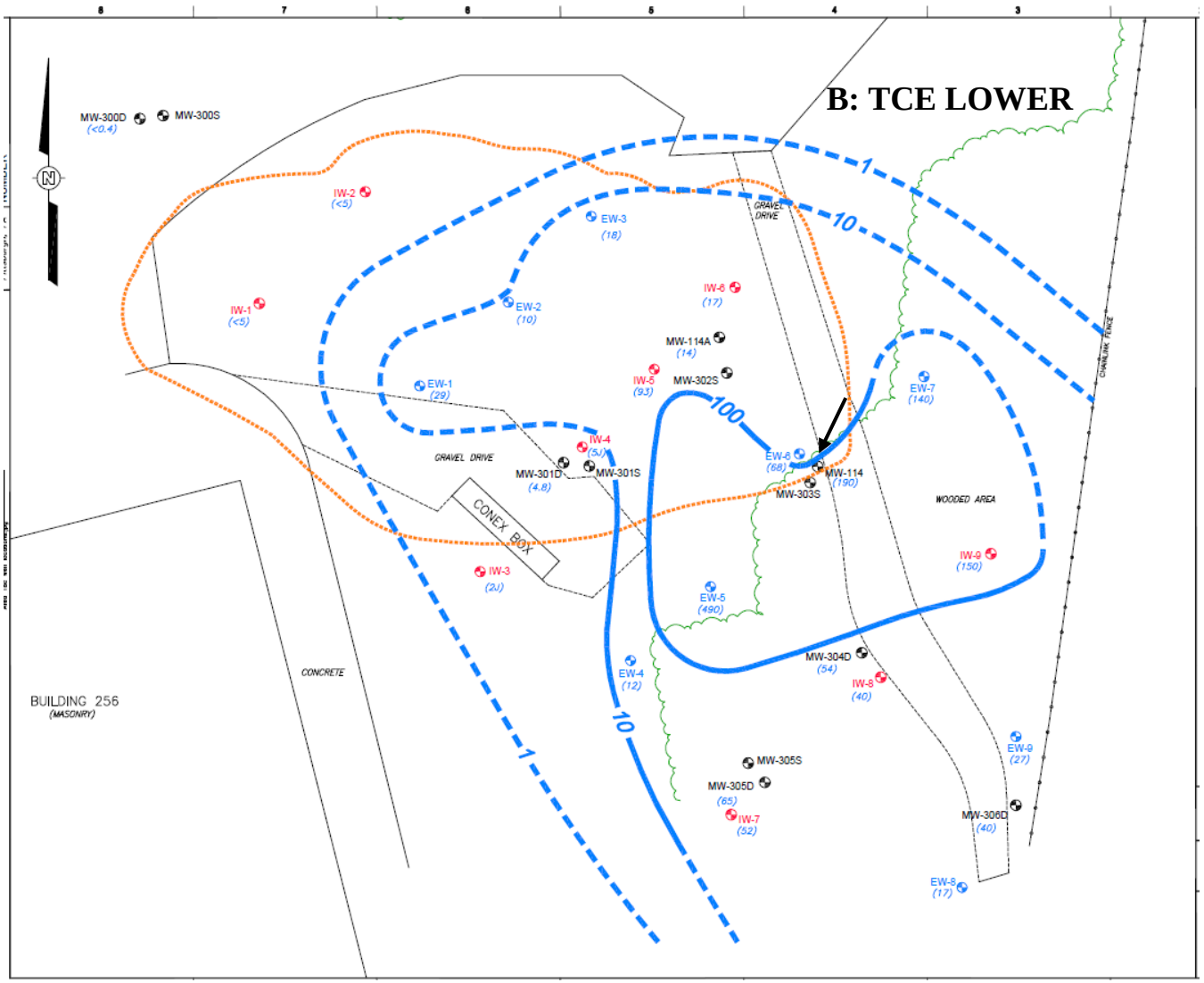
a: Cytochrome P450 (ABE47160.1) from *Polaromonas* JS666 is not a RDase but catalyzes the initial step of cDCE degradation.

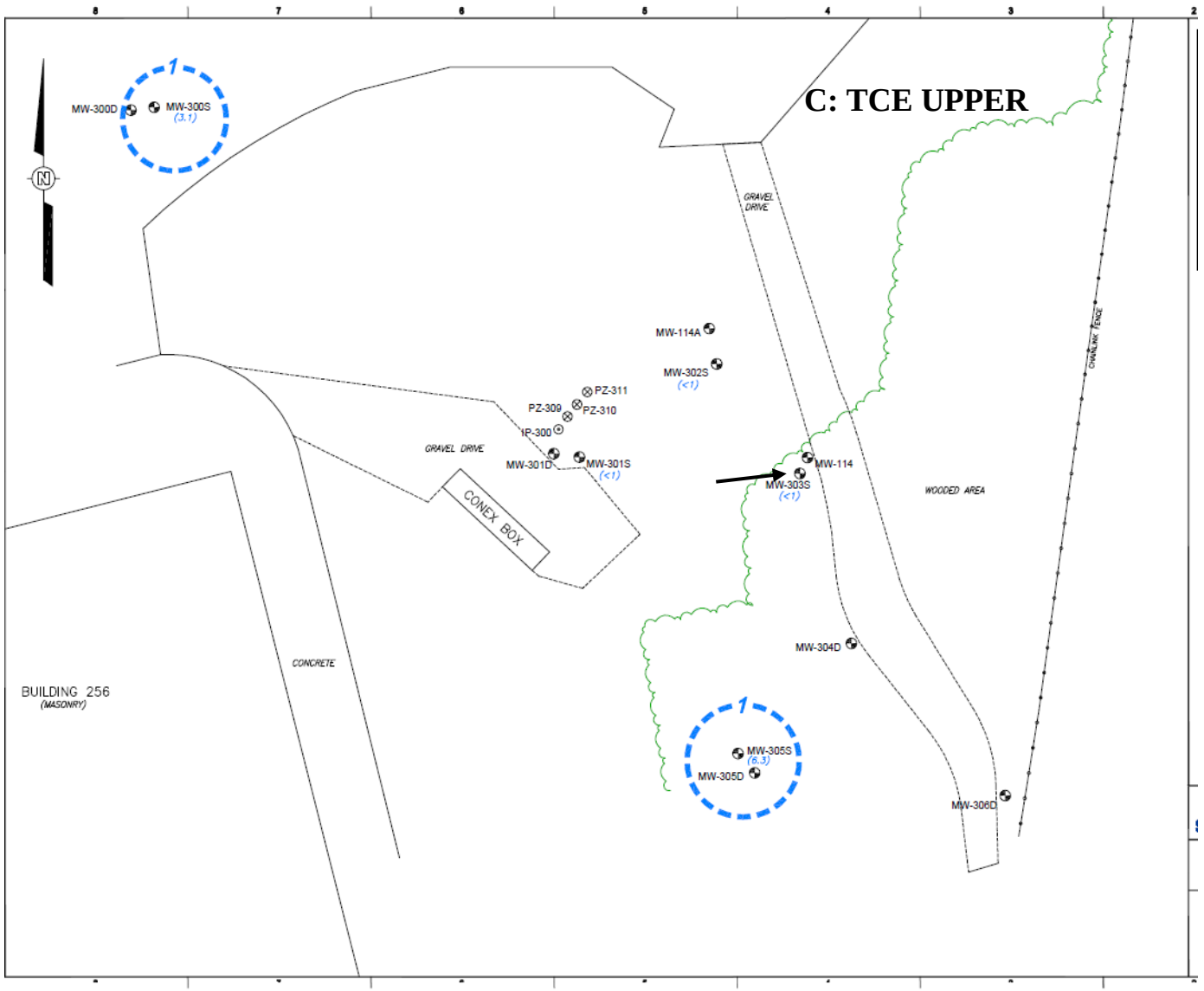
Supplementary Table 7.4. Number of collected genomes and dereplicated RDases.

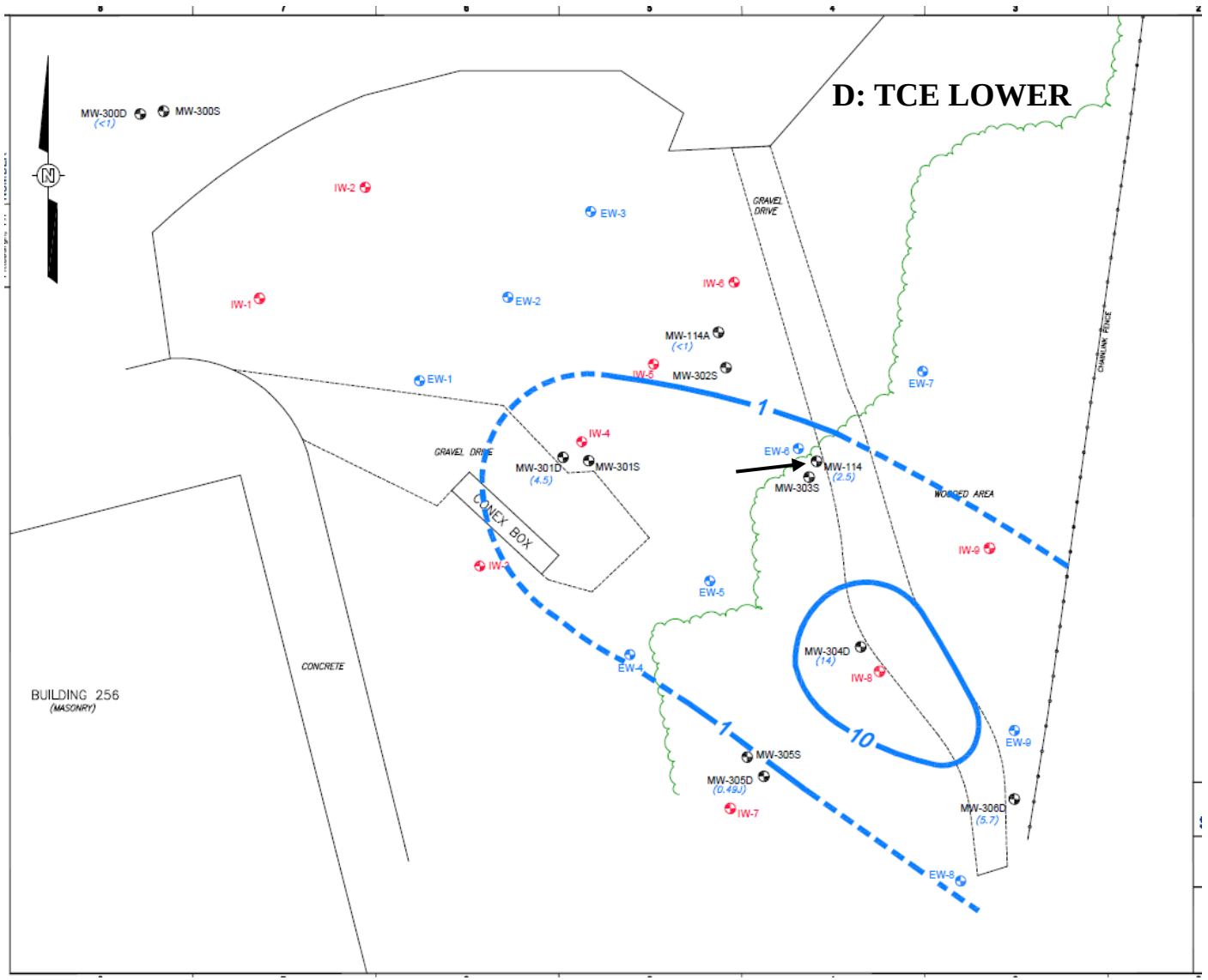
Microorganism	Number of collected genome	Dereplicated RDase number
Dehalococcoides mccartyi	30	317
Dehalogenimonas	4	91
Anaeromyxobacter	1	2
Dehalobacter	7	103
Geobacter	1	2
Sulfurospirillum	5	6
Desulfitobacterium	9	36
Polaromonas	1	1 (not an RDase)

Supplementary Figure 7.1. TCE plume maps for the Edison, NJ site. TCE contour maps for the site prior to addition of emulsified oil and dehalogenating culture SDC-9 in 2009 are provided for the shallow zone (A) and deep zone at the site (B). Well 303S is located in the shallow zone and well 114 is located in the deep zone. Post-treatment contour maps in 2010 for the shallow zone (C) and deep zone (D) are also provided. All values are in µg/L. The wells from which samples were collected and analyzed are indicated with arrows.

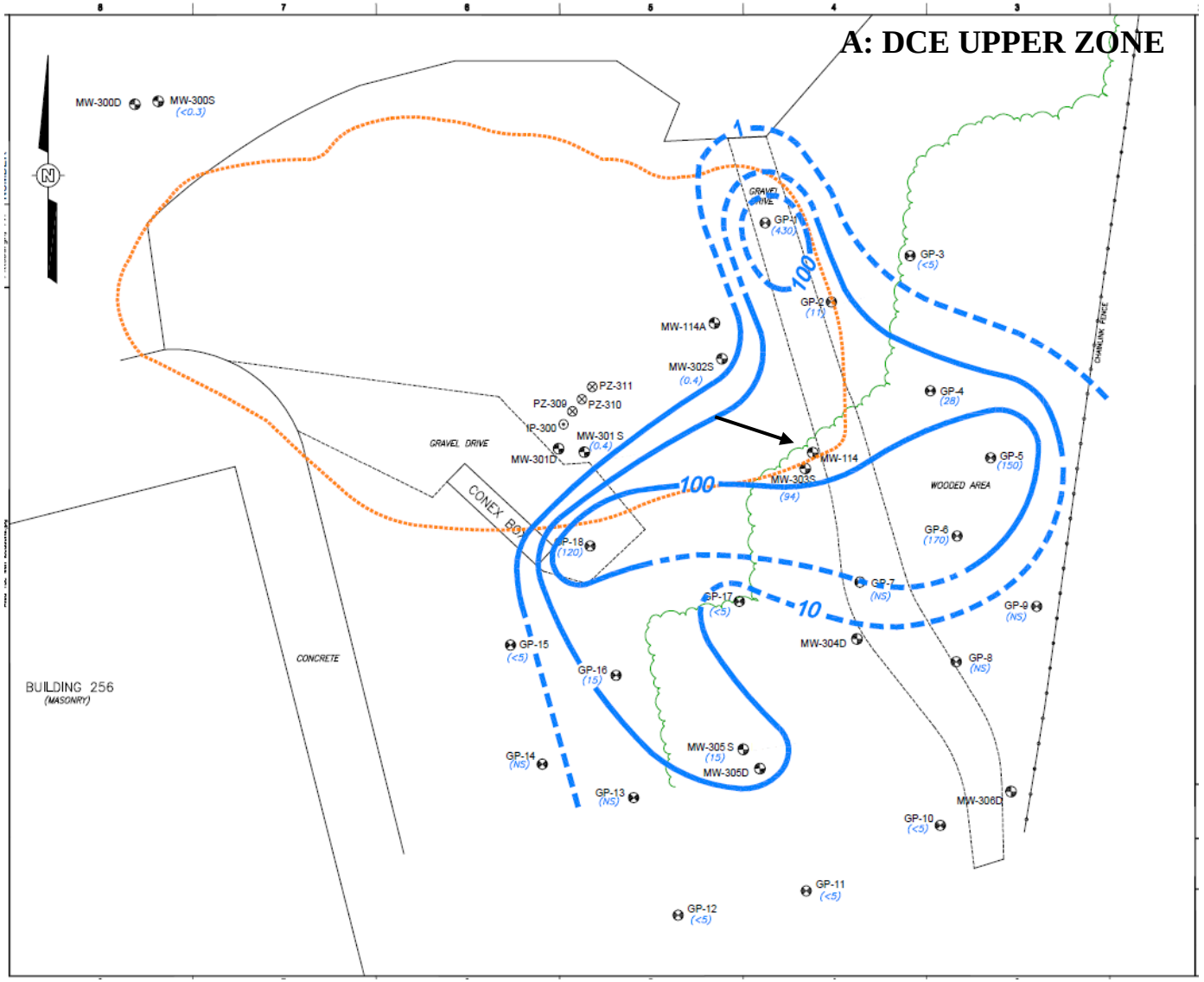


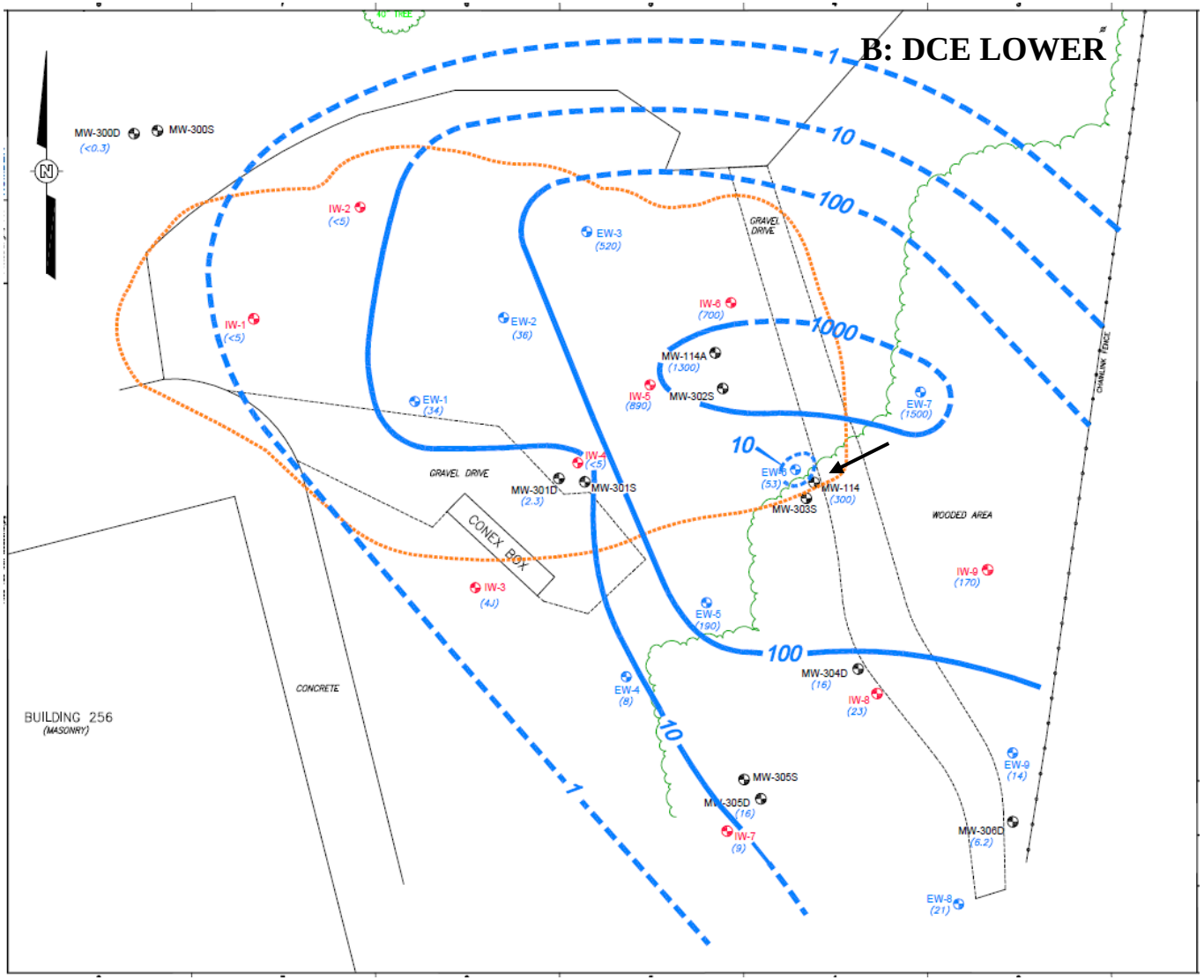

B: TCE LOWER
MW-300D
(<0.4)
MW-300S
IW-2
(<5)
EW-3
(18)
GRAVEL DRIVE
IW-1
(<5)
EW-2
(10)
IW-6
(17)
MW-114A
(14)
IW-5
(93)
MW-302S
EW-1
(29)
EW-7
(140)
CHAINLINK FENCE
1
10
100
IW-4
(5J)
GRAVEL DRIVE
MW-301D
(4.8)
MW-301S
EW-6
(68)
MW-114
(190)
MW-303S
WOODED AREA
CONEX BOX
IW-9
(150)
IW-3
(2J)
EW-5
(490)
MW-304D
(54)
EW-4
(12)
IW-8
(40)
CONCRETE
BUILDING 256
(MASONRY)
EW-9
(27)
MW-305S
MW-305D
(65)
IW-7
(52)
MW-306D
(40)
EW-8
(17)

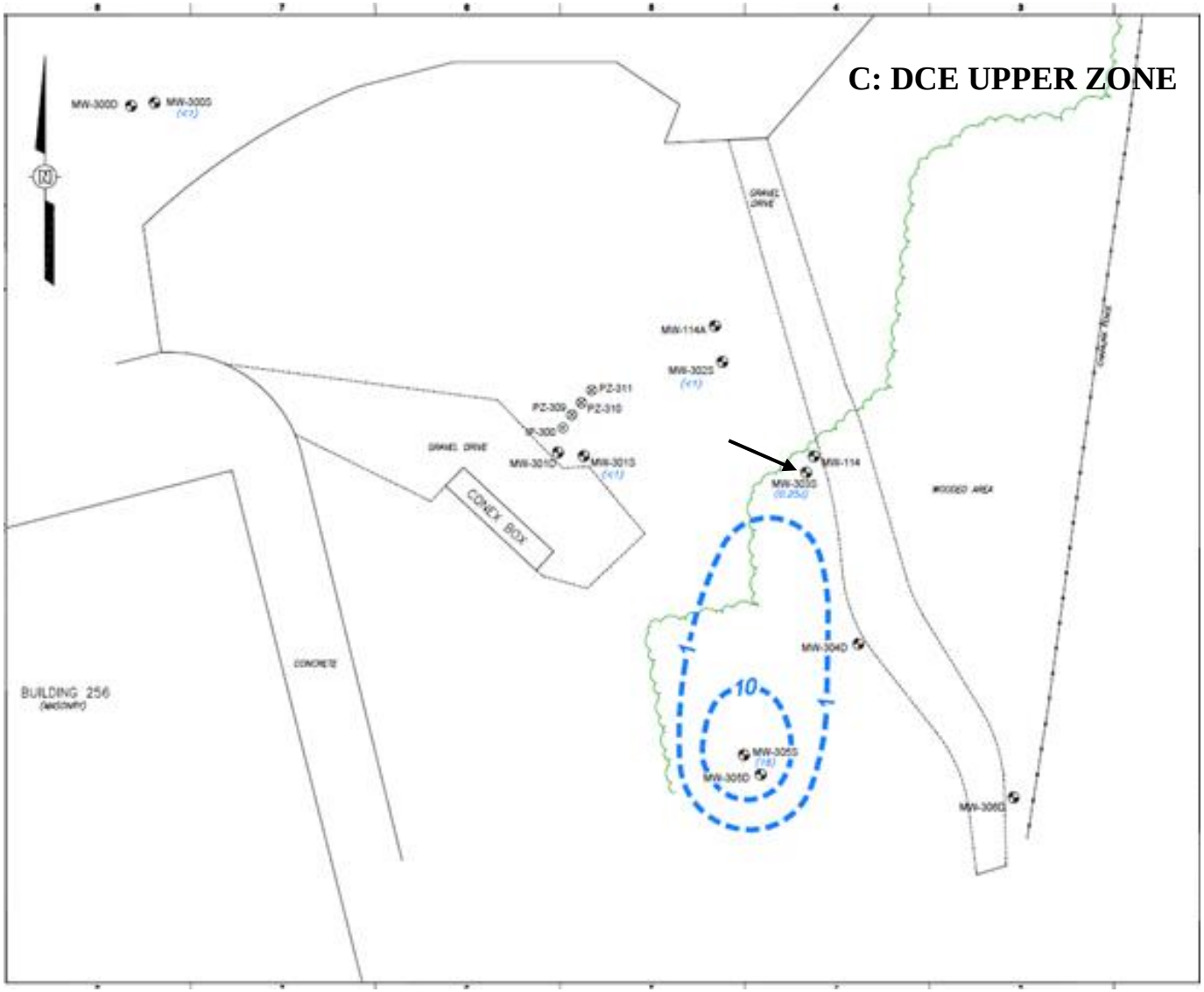

C: TCE UPPER
MW-300D
MW-300S
(3.1)
MW-114A
MW-302S
(<1)
PZ-311
PZ-309
PZ-310
IP-300
MW-301D
MW-301S
(<1)
GRAVEL DRIVE
CONEX BOX
MW-114
MW-303S
(<1)
WOODED AREA
GRAVEL DRIVE
CHAIN LINK FENCE
MW-304D
CONCRETE
BUILDING 256
(MASONRY)
MW-305S
(6.3)
MW-305D
MW-306D

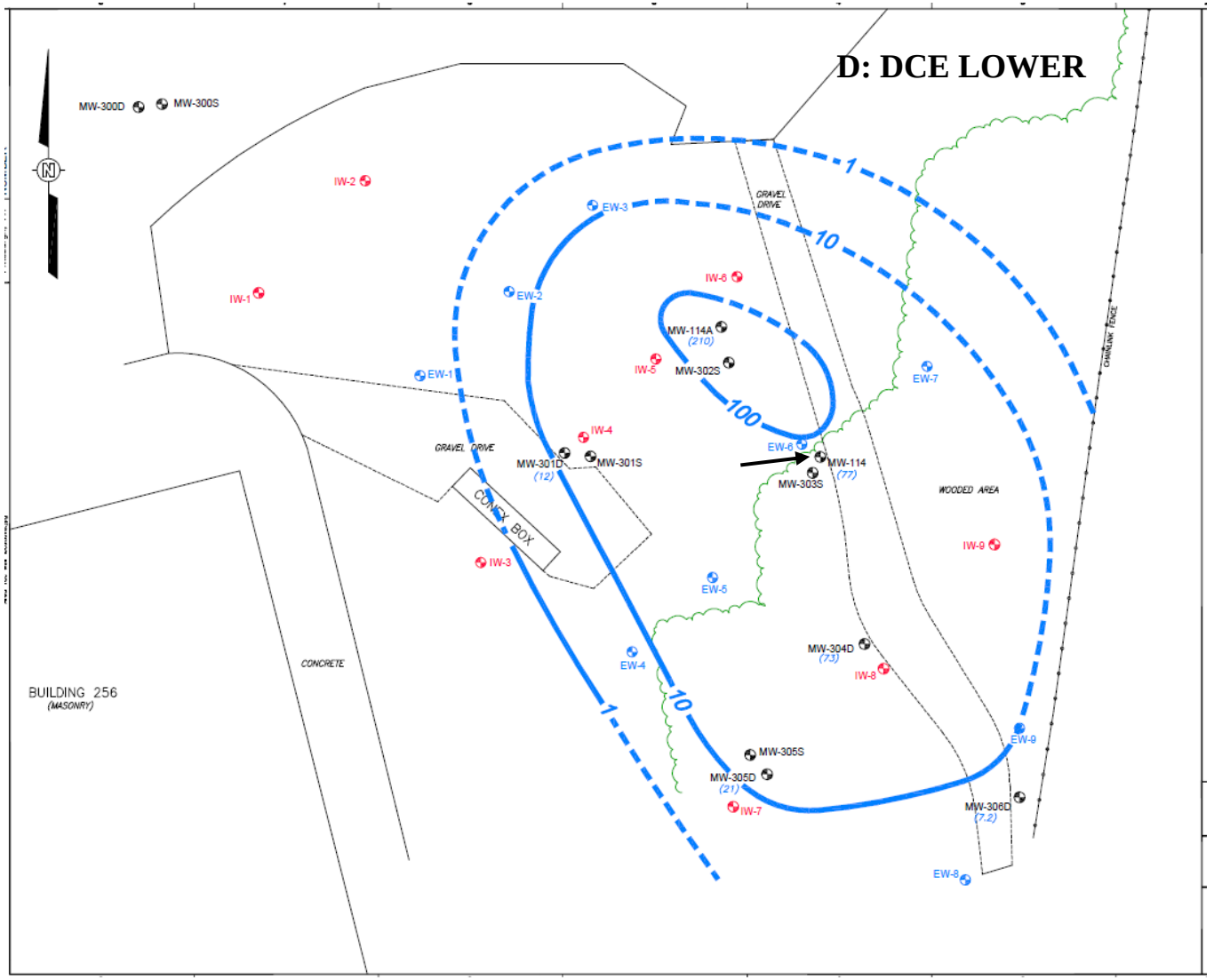

D: TCE LOWER
MW-300D
(<1)
MW-300S
IW-2
EW-3
IW-1
EW-2
IW-6
MW-114A
(<1)
IW-5
MW-302S
EW-1
EW-7
IW-4
GRAVEL DRIVE
MW-301D
(4.5)
MW-301S
EW-6
MW-114
(2.5)
MW-303S
CONEX BOX
IW-3
IW-9
EW-5
MW-304D
(14)
IW-8
EW-4
CONCRETE
BUILDING 256
(MASONRY)
MW-305S
MW-305D
(0.49J)
IW-7
EW-9
MW-306D
(5.7)
EW-8
1
10
CHAINLINK FENCE
WOODED AREA

Supplementary Figure 7.2. Cis-DCE Plume maps for the Edison, NJ site. Cis-DCE contour maps for the site prior to addition of emulsified oil and dehalogenating culture SDC-9 in 2009 are provided for the shallow zone (A) and deep zone at the site (B). Well 303S is located in the shallow zone and well 114 is located in the deep zone. Post-treatment contour maps in 2010 for the shallow zone (C) and deep zone (D) are also provided. All values are in µg/L. The wells from which samples were collected and analyzed are indicated with arrows.

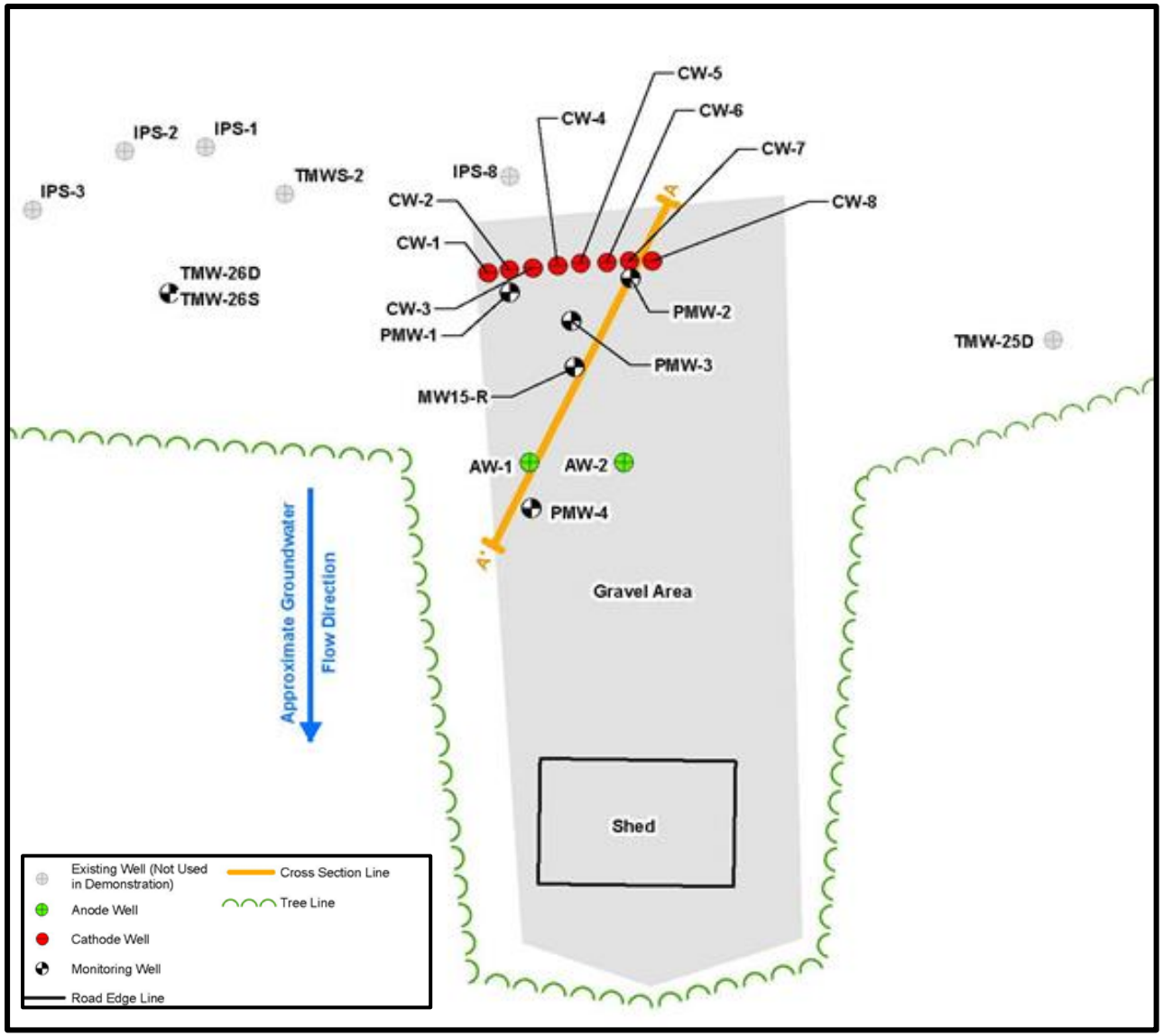



B: DCE LOWER
MW-300D
(<0.3)
MW-300S
IW-2
(<5)
EW-3
(520)
GRAVEL DRIVE
IW-1
(<5)
EW-2
(36)
IW-6
(700)
MW-114A
(1300)
IW-5
(890)
MW-302S
EW-7
(1500)
EW-1
(34)
IW-4
(<5)
MW-301D
(2.3)
MW-301S
EW-6
(53)
MW-114
(300)
MW-303S
GRAVEL DRIVE
CONEX BOX
WOODED AREA
IW-3
(4J)
IW-9
(170)
EW-5
(190)
MW-304D
(16)
IW-8
(23)
EW-4
(8)
CONCRETE
BUILDING 256
(MASONRY)
MW-305S
MW-305D
(16)
IW-7
(9)
EW-9
(14)
MW-306D
(6.2)
EW-8
(21)
CHAINLINK FENCE
1
10
100
1000

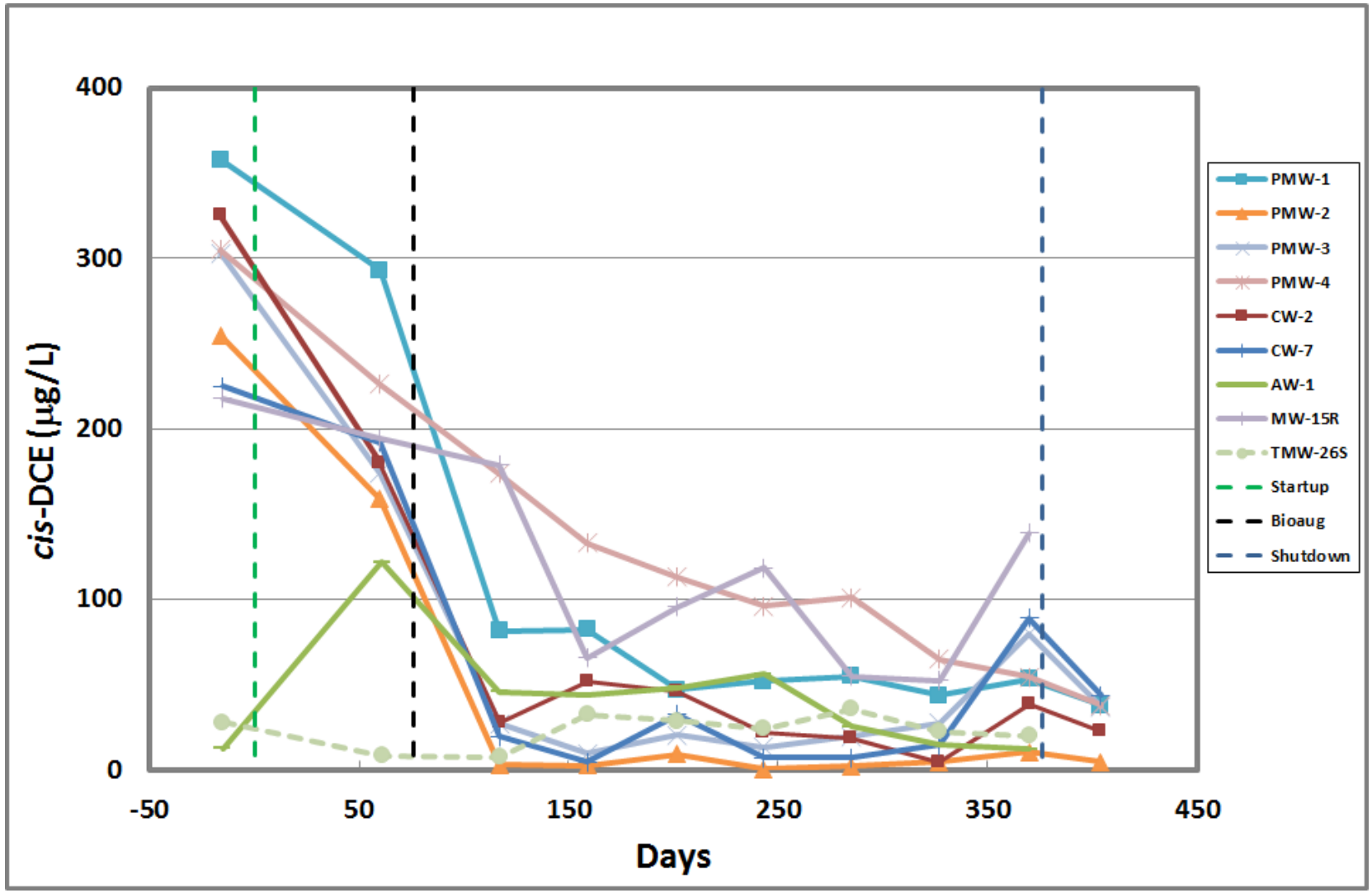

C: DCE UPPER ZONE
MW-300D
MW-300S
(x1)
N
GRAVEL DRIVE
MW-114A
MW-302S
(x1)
PZ-311
PZ-309
PZ-310
MW-302
GRAVEL DRIVE
MW-301D
MW-301S
(x1)
CONEX BOX
MW-114
MW-303S
(0.25x)
WOODED AREA
CONCRETE
BUILDING 256
(welcome)
MW-304D
1
10
1
MW-305S
(1x)
MW-305D
MW-306D


D: DCE LOWER
MW-300D
MW-300S
IW-2
IW-1
EW-3
EW-2
EW-1
GRAVEL DRIVE
IW-6
MW-114A
(210)
IW-5
MW-302S
EW-7
CHAINLINK FENCE
100
10
1
IW-4
GRAVEL DRIVE
MW-301D
(12)
MW-301S
EW-6
MW-114
(77)
MW-303S
WOODED AREA
CONCRETE BOX
IW-3
IW-9
EW-5
MW-304D
(73)
IW-8
EW-4
CONCRETE
BUILDING 256
(MASONRY)
MW-305S
MW-305D
(21)
IW-7
EW-9
MW-306D
(7.2)
EW-8

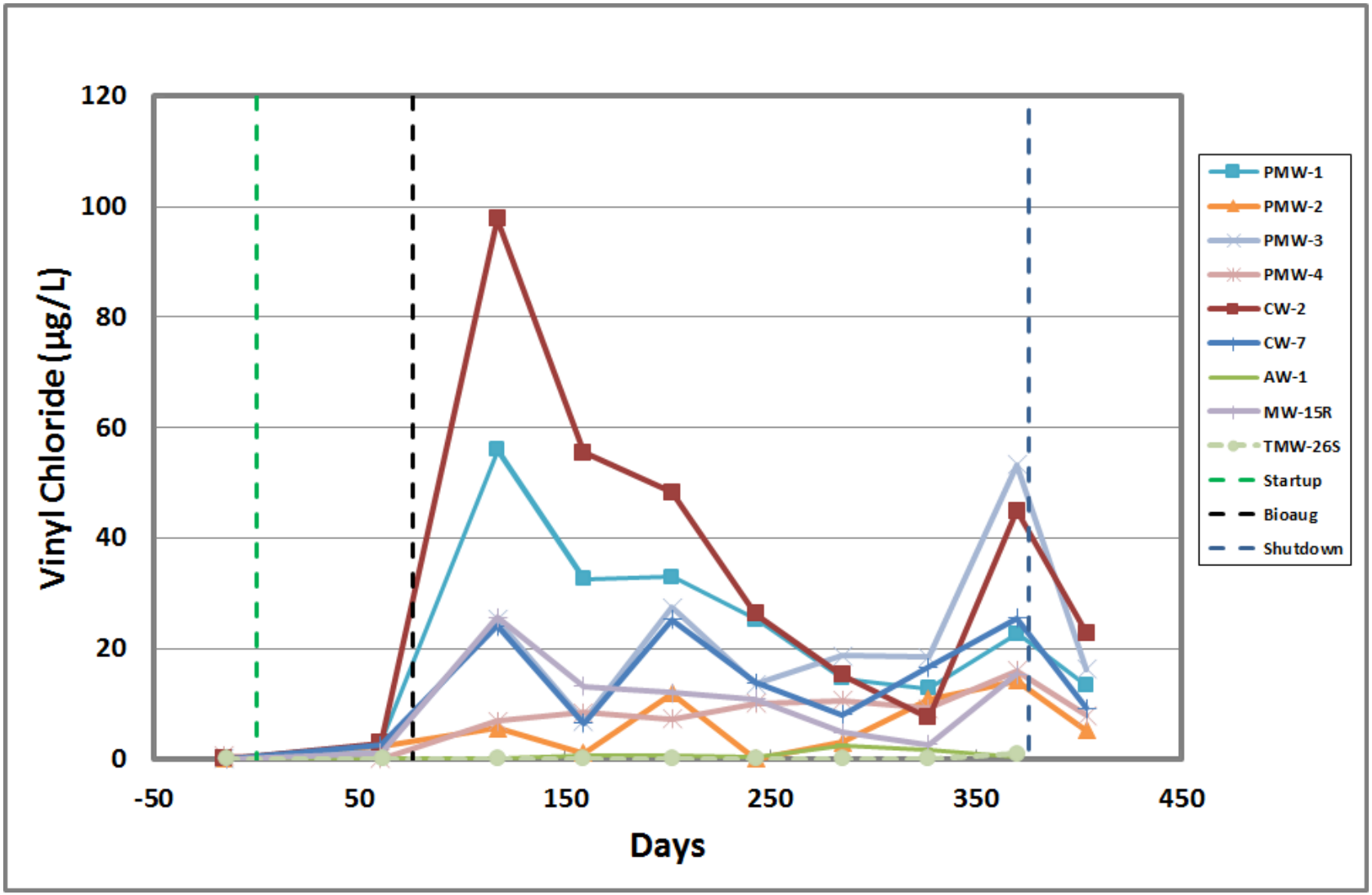
Supplementary Figure 7.3. Demonstration plot layout at the Quantico, VA site. The cathode and anode wells are indicated by red and green symbols, respectively. This system was used to supply H_2 to support reductive dechlorination of cis-DCE downgradient of a landfill. See data in Supplementary Figures 17-19.



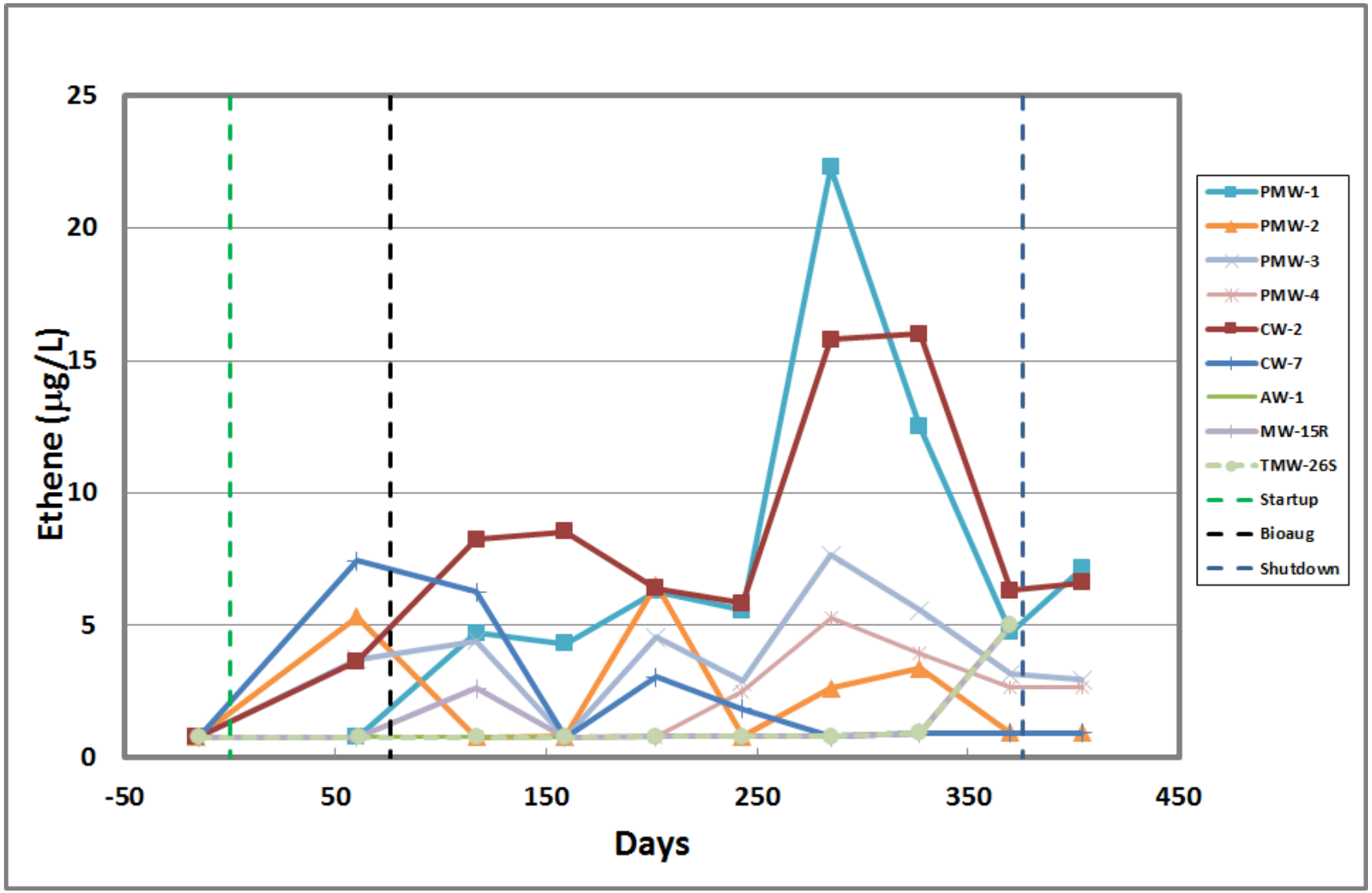
Supplementary Figure 7.4. Concentration data for cis-DCE at the Quantico, VA site. The groundwater samples were collected on Day 243 from wells CW-2, PMW-2, CW-2, AW-1, MW-15R, and PMW-4.



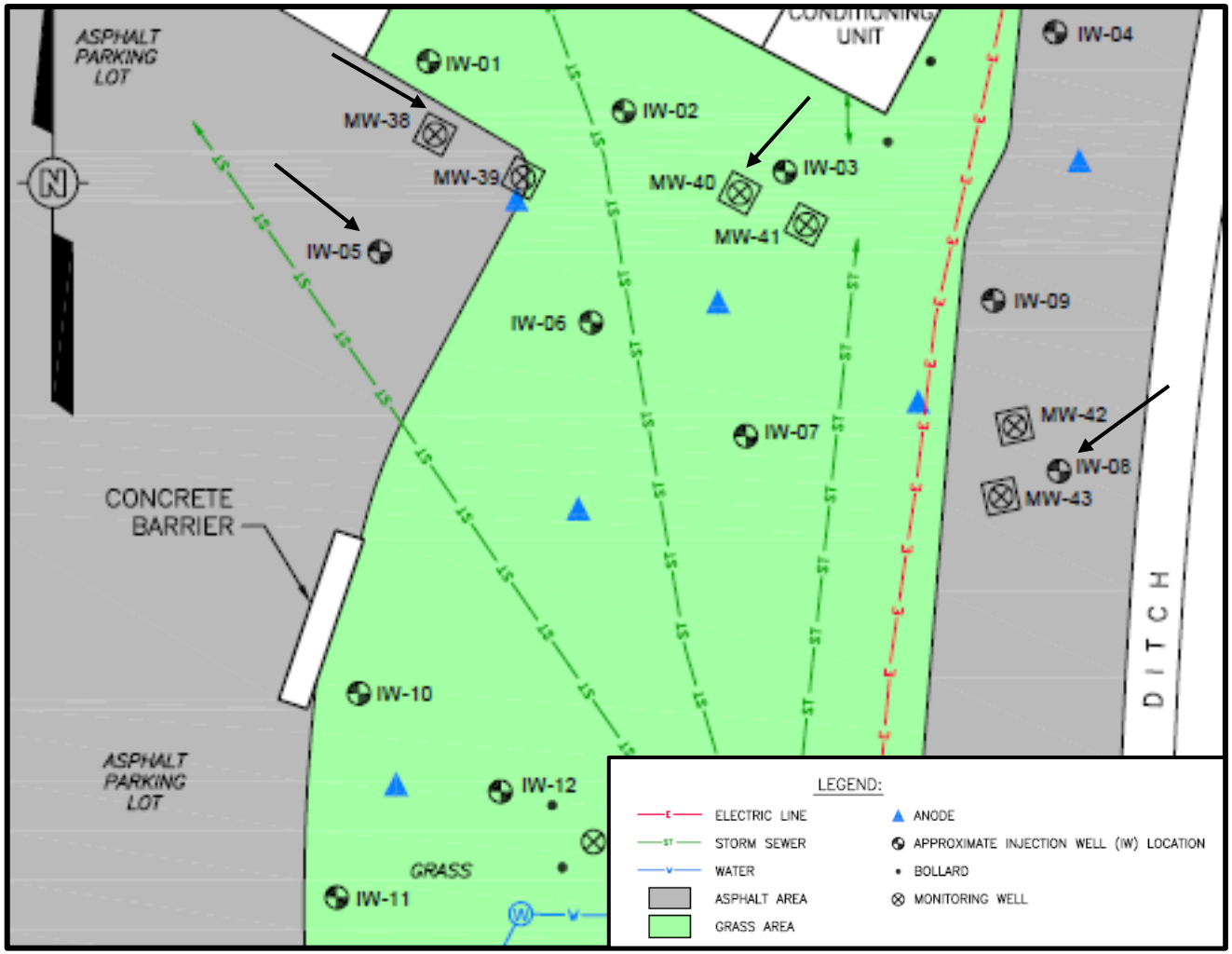
Supplementary Figure 7.5. Concentration data for vinyl chloride at the Quantico, VA site. The groundwater samples were collected on Day 243 from wells CW-2, PMW-2, CW-2, AW-1, MW-15R, and PMW-4.



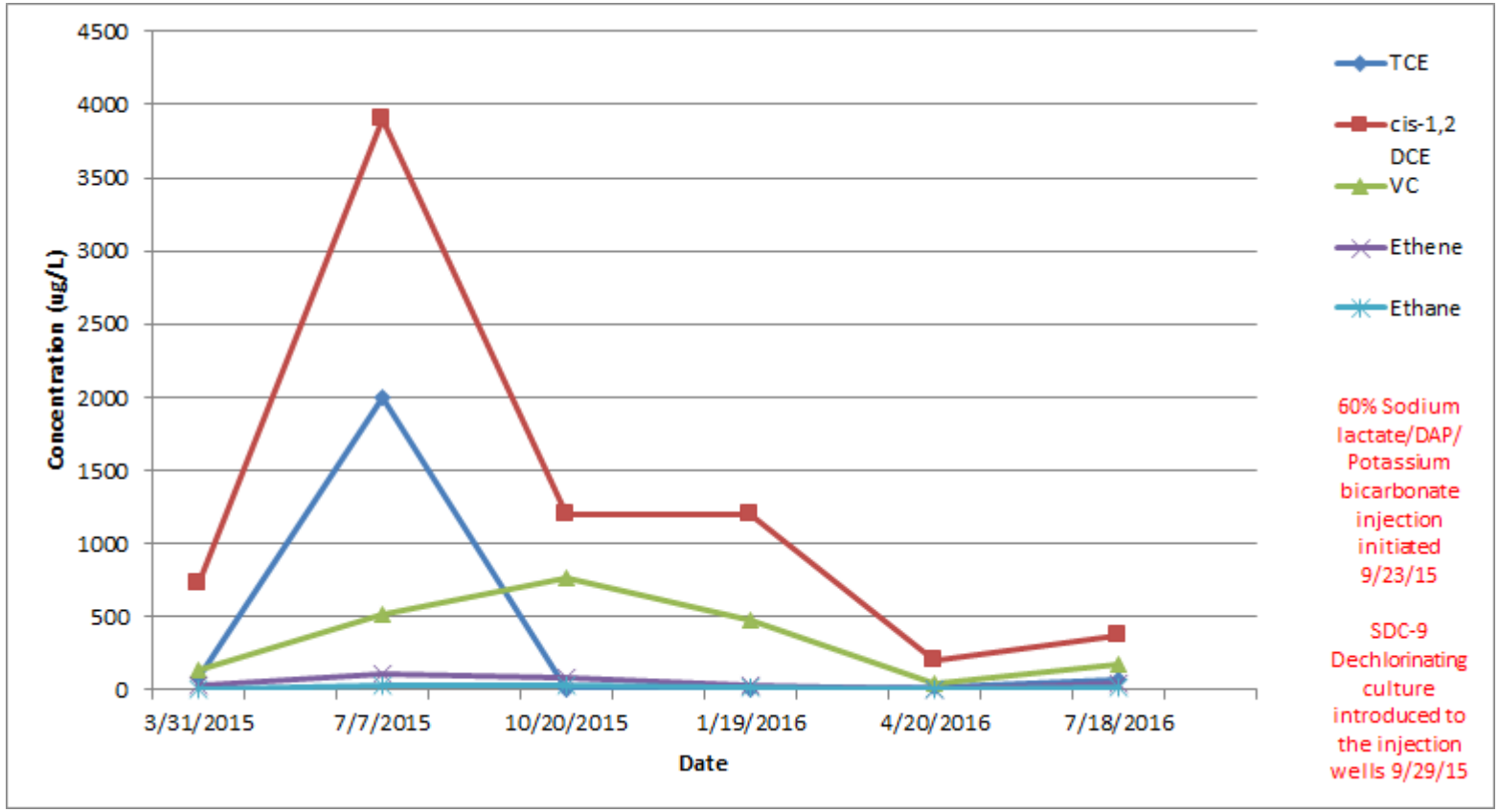
Supplementary Figure 7.6. Concentration data for ethene at the Quantico, VA site. The groundwater samples were collected on Day 243 from wells CW-2, PMW-2, CW-2, AW-1, MW-15R, and PMW-4.



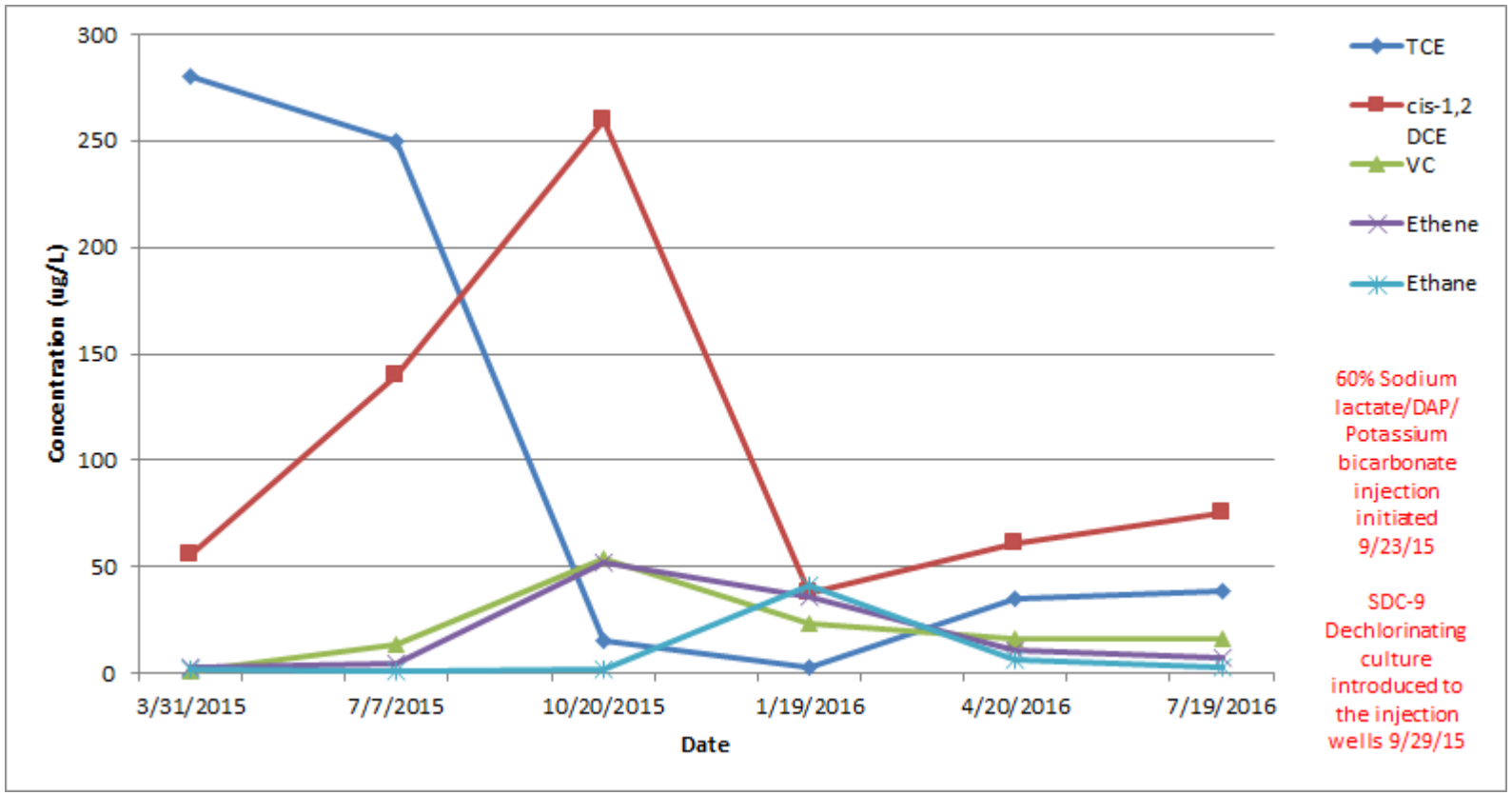
Supplementary Figure 7.7. Demonstration plot layout at the Indian Head, Md site. Injection wells (IWs) were amended with lactate, diammonium phosphate, potassium bicarbonate (for pH adjustment) and dehalogenating culture SDC-9. Monitoring wells (MWs) were used to measure system performance. A low voltage was used to maintain system pH. Anodes for this system are shown in the figure. Wells that were sampled are indicated by arrows. See MW data in Supplementary Figures 21-22. No analytical data are available for the IWs.



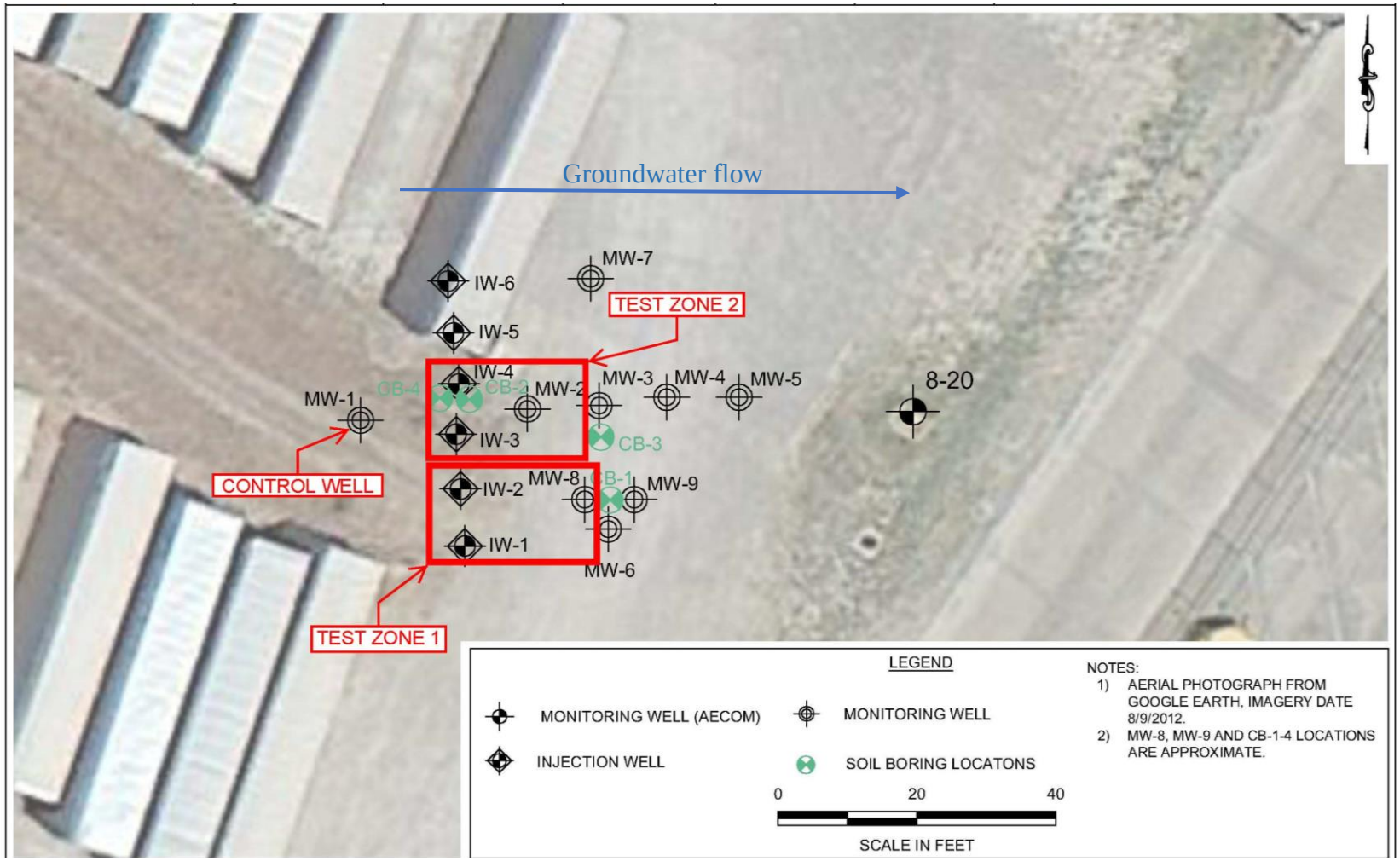
Supplementary Figure 7.8. Concentration data for cVOCs, ethene and ethane in well MW38 at the Indian Head, Md site. The groundwater samples were collected on 6/22/16.



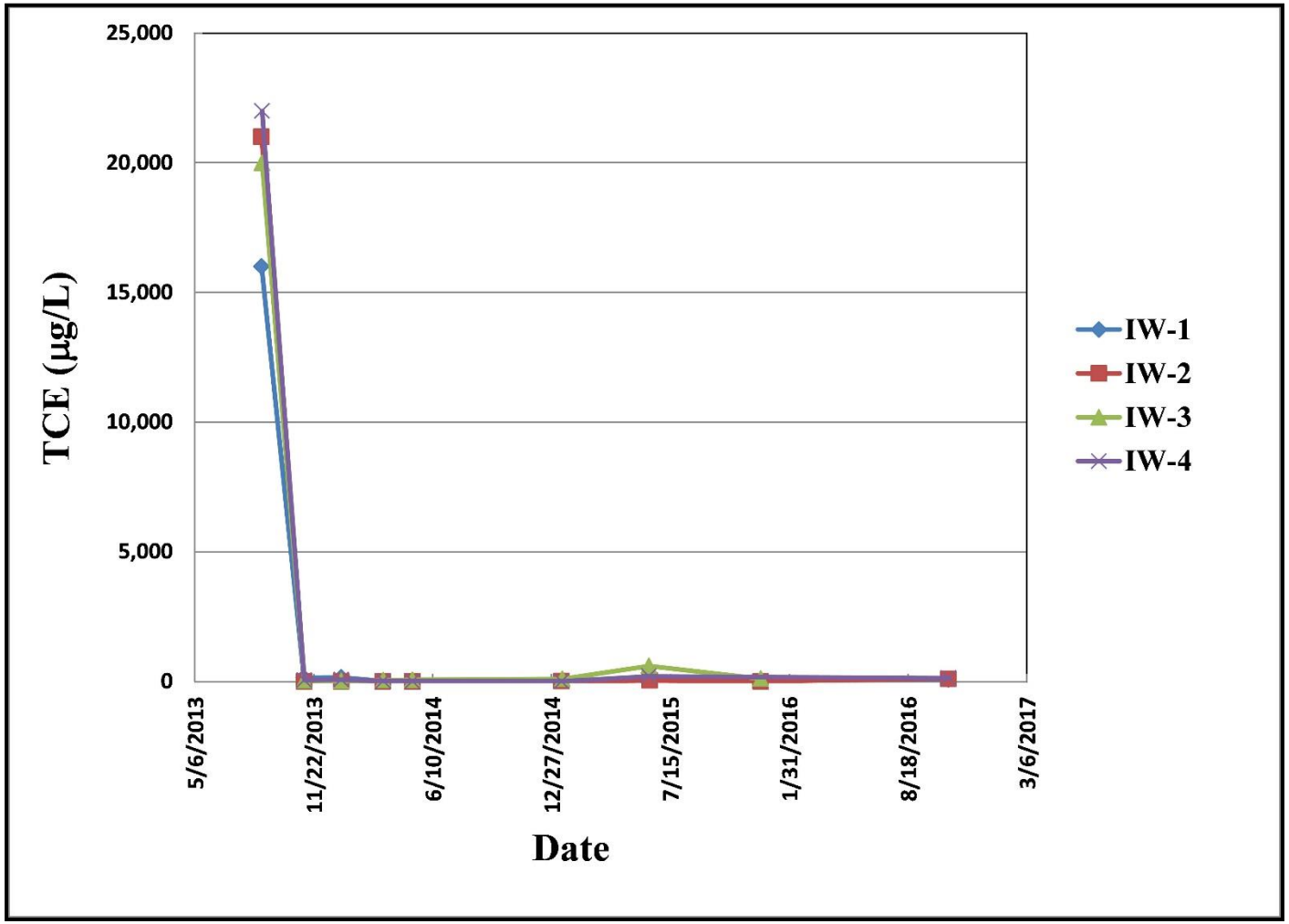
Supplementary Figure 7.9. Concentration data for cVOCs, ethene and ethane in well MW40 at the Indian Head, Md site. The groundwater samples were collected on 6/22/16.



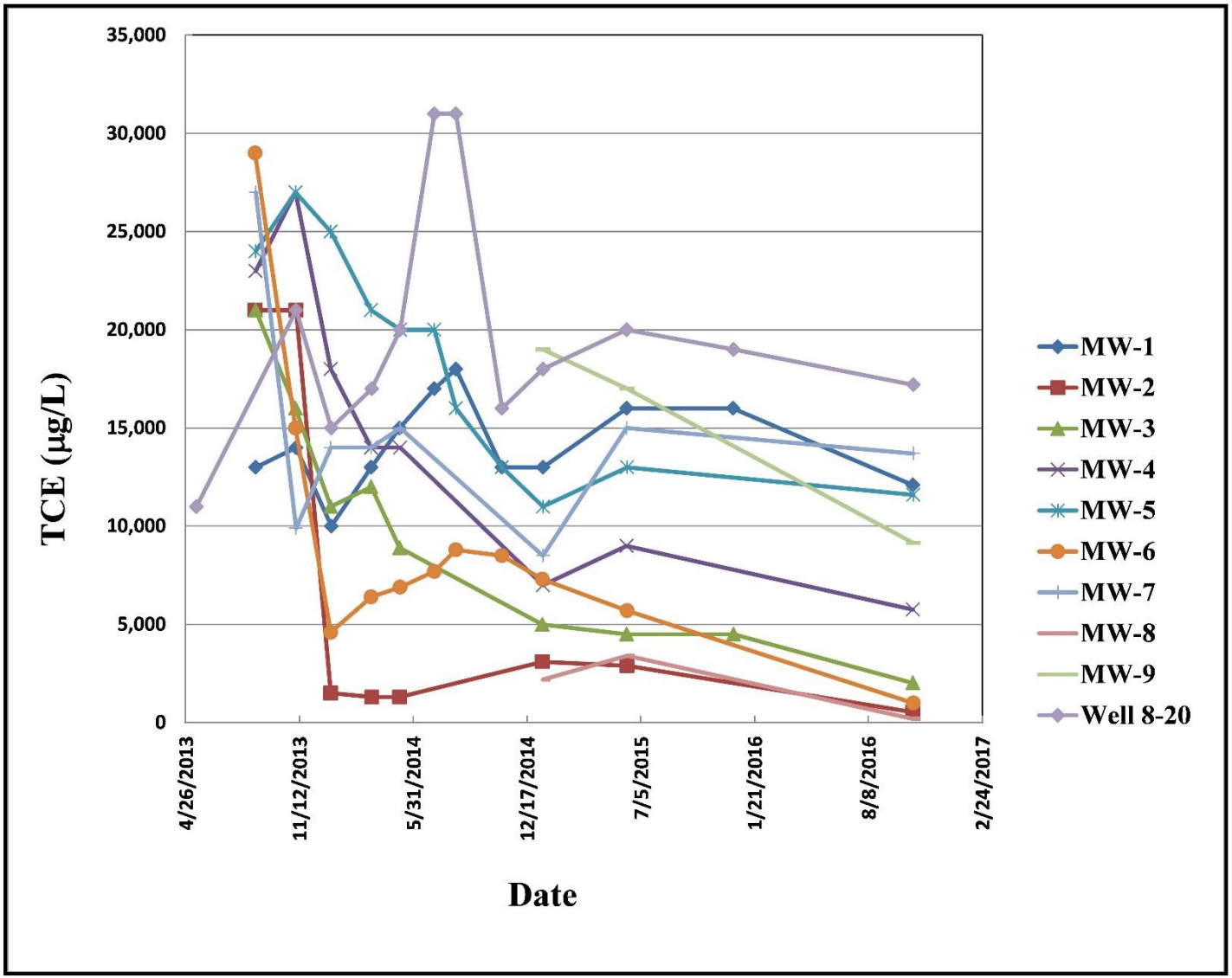
Supplementary Figure 7.10. Demonstration Plot layout at the Tulsa, Ok site. IWs are emulsified oil and dehalogenating culture SDC-9 injection wells and MWs are groundwater monitoring wells. See data in Supplementary Figures 24-26.



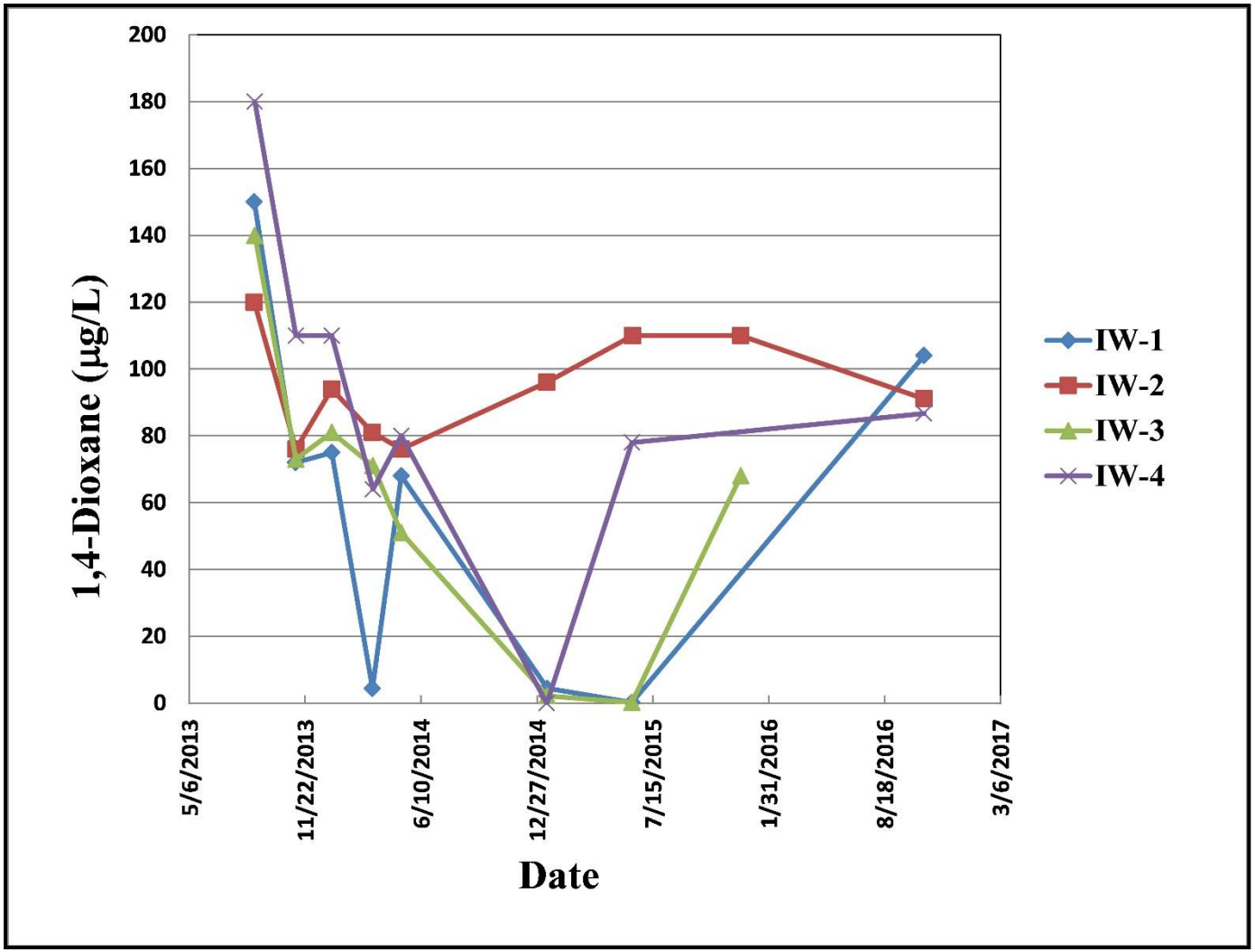
Supplementary Figure 7.11. Concentration data for TCE in injection wells (IWs) at the Tulsa, OK Site. The groundwater samples were collected on 6/09/15.



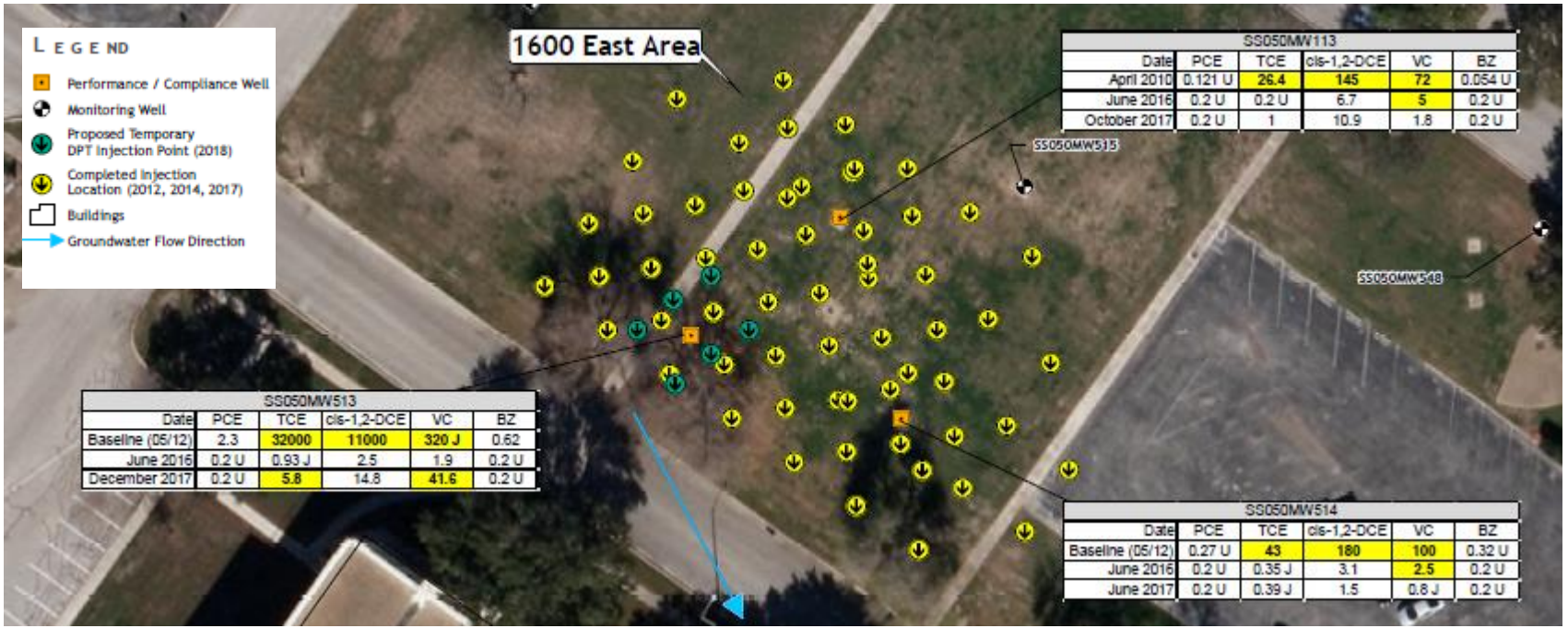
Supplementary Figure 7.12. Concentration data for TCE in monitoring wells (MWs) at the Tulsa, OK Site. The groundwater samples were collected on 6/09/15.



Supplementary Figure 7.13. Concentration data for 1,4-dioxane in injection wells (IWs) at the Tulsa, OK Site. The groundwater samples were collected on 6/09/15.



Supplementary Figure 7.14. Injection points and locations of monitoring wells SS050MW113 (113) and SS050MW514 (514) at the San Antonio, TX, Site. Analytical data are provided for each well. Groundwater samples were collected on 7/28/16. BZ = benzene.



SS050MW113

Date	PCE	TCE	cis-1,2-DCE	VC	BZ
April 2010	0.121 U	26.4	145	72	0.054 U
June 2016	0.2 U	0.2 U	6.7	5	0.2 U
October 2017	0.2 U	1	10.9	1.8	0.2 U

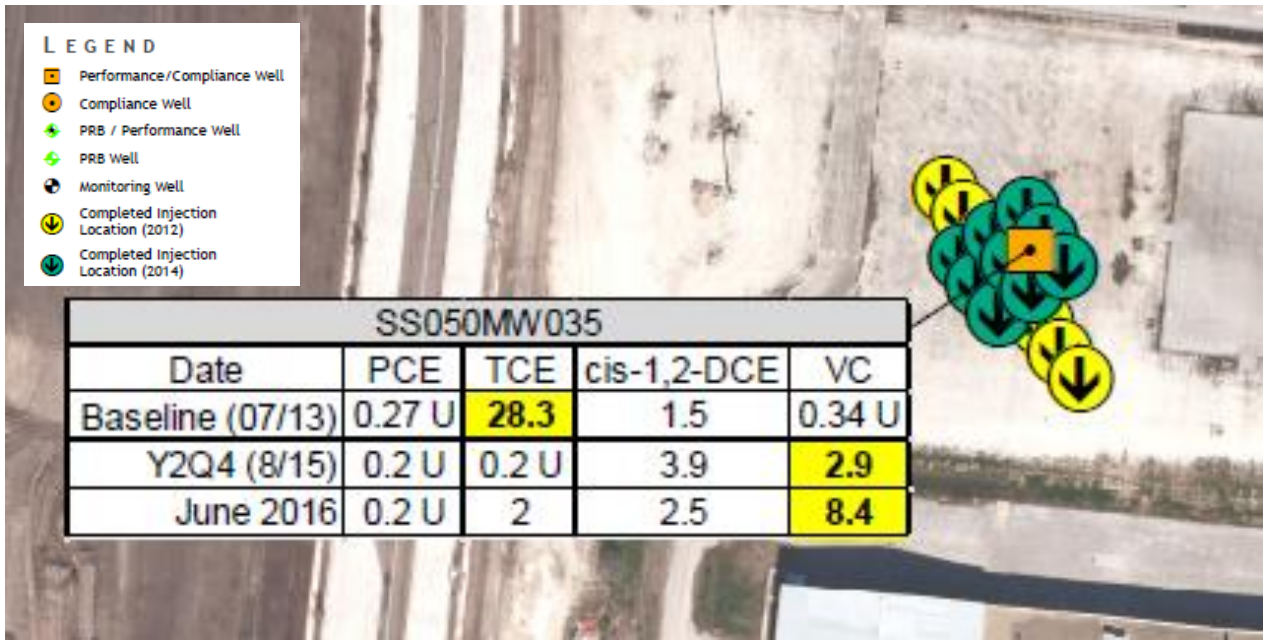
SS050MW513

Date	PCE	TCE	cis-1,2-DCE	VC	BZ
Baseline (05/12)	2.3	32000	11000	320 J	0.62
June 2016	0.2 U	0.93 J	2.5	1.9	0.2 U
December 2017	0.2 U	5.8	14.8	41.6	0.2 U

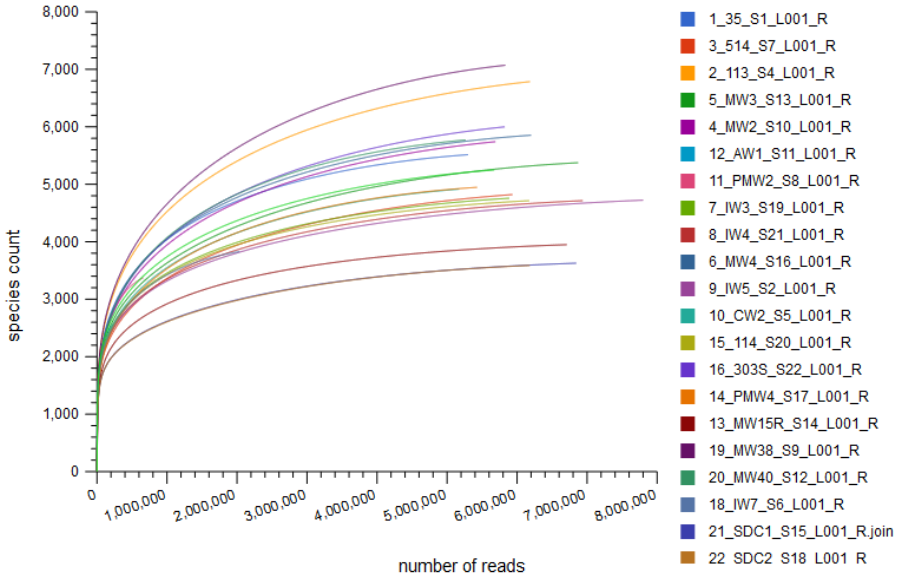
SS050MW514

Date	PCE	TCE	cis-1,2-DCE	VC	BZ
Baseline (05/12)	0.27 U	43	180	100	0.32 U
June 2016	0.2 U	0.35 J	3.1	2.5	0.2 U
June 2017	0.2 U	0.39 J	1.5	0.8 J	0.2 U

Supplementary Figure 7.15. Injection points and location of monitoring well SS050MW035 (35) at the San Antonio, TX, Site. Analytical data are provided. Groundwater samples were collected on 7/28/16.



SS050MW035				
Date	PCE	TCE	cis-1,2-DCE	VC
Baseline (07/13)	0.27 U	**28.3**	1.5	0.34 U
Y2Q4 (8/15)	0.2 U	0.2 U	3.9	**2.9**
June 2016	0.2 U	2	2.5	**8.4**



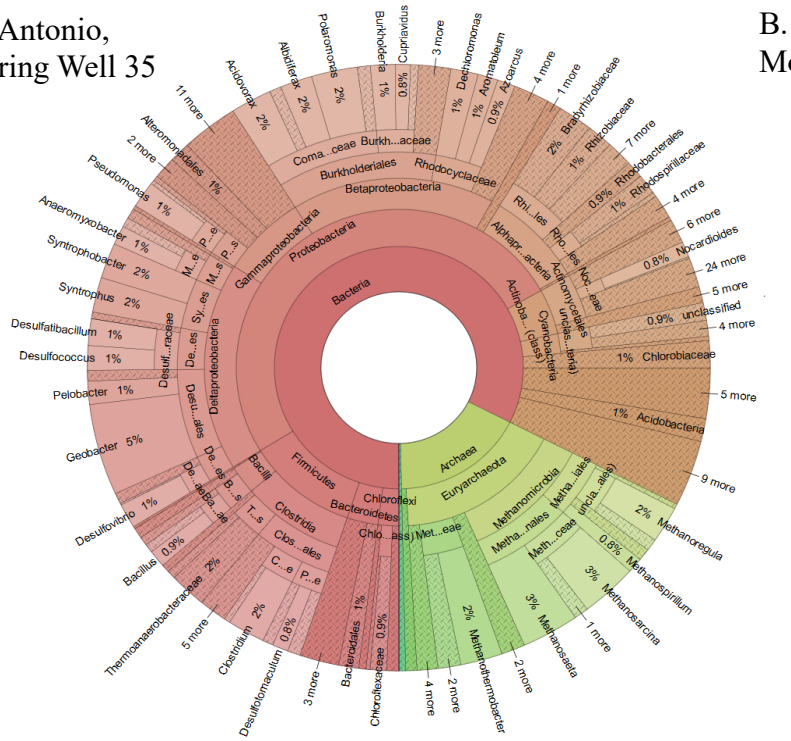
Supplementary Figure 7.16. Rarefaction curves for microbial communities in groundwater and in SDC-9.

A. SDC-9, replicate 1

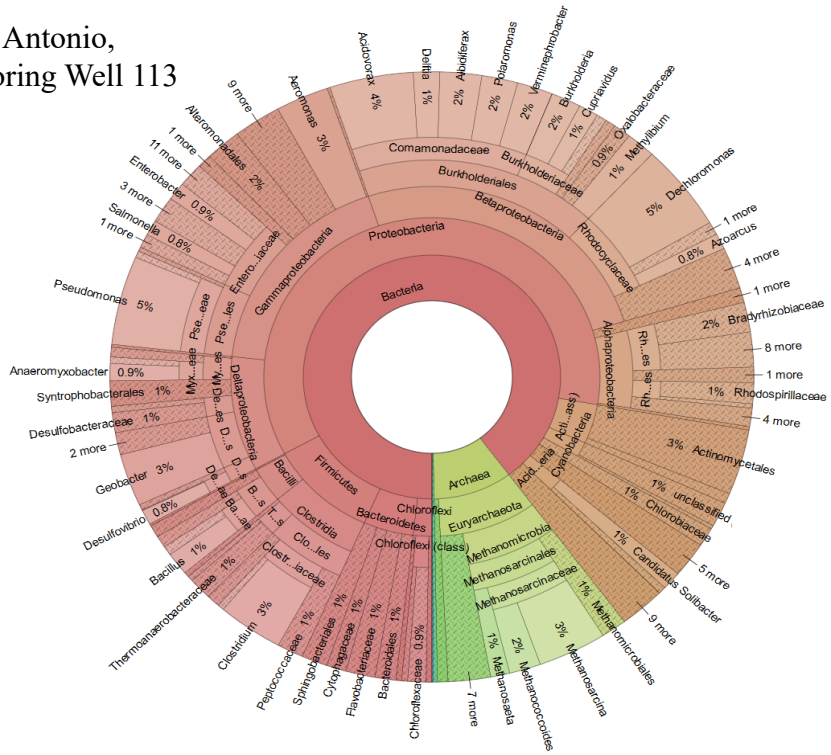
B. SDC-9, replicate 2

Supplementary Figure 7.17. Classification of microbial communities in two samples of SDC-9 (data analyzed with MG-RAST).

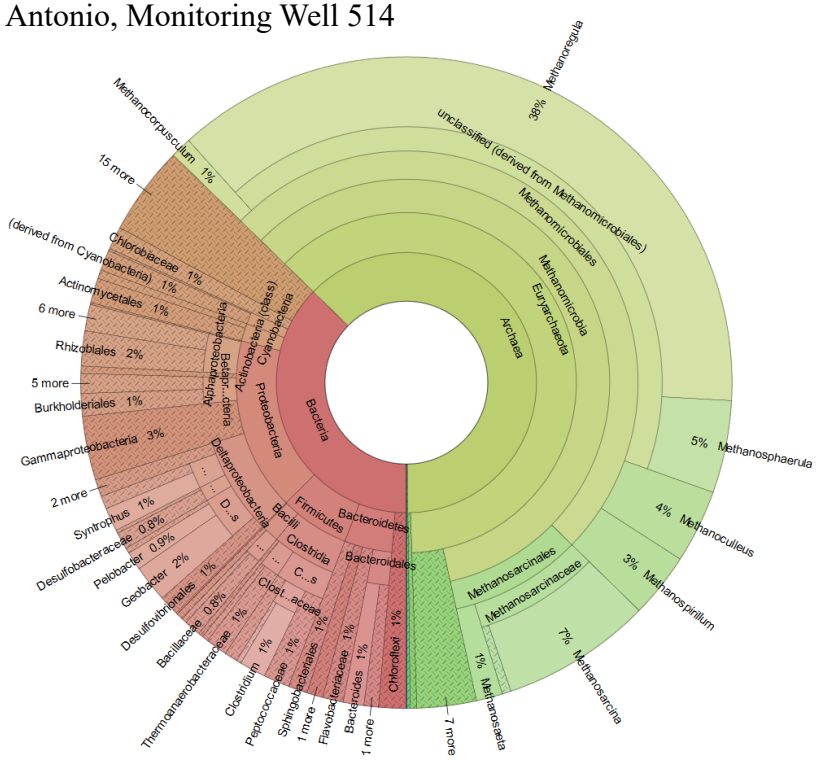
A. San Antonio, Monitoring Well 35



B. San Antonio, Monitoring Well 113

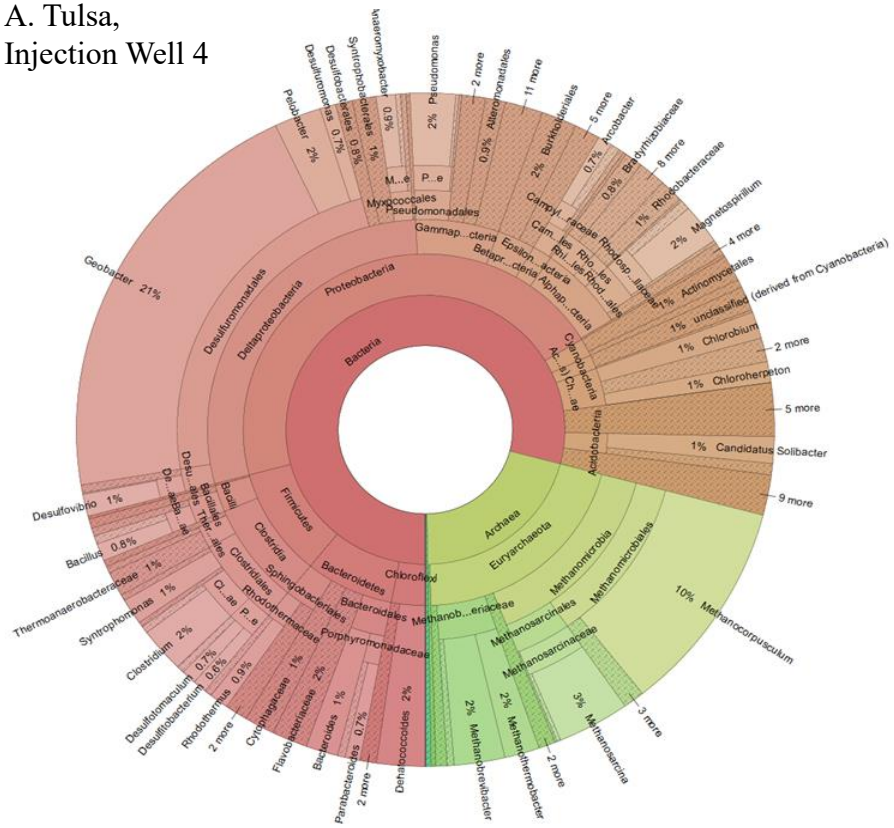


C. San Antonio, Monitoring Well 514

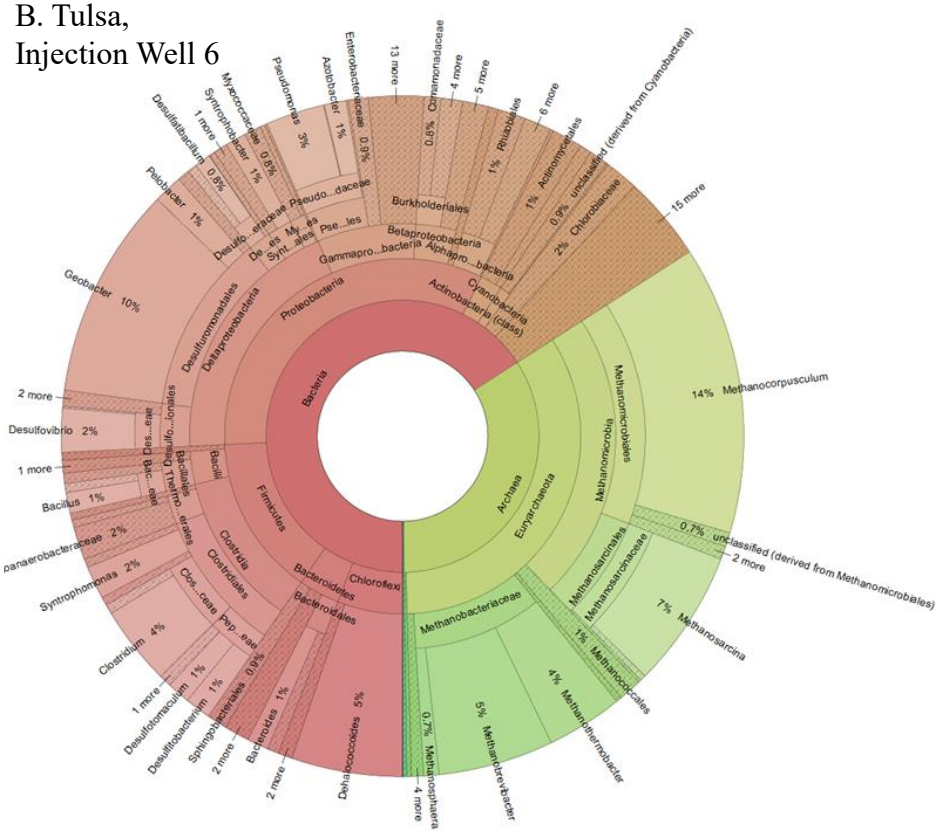


Supplementary Figure 7.18. Classification of microbial communities in three monitoring well groundwater samples from San Antonio (data analyzed with MG-RAST).

A. Tulsa,
Injection Well 4

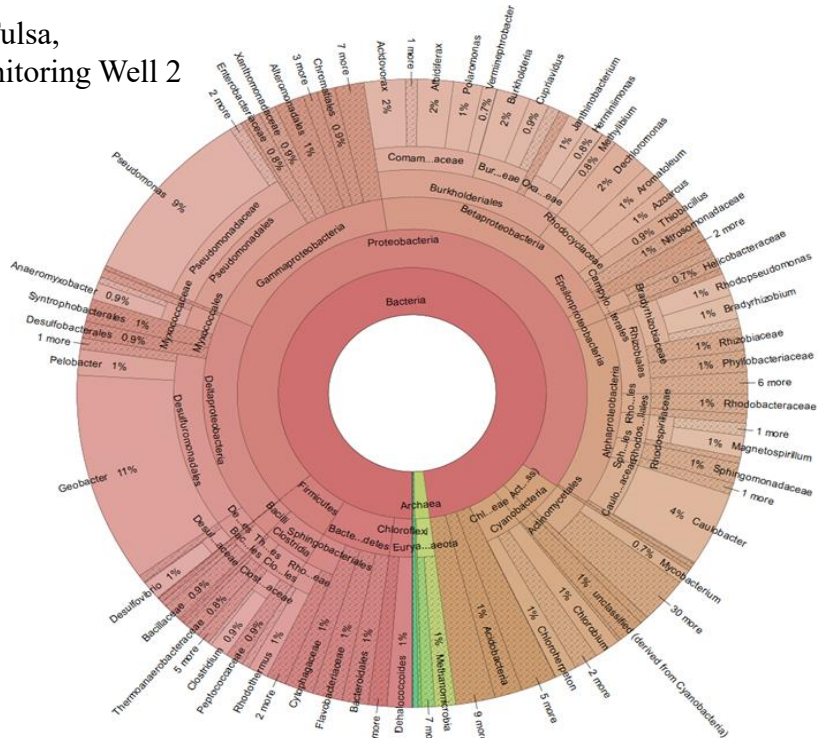


B. Tulsa,
Injection Well 6

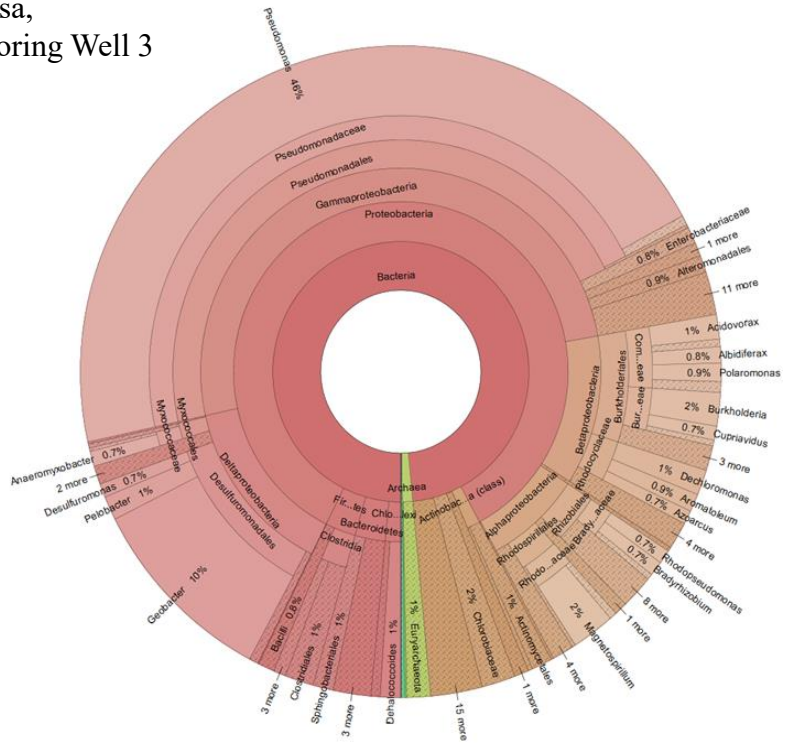


Supplementary Figure 7.19. Classification of microbial communities in injection well (A and B) and monitoring well (C, D and E) groundwater samples from Tulsa (data analyzed with MG-RAST).

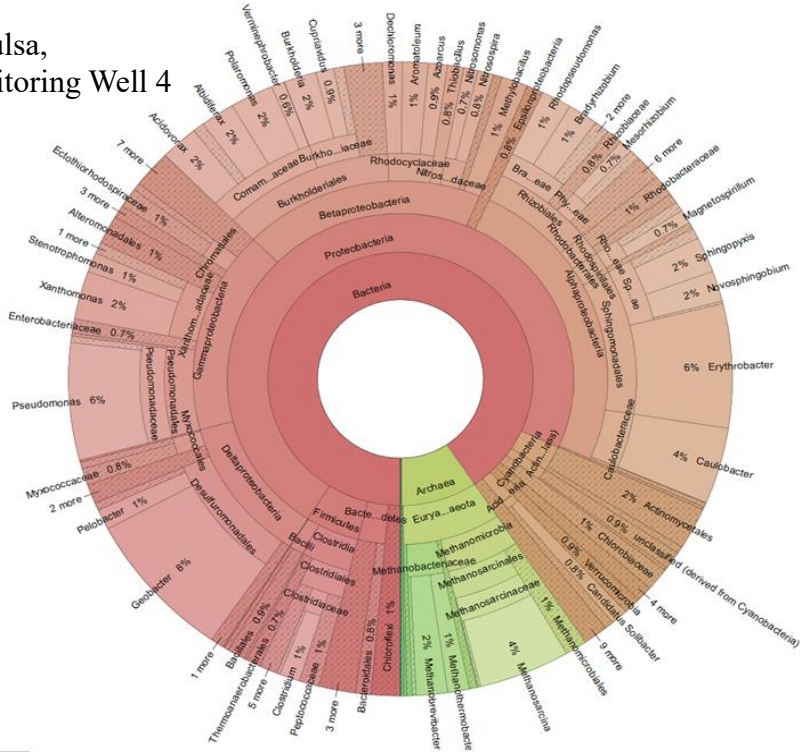
C. Tulsa,
Monitoring Well 2

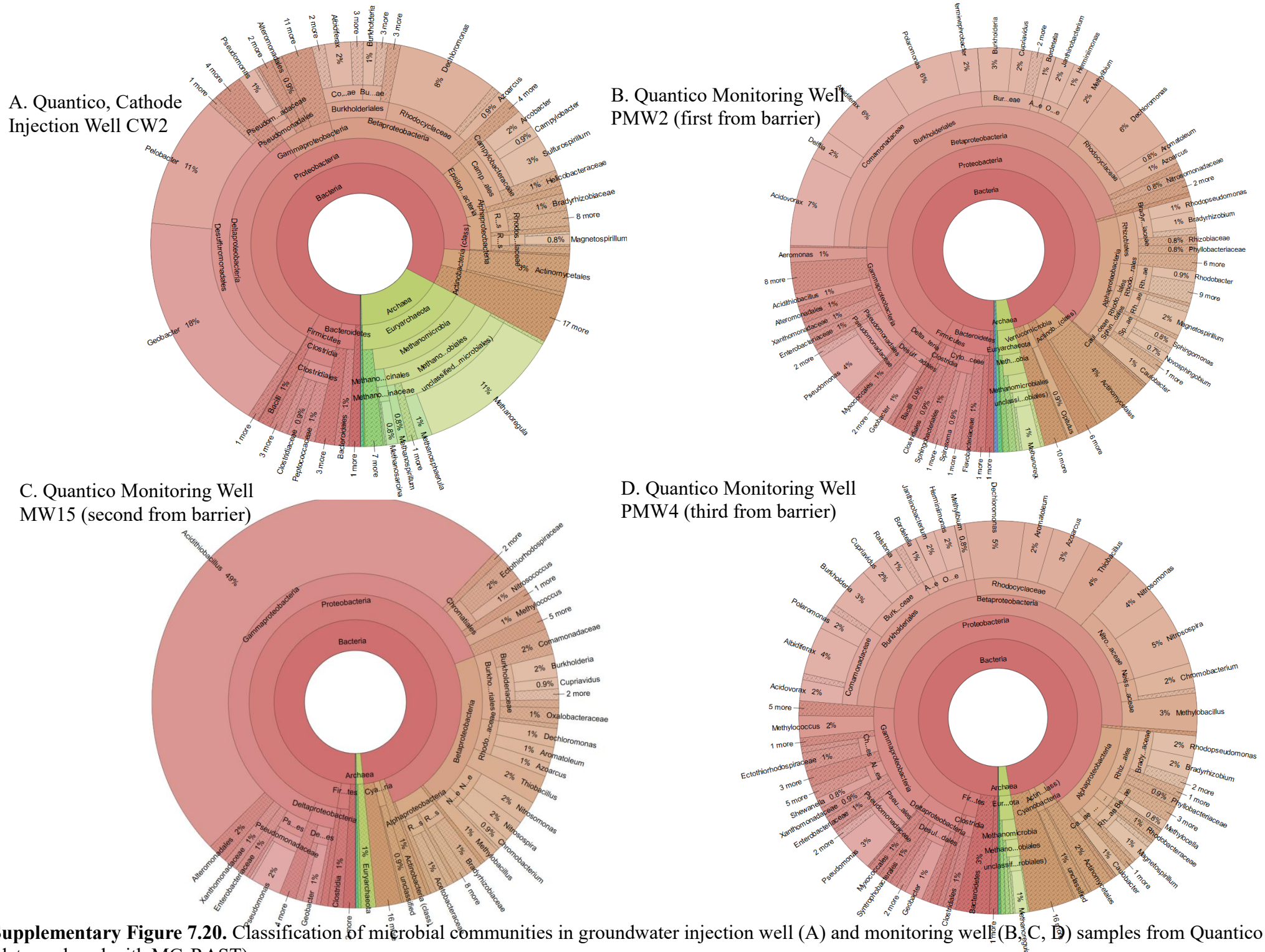


D. Tulsa,
Monitoring Well 3



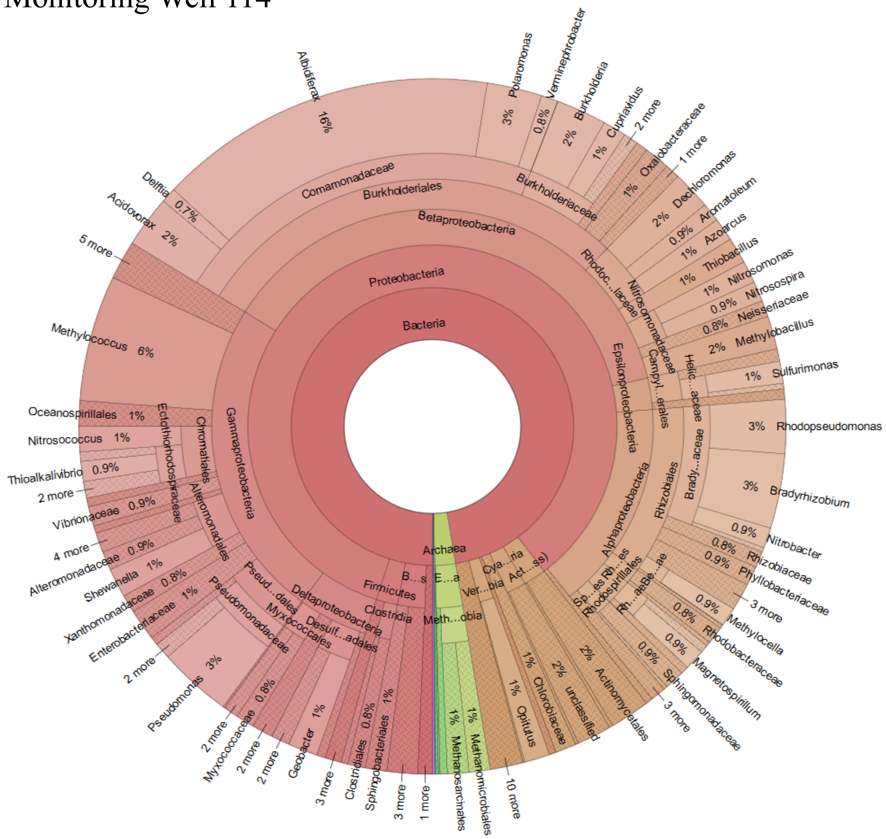
E. Tulsa,
Monitoring Well 4



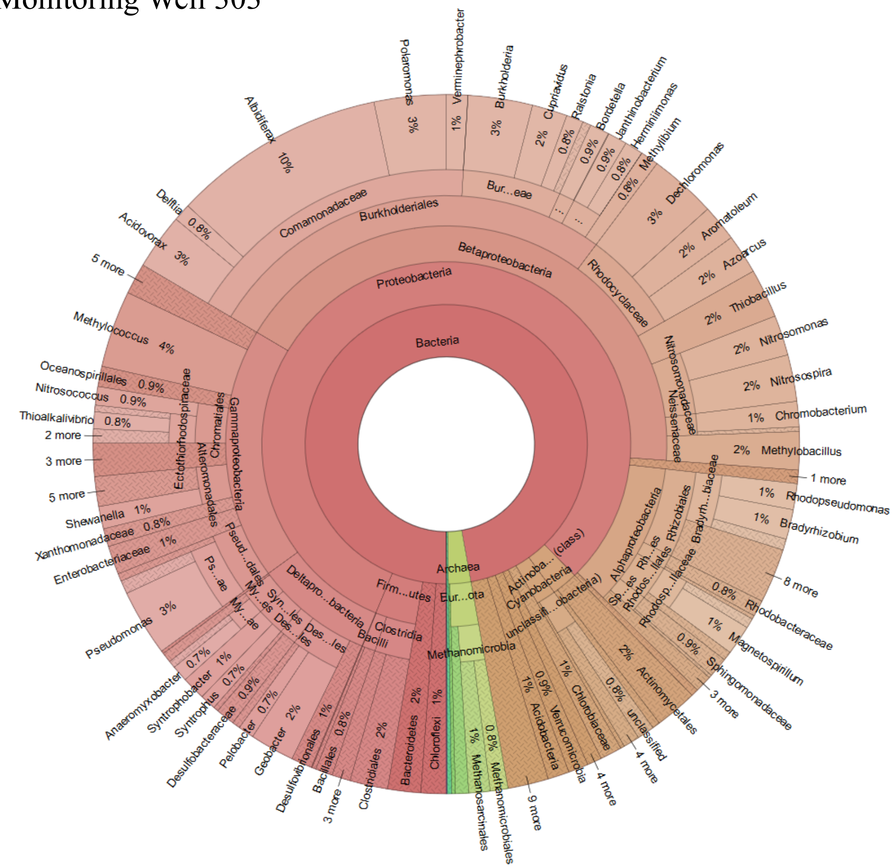


Supplementary Figure 7.20. Classification of microbial communities in groundwater injection well (A) and monitoring well (B, C, D) samples from Quantico (data analyzed with MG-RAST).

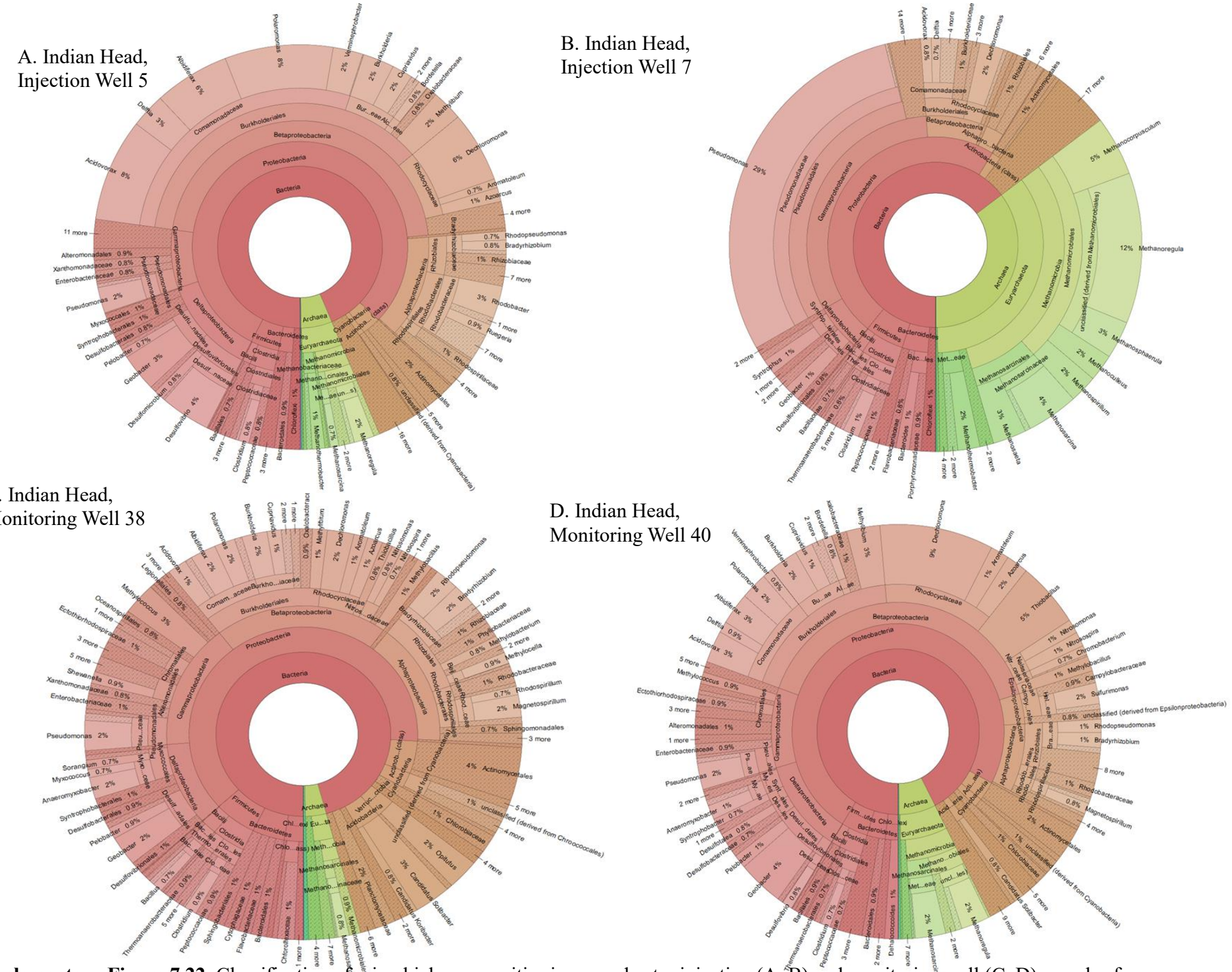
A. Edison,
Monitoring Well 114



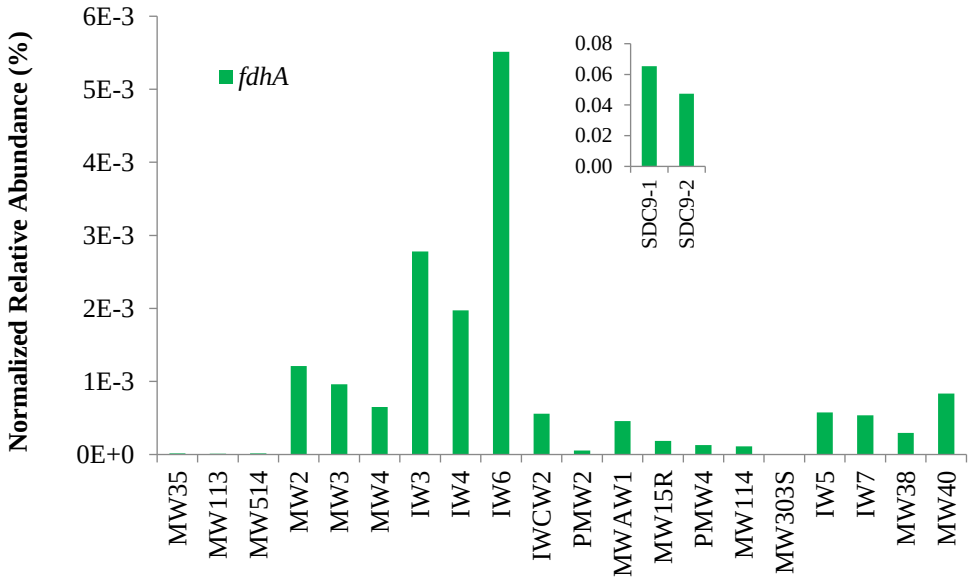
B. Edison,
Monitoring Well 303



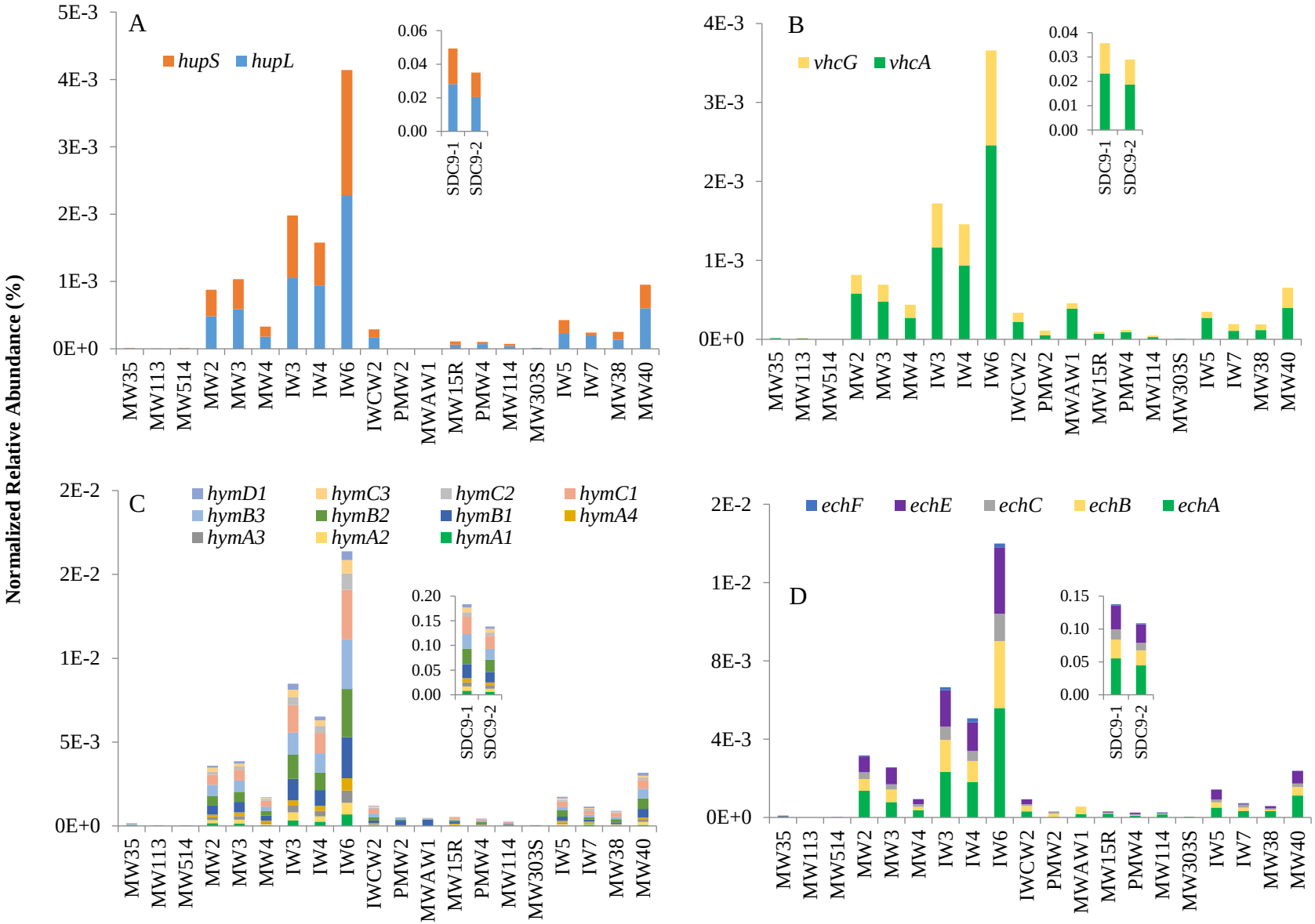
Supplementary Figure 7.21. Classification of microbial communities in groundwater monitoring well samples from Edison (data analyzed with MG-RAST).



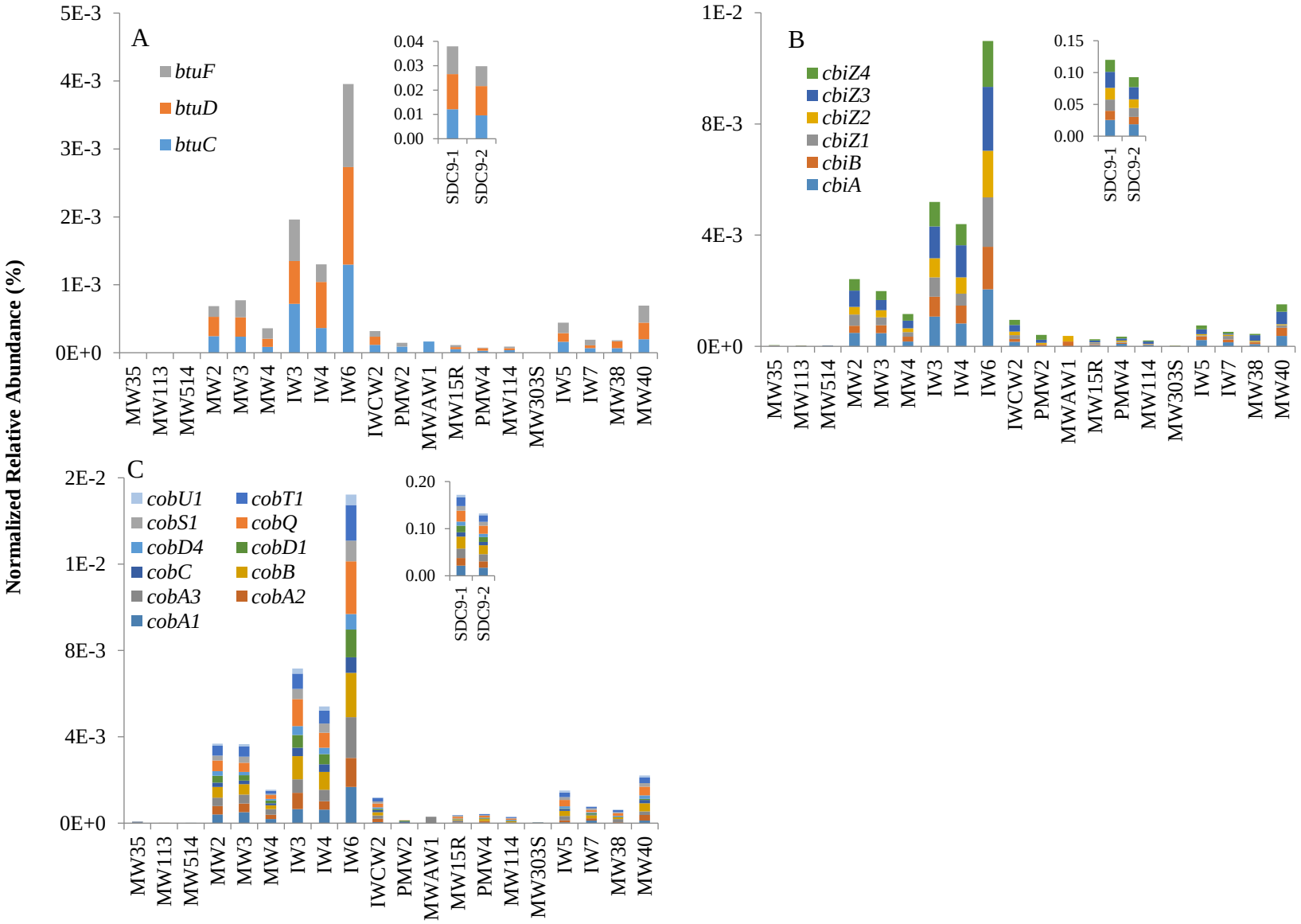
Supplementary Figure 7.22. Classification of microbial communities in groundwater injection (A, B) and monitoring well (C, D) samples from Indian Head (data analyzed with MG-RAST).



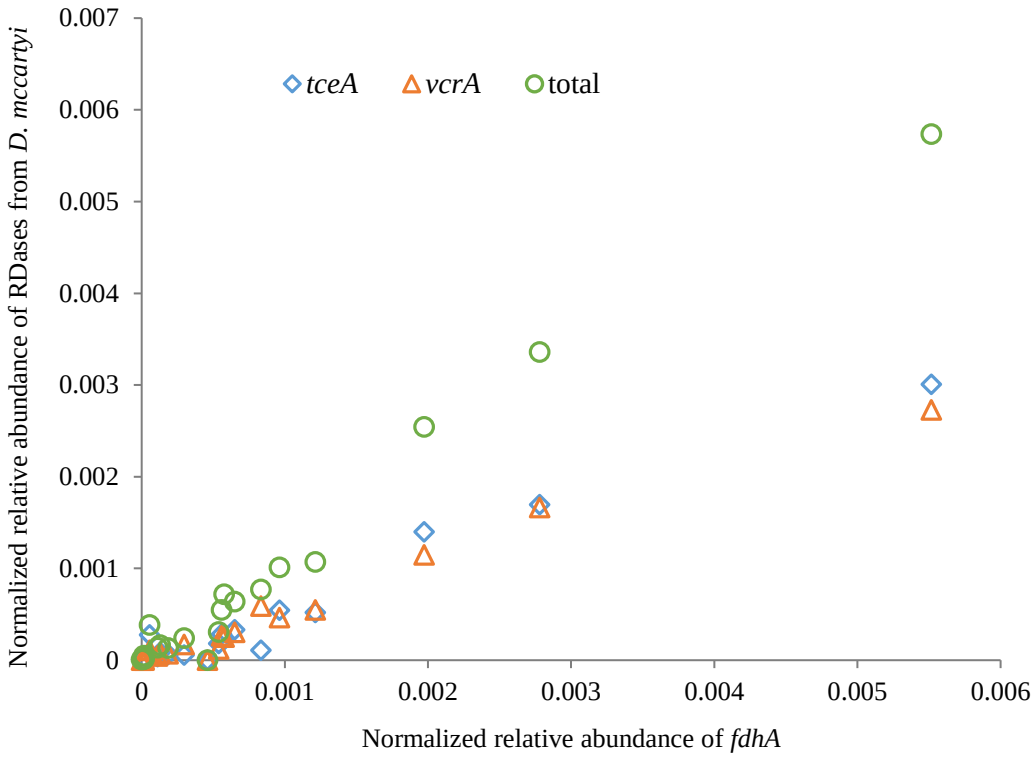
Supplementary Figure 7.23. Normalized relative abundance (%) of *fdhA* in SDC-9 (insert) and in groundwater from the five chlorinated solvent sites (data analyzed with DIAMOND).



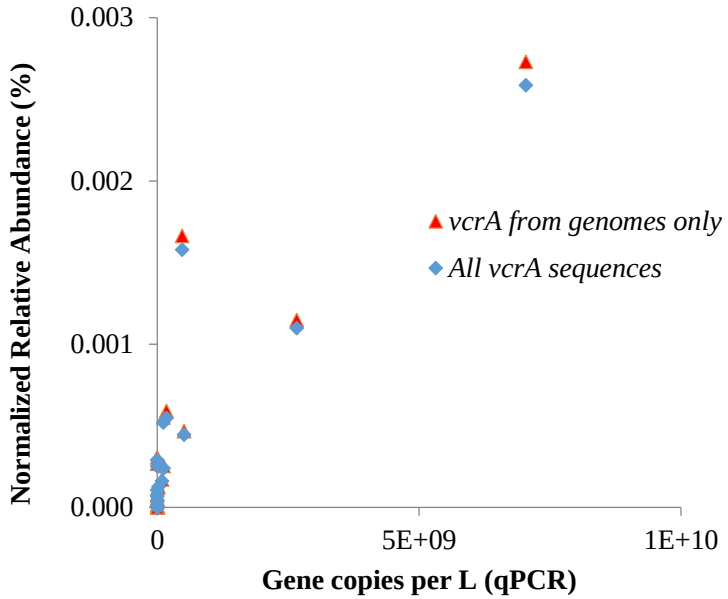
Supplementary Figure 7.24. Normalized relative abundance (%) of *Dehalococcoidies mccartyi* hydrogenase genes *hupLS* (A), *vhcAG* (B), *hymABCD* (C) and *echABCEF* (D) in SDC-9 (inserts) and in groundwater from the five chlorinated solvent sites (data analyzed with DIAMOND).



Supplementary Figure 7.25. Normalized relative abundance (%) of *Dehalococcoidies mccartyi* corrinoid metabolism genes *btuFCD* (A), *cbiA*, *cbiB*, *cbiZ* (B) and *cobA*, *cobB*, *cobC*, *cobD*, *cobQ*, *cobS*, *cobT*, *cobU* (C) in SDC-9 (inserts) and in groundwater from the five chlorinated solvent sites (data analyzed with DIAMOND).



Supplementary Figure 7.26. Comparison between normalized relative abundance of *vcrA*, *tceA* and sum of RDases to *fdhA* (data analyzed with DIAMOND).



Supplementary Figure 7.27. Comparison between *vcrA* gene copies (per L) determined via qPCR and shotgun sequencing (normalized relative abundance, %, MG-RAST). The results from two shotgun sequencing quantification methods are shown (as discussed in the text).

B. List of Scientific/Technical Publications

Articles Peer-Reviewed Journals

1. Dang, H., Kanitkar, Y. H., Stedtfeld, R. D., Hatzinger, P. B., Hashsham, S. A. and A. M. Cupples. 2018. Abundance of chlorinated solvent and 1,4-dioxane degrading microorganisms at five chlorinated solvent contaminated sites determined via shotgun sequencing, Environmental Science and Technology. 52 (23): 13914–13924.
2. Kanitkar, Y. H., Stedtfeld, R. D., Hatzinger. P. B., Hashsham, S. A. and A. M. Cupples. 2017. Development and application of a rapid, user-friendly and inexpensive method to detect *Dehalococcoides* sp. reductive dehalogenase genes from groundwater. Applied Microbiology and Biotechnology. 101: 4827–4835.
3. Kanitkar, Y. H., Stedtfeld, R. D., Hatzinger. P. B., Hashsham, S. A. and A. M. Cupples. 2017. Most probable number with visual based LAMP for the quantification of reductive dehalogenase genes in groundwater samples. Journal of Microbiological Methods. 143:44-49.
4. Stedtfeld, R. D., T. M. Stedtfeld, F. Samhan, Y. H. Kanitkar, P. B. Hatzinger, A. M. Cupples, and S. A. Hashsham. 2016. Direct loop mediated isothermal amplification on filters for quantification of *Dehalobacter* in groundwater. Journal of Microbiological Methods. 131: 61-67.
5. Kanitkar, Y. H., Stedtfeld, R. D., Steffan, R. J., Hashsham, S. A. and A. M. Cupples. 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.
6. Stedtfeld, R., Stedtfeld, T., Kronlein, M., Seyrig, G., Steffan, R., Cupples, A.M. and S. A. Hashsham. 2014. DNA extraction-free quantification of *Dehalococcoides* spp. in groundwater using a hand-held device. Environmental Science and Technology, 48: 13855-13863.

Conference Papers

Dang, H., Y. H. Kanitkar, R. D. Stedtfeld, S. A. Hashsham, P. B. Hatzinger and A. M. Cupples. 2018. Microbial community characterization at five chlorinated solvent sites following bioaugmentation with *Dehalococcoides* enriched culture, SDC-9. The Eleventh International Conference on Remediation of Chlorinated and Recalcitrant Compounds, April 8-12, 2018, Palm Springs, California

Presentations

1. Dang, H., Y. H. Kanitkar, R. D. Stedtfeld, S. A. Hashsham, P. B. Hatzinger and A. M. Cupples. 2018. Taxonomic and functional microbial community characterization at five chlorinated solvent sites following bioaugmentation. SERDP & ESTCP Symposium 2018: Enhancing DoD's Mission Effectiveness, November 27 - 29, 2018, Washington DC.
2. Dang, H., Y. H. Kanitkar, R. D. Stedtfeld, S. A. Hashsham, P. B. Hatzinger and A. M. Cupples. 2018. Microbial community characterization at five chlorinated solvent sites following bioaugmentation with *Dehalococcoides* enriched culture, SDC-9. College of Engineering Graduate Symposium. Breslin Center, MSU. 29th March 2018.
3. Dang, H., Y. H. Kanitkar, R. D. Stedtfeld, S. A. Hashsham, P. B. Hatzinger and A. M. Cupples. 2018. Microbial community characterization at five chlorinated solvent sites following bioaugmentation with *Dehalococcoides* enriched culture, SDC-9. The Eleventh International Conference on Remediation of Chlorinated and Recalcitrant Compounds, April 8-12, 2018, Palm Springs, California.
4. Kanitkar, Y., R. D. Stedtfeld, S.A. Hashsham, P. Hatzinger, A.M. Cupples. 2017. The application of loop mediated isothermal amplification (LAMP) for rapid detection of *vcrA* and *tceA* in groundwater samples. College of Engineering Graduate Symposium. Breslin Center, MSU. 30th March 2017.
5. Kanitkar, Y., R. D. Stedtfeld, S.A. Hashsham, P. Hatzinger, A.M. Cupples. 2017. Development and application of a rapid, user-friendly and inexpensive method to detect *Dehalococcoides* reductive dehalogenase genes from groundwater. Association of Environmental Engineering and Science Professors (AEESP) Conference, Ann Arbor, MI, 21st June 2017.

6. Cupples, A. M. 2017. The application of molecular methods to identify and quantify contaminant degrading microorganisms. School of Chemical, Biological and Environmental Engineering. Oregon State University, Corvallis, OR. May 31st 2017.
7. Cupples, A. M. Y. Kanitkar, H. Dang, R.D. Stedtfeld, S.A. Hashsham and P. Hatzinger. 2017. Application of loop mediated isothermal amplification and shotgun sequencing to detect chlorinated solvent degrading microorganisms in contaminated groundwater. SERDP ESTCP Symposium Enhancing DoD's Mission Effectiveness. 28-30th November, 2017.
8. Y. Kanitkar, R.D. Stedtfeld, S.A. Hashsham, P. Hatzinger and Cupples, A. M. 2017. Development and application of a rapid, user-friendly and inexpensive method to detect *Dehalococcoides* spp. reductive dehalogenase genes from groundwater. The Fourth International Symposium on Bioremediation & Sustainable Environmental Technologies, Miami, Fl. May 22nd 2017.
9. Kanitkar, Y., R. D. Stedtfeld, S.A. Hashsham, P. Hatzinger, A.M. Cupples. 2016. The application of loop mediated isothermal amplification (LAMP) for the rapid detection of *vcrA, bvcA* and *tceA* in groundwater samples. College of Engineering Graduate Symposium. Breslin Center, MSU. 31st March 2016.
10. Kanitkar, Y., R. D. Stedtfeld, S.A. Hashsham, P. Hatzinger, A.M. Cupples. 2016. The application of loop mediated isothermal amplification (LAMP) for rapid detection of *vcrA, bvcA* and *tceA* in groundwater samples. Fate of the Earth Symposium, Environmental Science and Policy Program, MSU April 6-7th 2016
11. Kanitkar, Y., R. D. Stedtfeld, S.A. Hashsham, P. Hatzinger, A.M. Cupples. 2016. The application of loop mediated isothermal amplification (LAMP) for the rapid detection of *vcrA, bvcA* and *tceA* in groundwater samples. The Tenth International Conference on Remediation of the Chlorinated Solvents and Recalcitrant Compounds. Palm Springs, CA, 25th May 2016
12. Cupples, A. M., Y. Kanitkar, R.D. Stedtfeld, S.A. Hashsham, P. Hatzinger. 2016. Development of field methodology to rapidly detect *Dehalococcoides* and *Dehalobacter* Spp. genes on site. SERDP In Progress Review (IRP) Meeting. May 17th 2016. Arlington, VA.
13. Kanitkar, Y., R. D. Stedtfeld, S.A. Hashsham, P. Hatzinger, A.M. Cupples. 2015**.** Development of loop mediated isothermal amplification (LAMP) for rapid detection and quantification of *Dehalococcoides* spp. in groundwater samples. Third International

Symposium on Bioremediation & Sustainable Environmental Technologies, Miami, Fl. May 20th 2015.

14. Kanitkar, Y., R. D. Stedtfeld, S.A. Hashsham, P. Hatzinger, A.M. Cupples. 2015. Development of loop mediated isothermal amplification (LAMP) for rapid detection and quantification of *Dehalococcoides* spp. in groundwater samples. College of Engineering Graduate Symposium. Breslin Center, MSU. April 2015.
15. Cupples, A. M., Y. Kanitkar, R.D. Stedtfeld, S.A. Hashsham, P. Hatzinger. 2015. Development of field methodology to rapidly detect *Dehalococcoides* and *Dehalobacter* Spp. Genes on Site. SERDP IPR Meeting. May 6th 2015. Arlington, VA.
16. Kanitkar, Y., R.D. Stedtfeld, T.M. Stedtfeld, S.A. Hashsham, R.J. Steffan, and AM Cupples. 2014. DNA-extraction free loop mediated isothermal amplification (LAMP) of *Dehalococcoides* spp. Engineering Graduate Research Symposium, March 24th, 2014, Breslin Center.
17. Kanitkar, Y., R. Stedtfeld, S. Hashsham, R. Steffan, and A. M. Cupples. 2014. DNA extraction free loop mediation isothermal amplification (LAMP) of *Dehalococcoides* and *Dehalobacter* spp. May 20th, 2014. Ninth International Conference on Remediation of Chlorinated and Recalcitrant Compounds. Monterey, CA, May 19-22, 2014
18. Cupples, A. M., Y. Kanitkar, R.D. Stedtfeld, S.A. Hashsham, R.J. Steffan. 2014. Development of field methodology to rapidly detect *Dehalococcoides* & *Dehalobacter* spp. genes. Chlorinated Solvents Technical Exchange Meeting, Arlington, VA, December 10th 2014.
19. Cupples, A. M., Y Kanitkar, R.D. Stedtfeld, T.M. Stedtfeld, S.A. Hashsham, RJ Steffan. 2014. Development of LAMP to detect *Dehalococcoides* & *Dehalobacter* spp. genes without DNA extraction. SERDP IPR Meeting, Arlington, VA, May 6th 2014.
20. Cupples, A. M., R. Stedtfeld, S. A. Hashsham and R. Steffan. 2012. Development of LAMP to detect *Dehalococcoides* & *Dehalobacter* spp. genes without DNA extraction. Brief to SERDP Scientific Advisory Board. October 23rd, 2012. Arlington, VA.