GUIDANCE DOCUMENT

Validation of Biotechnology for Quantifying the Abundance and Activity of Vinyl-chloride Oxidizers in Contaminated Groundwater

ESTCP Project ER-201425

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The University of Iowa

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The purpose of this guidance document is to provide a detailed technical protocol for conducting Quantitative PCR (qPCR) and Reverse transcription quantitative PCR (RT-qPCR) assays for Vinyl chloride (VC)-oxidizing bacteria in groundwater and sediment samples. Additional guidance on interpreting molecular data in the context of MNA processes and associated statistical analyses will be provided. This guidance document could be useful for site managers interested in utilizing qPCR/ RT-qPCR technology at their site or by a commercial laboratories interesting in transferring the technology for commercial applications.

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Executive summary

Ethene, the by-product of vinyl chloride (VC) reduction, commonly co-migrates along with VC from anaerobic zones. Ethene stimulates the activity of ethene-assimilating bacteria (etheneotrophs), which can also co-metabolize VC in the presence of oxygen. In a monitored natural attenuation (MNA) context, intrinsic aerobic biodegradation processes could account for significant mass removal. Yet despite scientific advances, the potential contribution of etheneotrophs and VC-assimilators to natural attenuation of VC is still not well understood or quantified. The ultimate goal of this project was to develop quantitative evidence of this contribution.

Etheneotrophs and methanotrophs are two major bacterial groups that could contribute significantly to aerobic VC biodegradation in contaminated groundwater systems. The genes *etnC* and *etnE* and their transcripts are targeted as indicators of the abundance and activity of etheneotrophs. Methanotrophs, which can cometabolize VC in the presence of methane, are present and likely active at most VC-contaminated sites because methane is commonly generated under the same conditions favorable for reductive dechlorination of chlorinated ethenes. Methanotrophs are well known for their ability to cometabolize VC, yet, reports demonstrating the contributions of methanotrophs to VC cometabolism in the field are not abundant in the literature.

The purpose of this project was to validate the utility of qPCR-based molecular diagnostic tools in demonstrating the attenuation contribution of VC-oxidizing bacteria (particularly etheneotrophs) in groundwater and aquifer samples from several DoD sites. This method involved employs degenerate oligonucleotide primers that target functional genes, which are involved in the aerobic VC biodegradation pathway employed by etheneotrophs.

Over the course of the project, 95 groundwater samples were collected from monitoring wells located in 7 VC plumes at 6 contaminated sites. DNA and RNA were extracted from each of these samples in duplicate generating 190 DNA and 188 RNA extracts for qPCR analysis. The data generated were used to investigate whether functional genes associated with VC oxidation processes were present and expressed at VC-contaminated sites under different geochemical conditions (including nominally anaerobic conditions) at sites featuring a wide range of VC concentrations.

During this project, field demonstration of these tools in a variety of different groundwater environments (e.g., different VC concentrations, different hydrogeological conditions, natural attenuation vs. biostimulation scenarios) established relationships between VC-oxidizer functional gene abundance and expression with VC concentration as well as other geochemical parameters (Liang et al., 2017b). Relationships between functional genes associated with VC biodegradation and geochemical parameters as well as the bulk VC attenuation rate at contaminated sites were determined. This revealed that functional genes associated with both VC oxidation and VC reductions are often present and expressed in groundwater samples.

The following are offered as lessons learned from the controlled evaluation:

1. The RT-qPCR/qPCR technology for etheneotrophs is an innovative and rapid means of revealing information about the potential for in situ microbial aerobic VC oxidation at contaminated sites.

2. Of the 95 samples analyzed during this effort, functional genes from etheneotrophs and methanotrophs and anaerobic VC-dechlorinators were frequently present (99%) and expressed (59%) in the same groundwater samples the majority of which featured low DO and ORP levels.

3. Although confident quantitative relationships between bulk VC attenuation rates and functional gene abundance and expression could not be made, data analysis shown in Table 5, revealed that both etheneotroph functional gene (*etnC* and *etnE*) abundances are significantly correlated with categorical bulk VC attenuation rates. This relationship was also seen for VC reductive dehalogenase gene *vcrA* and the sum of VC reductive dehalogenase genes *bvcA* and *vcrA*. In contrast, methanotroph functional genes *pmoA* and *mmoX* were not correlated with categorical bulk VC attenuation rates.

4. Strong positive relationships using both Spearman's correlation analysis (Liang et al., 2017b) and multilevel modeling were observed between VC concentrations and the abundance and expression of functional genes from etheneotrophs and anaerobic VC-dechlorinators (Fig. 7). However, this relationship with VC concentration was not observed with methanotroph functional genes (Fig. 7). This is an important observation with respect to etheneotrophs, and indicates the abundance of etheneotrophs is greater when VC concentrations are greater irrespective of the dissolved oxygen concentration. Functional gene abundance for etheneotrophs will be proportional to VC concentration irrespective of measured DO concentrations in the groundwater.

5. During the project, we also found a high level of co-occurrence of etheneotroph functional genes and VC reductive dehalogenase genes in groundwater samples. Therefore, we concluded from this analysis that etheneotrophs have a strong potential to contribute to VC biodegradation in groundwater when VC concentrations are high and in areas of the aquifer that may be considered anaerobic. Therefore, it is recommended that qPCR analyses for aerobic VC oxidizers always be conducted concurrently with those for anaerobic VC dechlorinators during long term groundwater monitoring, irrespective of DO concentrations, so as to build lines of evidence for natural attenuation of VC for the entire site.

The spatial resolution of A) total 16S rRNA genes, B) *etnC* and *etnE* (etheneotroph functional genes), C) *mmoX* and *pmoA* (methanotroph functional genes), and D) *bvcA* and *vcrA* (VC reductive dehalogenase genes) in an aquifer sediment core, frozen in place with liquid nitrogen, obtained from Parris Island Site 45 showed that indeed, functional genes from etheneotrophs and anaerobic VC-dechlorinators coexist within small (~0.25 g) sediment samples as deep as 18 feet below ground surface in this particular sediment core. This suggests that there is the potential for essentially simultaneous aerobic and anaerobic VC biodegradation at this site, even at a substantial depth.

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List of Acronyms

AkMO	Alkene monooxygenase
BSA	Bovine Serum Albumin
cDCE	*cis*-1,2-Dichloroethene
cDNA	Complementary DNA
C_t	Threshold cycle
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
DO	Dissolved oxygen
DoD	Department of Defense
EaCoMT	Epoxyalkane:Coenzyme M transferase
EPA	Environmental Protection Agency
EZVI	Emulsified zero-valent iron
FISH	Fluorescence *in situ* hybridization
IFC	integrated fluidic circuit
ISCO	in situ chemical oxidation
LOD	Level of detection
LOQ	Level of quantification
MCL	Maximum contaminant level
MCRD	Marine corps recruit depot
MNA	Monitored natural attenuation
mRNA	Message RNA
NTC	No template control
ORP	Oxidation-reduction potential
PCE	Tetrachloroethene
PCR	Polymerase chain reaction
pMMO	Particulate methane monooxygenase
qPCR	Quantitative PCR
QA/QC	Quality assurance/quality control
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription quantitative PCR
SDIMO	Soluble di-iron monooxygenase alpha subunit
SERDP	Strategic Environmental Research and Development Program
sMMO	Soluble methane monooxygenase
TCE	Trichloroethene
TeCA	Tetrachloroethane
VC	Vinyl chloride (monochloroethene)

1.0 INTRODUCTION

1.1 BACKGROUND

Contamination of groundwater by the widely used chlorinated solvents tetrachloroethene (PCE) and trichloroethene (TCE) is a pervasive environmental problem at Department of Defense (DoD) installations. In addition, certain DoD installations face groundwater contamination issues with chlorinated ethanes such as 1,1,2,2 tetrachloroethane (TeCA), among other contaminants.

A very popular and robust groundwater clean-up strategy for chlorinated ethenes (and chlorinated ethanes) is anaerobic reductive dechlorination. In many cases, injection of electron donor amendments to a contaminated aquifer is employed to develop anaerobic conditions conducive to reductive dechlorination of PCE and TCE and 1,1,2,2-tetrachloroethane (TeCA). This leads to the production of lesser chlorinated and even non-chlorinated daughter products (i.e. ethene and ethane). In some cases, bioaugmentation with cultures containing specialized anaerobic strains can accelerate the process of generating the desired end-products, ethene and ethane.

Vinyl chloride (VC) is a daughter product of both chlorinated ethene and chlorinated ethane reduction. VC is most commonly generated in groundwater by reductive dechlorination of *cis*-dichloroethene (cDCE). VC can also be formed by dihaloelimination of 1,1,2-trichloroethane (a daughter product of TeCA dechlorination).

Although *in situ* bioremediation via anaerobic reductive dechlorination is a robust biotechnology for clean-up of chlorinated ethenes and certain chlorinated ethanes, VC often accumulates in groundwater at sites where this strategy is employed. VC plumes are therefore present at many DoD sites. This is problematic from a regulatory standpoint in that VC is a known human carcinogen with a low Environmental Protection Agency (EPA) maximum contaminant level (MCL) of 2 ppb. There are significant costs associated clean-up of VC plumes, primarily because they are can resist anaerobic dechlorination and often evolve into large, dilute plumes such that a large volume of VC-contaminated water must be treated. As a result, monitored natural attenuation (MNA) represents an attractive, cost-effective treatment option for VC plumes.

In the context of MNA, VC plumes formed under anaerobic conditions have often migrated (or have the potential to migrate) into zones where sufficient oxygen is present for oxidative degradation processes to become significant. Under this scenario, intrinsic aerobic biodegradation processes could account for significant VC mass removal. For instance, the co-migration of methane along with VC into aerobic groundwater could promote the activity of methanotrophic bacteria, which are known to cometabolize VC. Ethene, the by-product of VC reduction, also commonly co-migrates with VC from anaerobic zones. Ethene can stimulate the activity of ethene-assimilating bacteria (i.e. etheneotrophs), which also co-metabolize VC while utilizing ethene as a primary substrate. Several etheneotrophic strains are known to switch from a cometabolic VC oxidation mode to a growth-coupled VC oxidation mode. Growth-coupled VC oxidation is an especially attractive approach to VC bioremediation (at moderate to high concentrations of VC) because it represents a sustainable process not subject to the toxicity issues and requirements for a primary substrate associated with cometabolic VC processes.

However, in very dilute VC plumes cometabolic VC oxidation processes are expected to dominate over growth-coupled VC oxidation processes.

Although methanotrophs are well known for their ability to cometabolize VC, reports demonstrating the contributions of methanotrophs to VC cometabolism in the field are not abundant in the literature (Semprini, *et al.*, 1991) . Several studies providing evidence that etheneotrophs cometabolize VC in the field have recently been published (Begley, *et al.*, 2012, Atashgahi, *et al.*, 2013, Patterson, *et al.*, 2013, Mattes, *et al.*, 2015). These recent small-scale field studies suggest that etheneotrophs could contribute significantly to aerobic natural attenuation of VC plumes.

Despite these scientific advances, the potential contribution of etheneotrophs and VC-assimilators to natural attenuation of VC is still not well understood or quantified. Perhaps a roadblock to greater understanding is that until recently rapid and cost-effective methods for providing microbiological lines of evidence for VC oxidation in aquifers was lacking. Our SERDP-supported research (Project ER-1683) has addressed several of these knowledge and technology gaps. We have developed quantitative PCR (qPCR) and reverse transcription quantitative PCR (RT-qPCR) methods that could provide rapid, useful and cost-effective information about the abundance and activity of VC-oxidizing etheneotrophs in the context of a long-term MNA strategy. We also adapted existing qPCR methods for methanotrophs from primers in the literature and showed that these primers could also be used in RT-qPCR experiments (Mattes, *et al.*, 2015).

There also appears to be the general perception that oxidative processes are not present or insignificant under the typical geochemical conditions in contaminated aquifers. However, other recent publications suggest that aerobic VC-oxidizers could be active in regions of the aquifer that are considered anoxic or anaerobic (Gossett, 2010, Fullerton, *et al.*, 2014). Thus, the complex subsurface environment should really not be considered to be either completely aerobic or completely anaerobic, and monitoring for aerobic VC biodegradation processes should be conducted alongside monitoring for anaerobic chlorinated ethene reductive dechlorination processes to assess all potential pathways for VC removal at contaminated sites.

The purpose of ER-201425 was to demonstrate and validate the application of qPCR-based molecular diagnostic tools for VC-oxidizing bacteria (particularly etheneotrophs) in groundwater and aquifer sediment samples from several DoD sites. Over the course of the project 95 groundwater samples were collected from monitoring wells located in 7 VC plumes at 6 contaminated sites. DNA and RNA were extracted from each of these samples in duplicate generating 190 DNA and 188 RNA extracts for qPCR analysis. The data generated were used to investigate whether functional genes associated with VC oxidation processes are present and expressed at VC-contaminated sites under different geochemical conditions (including nominally anaerobic conditions) at sites featuring a wide range of VC concentrations. Relationships between functional genes associated with VC biodegradation and geochemical parameters as well as the bulk VC attenuation rate at contaminated sites were determined. This revealed that functional genes associated with both VC oxidation and VC reduction are often present and expressed in the sample groundwater sample. The results of this effort were recently published (Liang, *et al.*, 2017a, Liang, *et al.*, 2017b).

1.2 OBJECTIVE OF THIS GUIDANCE DOCUMENT

The purpose of this guidance document is to provide a detailed technical protocol for conducting qPCR and RT-qPCR assays for VC-oxidizing bacteria in groundwater and sediment samples. Additional guidance on interpreting molecular data in the context of MNA processes and associated statistical analyses will be provided. This guidance document could be useful for site managers interested in utilizing qPCR/RT-qPCR technology at their site or by a commercial laboratories interesting in transferring the technology for commercial applications. This demonstration and validation project has shown that application of environmental molecular diagnostic tools such as qPCR during long term monitoring of VC-contaminated groundwater yields useful information for site managers who are interested in better understanding the potential for natural attenuation of VC. Application of these environmental molecular diagnostic tools could lead to cost savings if lines of evidence lead to regulatory approval for MNA of VC, rather than a more intensive interventions with electron donor injections and bioaugmentation of anaerobic VC dechlorinating bacteria.

1.3 REGULATORY DRIVERS

The USEPA MCL for VC in drinking water is 2 μg/L. Although the goal of enhanced bioremediation of chloroethenes is complete conversion into ethene, this is rarely achieved in practice in field situations. Generation of VC plumes resulting from incomplete anaerobic reductive dechlorination of the more highly chlorinated ethene can significantly increase the costs and time of clean-up, particularly if the VC plume becomes large and dilute. During this project, we have improved the current understanding of the distribution and expression of functional genes associated with VC oxidation in several VC plumes with varying levels of VC concentrations. This new understanding will be useful when implementation of MNA is desirable to achieve cleanup goals and site closures. These findings are detailed in the following Guidance Protocol, which is aimed at assisting implementation of qPCR/RT-qPCR methods and interpreting the resulting data for the presence and expression of functional genes from VC-oxidizers at contaminated sites.

2.0 TECHNOLOGY

2.1 TECHNOLOGY DESCRIPTION

We have developed a SYBR green-based qPCR method that yields rapid information concerning presence, abundance, and expression of functional genes from aerobic VC-oxidizing microorganisms. This method employs degenerate oligonucleotide primers that target functional genes which are involved in the aerobic VC biodegradation pathway employed by etheneotrophs. The etheneotroph qPCR technology was developed, demonstrated, and validated in our laboratory (Jin & Mattes, 2010, Jin & Mattes, 2011, Mattes, *et al.*, 2015). This technology is innovative because it has revealed relationships between the abundance and functionality of aerobic VC-oxidizers at contaminated sites and the concentration of VC, as well as the bulk VC attenuation rate. How the technology provided this information will be discussed later in this report.

Etheneotrophs and methanotrophs are two major bacterial groups that could contribute significantly to aerobic VC biodegradation in contaminated groundwater systems. The genes *etnC* and *etnE* and their transcripts are targeted as indicators of the abundance and activity of etheneotrophs. The gene *etnC* encodes the alpha subunit of alkene monooxygenase (AkMO). The gene *etnE* encodes the epoxyalkane:coenzyme M transferase (EaCoMT). AkMO attacks VC and ethene and incorporates oxygen atoms into these compounds to form epoxide intermediates. EaCoMT detoxifies and/or transforms these epoxides into compounds that can enter central metabolic pathways (Mattes, *et al.*, 2010). Methanotrophs, which can cometabolize VC in the presence of methane, are present and likely to be active at most VC-contaminated sites because methane is commonly generated under the same conditions favorable for reductive dechlorination of chlorinated ethenes. Methanotroph functional genes *pmoA* (encodes the particulate methane monooxygenase alpha subunit) and *mmoX* (encodes the soluble methane monooxygenase subunit) and their transcripts are targeted to reveal the abundance and activity of methanotrophs (McDonald, *et al.*, 2008). For comparison purposes, we also include qPCR assays for anaerobic VC reductive dehalogenase genes (*bvcA* and *vcrA*) as these genes could be participating in simultaneous VC removal in groundwater systems alongside those from etheneotrophs and methanotrophs.

2.1.1 DESIGN AND LAYOUT OF TECHNOLOGY COMPONENTS

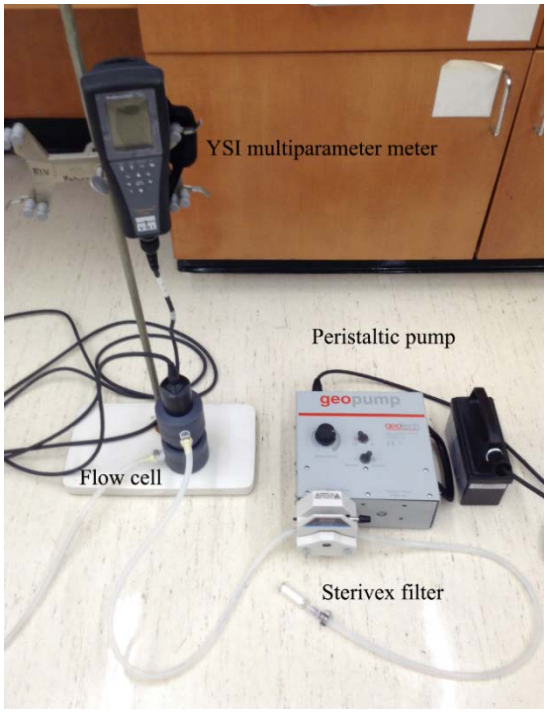

Figure 1. Set-up of our groundwater sampling equipment

The qPCR technology for estimating the abundance of functional genes from VC-oxidizing etheneotrophs was developed under SERDP (Project ER-1683). The qPCR technology, subsequent improvements, and applications have been described in several published articles (Jin, *et al.*, 2010, Jin & Mattes, 2011, Mattes, *et al.*, 2015). We use previously published primer sets to amplify methanotroph functional genes and their transcripts (Holmes, *et al.*, 1995, McDonald, *et al.*, 1995, Fuse, *et al.*, 1998, Kolb, *et al.*, 2003). We have recently published an article describing the RT-qPCR method extension with a small field-scale application (Mattes, *et al.*, 2015).

2.1.1.1 Groundwater sampling, filtering, and preservation

Because groundwater sampling from pre-existing monitoring wells is routinely performed by field technicians, bacteria (including VC-oxidizers) are therefore collected by sampling groundwater from wells along with other geochemical parameters of interest (e.g. chlorinated ethenes and dissolved gases (ethene and methane)). The sampling equipment include Geopump Peristaltic Pump Series I with easy-load I pump head or low flowrate groundwater sampling. A YSI Professional Plus handheld multiparameter meter (or

similar piece of equipment) was used to measure DO, pH, ORP, and conductivity of the groundwater prior to sampling for other chemical analyses or passed through Sterivex filters to collect groundwater biomass. The probes are inserted into a flow-through cell to facilitate these parameter measurements. The sampling equipment set-up is shown in Figure 1. A close-up of the fittings used to connect the Sterivex filter is shown in Figure 2.

Groundwater sampling follows USEPA 540/S-95/504 low-flow procedures (Puls & Barcelona, 1996). Prior to sampling, monitoring wells are purged at a flow rate <500 mL/min. Groundwater geochemical parameters are recorded in the field as described above. When readings stabilize (i.e. vary <10% within a minute), the flow-through cell is disconnected and groundwater is collected in 40 mL glass vials with Teflon-lined screw caps (VOA vials) and preserved with concentrated hydrochloric acid. VOA vials are placed on ice and shipped to a commercial laboratory for chlorinated ethenes (PCE, TCE, DCEs, VC) analysis using EPA method 8260B and dissolved gases (ethene and methane) using EPA method RSK 175.

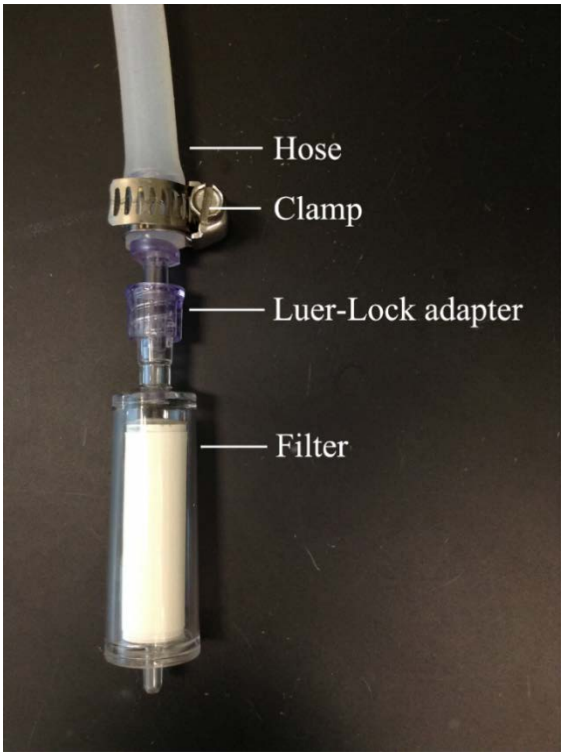

Figure 2. Close-up of the fitting used to connect the hose to the Sterivex filter.

After sample collection in VOA vials, microbial biomass samples for replicate DNA and RNA extractions are collected by passing groundwater through Sterivex-GP filters (pore size 0.22 µm, diameter 1.7 cm, Millipore, Germany)(Figure 2). Up to 3 liters groundwater is collected for RNA extraction and up to 1 liter groundwater is collected for DNA extraction. To preserve RNA for laboratory extraction, 3 mL of RNAlater (Ambion, Grand Island, NY) per filter is injected into Sterivex filters with a sterile syringe immediately after sampling. Filters are then placed into sterile 50 mL screw-capped Falcon tubes and shipped on ice by overnight courier to the laboratory. Filters with RNAlater preservation are subjected to RNA extraction immediately upon arrival. Filters for DNA extraction are stored at -80°C prior to extraction.

2.1.1.2 DNA and RNA extraction

Nucleic acid extraction is performed using commercially available DNA and RNA extraction kits. The MoBio PowerWater Sterivex DNA isolation kit and PowerWater RNA isolation kits (now provided by Qiagen) were used in this project, primarily because these kits are already commonly used in the industry. These kits utilize a mechanical cell lysis procedure (i.e. beadbeating) for releasing nucleic acids. DNA is extracted from filters without opening the filter housing according to the kit instructions. Prior to RNA extraction, the residual RNAlater left in the Sterivex filter is washed out by the buffer "PBS" (8 g/L NaCl, 0.2 g/L KCl, 1.44g/L Na_2HPO_4, 0.24 g/L KH_2PO_4, pH 7.4). After opening the filter cartridge with a PVC pipe cutter, the filter membrane is carefully excised with an autoclaved razorblade, removed with sterile

tweezers, inserted into kit-supplied bead tubes, and subjected to RNA extraction according to kit instructions. After extraction, RNase Inhibitor is added to RNA samples to prevent them from degrading. Extracted DNA and RNA is stored at -80°C prior to further analysis.

2.1.1.3 Addition of reference nucleic acids (RNA)

Before the cell lysis step in RNA extraction, known amounts of a reference nucleic acid (1 ng) (luciferase mRNA (1 ng) (GenBank accession No. X65316, Promega, Madison, WI) are added to the RNA samples after the lysis step to serve as internal controls for the efficiency of reverse transcription and other RNA losses throughout the remaining steps in the process (such as DNAse I treatment and RNA purification). To achieve this we used mRNA transcribed from the luciferase gene (ref mRNA), as described in a previous study (Johnson, *et al.*, 2005). Using reference mRNA allows us to more accurately estimate transcript abundance following qPCR.

The RNA recovery efficiency (%) is calculated as follows:

$$\%\,recovery = \frac{luciferase\ cDNA\ copies\ after\ qPCR}{total\ luciferase\ mRNA\ added} \times \frac{total\ cDNA\ vol\ after\ R.T.}{cDNA\ vol\ used\ for\ qPCR}$$

$$\times \frac{total\ RNA\ vol\ after\ RNA\ clean\ up}{RNA\ vol\ used\ for\ R.T.} \times 100\%$$

RNA recovery efficiencies are highly variable, ranging from 0.015-17.8% during this project. The reasons for the variability are unclear but there are many steps in the process where variability can be introduced (e.g. lysis, reverse transcription, and RNA purification).

2.1.1.4 Reverse transcription of RNA into cDNA

After extraction, RNA is subjected to DNA removal, RNA clean up, and converted to complementary DNA (cDNA) using the enzyme reverse transcriptase with random hexamer primers. Some RNA extract is kept as a control to test for any remaining DNA contamination in the RNA during qPCR. RNA samples are reversed transcribed into single stranded complementary DNA (cDNA) by SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) after contaminating DNA removal by DNase I (Biolab, Ipswich, MA) and purification by the RNeasy Mini Kit (Qiagen, Germantown, MD) according to manufacturer's protocol. For example, a 60 µL first strand cDNA synthesis reaction contains 30 µL purified RNA, 10 mM dNTP mix, and 2.25 µg random primers.

2.1.1.5 qPCR analysis of VC biodegradation functional genes and the luciferase reference gene, using both DNA and cDNA templates

Once DNA and RNA have been extracted, ref mRNA added, and RNA reversed transcribed (for RNA samples), then the samples are subjected to qPCR. Here we aim to estimate the gene and transcript abundances of *etnC*, *etnE*, *mmoX*, *pmo*A, *bvcA*, and *vcrA* using the primers in Table 2. For cDNA samples, the luciferase reference gene abundance is also quantified to measure the RNA loss during the experiment. DNA and cDNA samples (and DNA standards) are mixed with qPCR reagents (PCR Buffer containing Mg^{2+}, Taq polymerase, DNA building blocks (dNTPs),

oligonucleotide primers and SYBR Green dye), samples and calibration standards are loaded onto a plate (either 96 well or 384 well), and placed into an Applied Biosystems real-time PCR instrument. The overall workflow is depicted in Figure 3.

Each 20 µL qPCR contains 10 µL Power SYBR Green PCR Master Mix (Invitrogen, Carlsbad, CA) and variable primer and template (DNA and single-stranded cDNA) concentrations (Table 3). Bovine serum albumin (100 ng/µL) is added to each reaction to relieve possible PCR inhibition. PCR thermocycler conditions are as follows: 10 min at 95°C, followed by 40 cycles at 95°C (15 s) and 60°C (1 min).

During real-time PCR, the target genes are amplified and detected by increase in fluorescence as SYBR Green is a non-specific double-stranded DNA (dsDNA) binding dye. For each gene of interest, a range of standard DNA template concentrations must be included to develop a linear relationship between gene copy numbers and the Ct value (threshold cycle, the cycle number required for the generated fluorescence to reach the threshold detection line).

Following the real-time PCR thermocycling program, the temperature is raised incrementally, which will eventually lead to denaturation of the dsDNA PCR products. This is indicated by observing changes in the fluorescence intensity as SYBR Green dissociates from the melting PCR product. The software program produces a "melt curve" where peaks in the curve correspond to the sequence-dependent melting of PCR products as different temperatures. Non-specific amplification products and primer-dimers will impact quantitation since they cannot be distinguished from specific products. Thus the melt-curve is an important QA/QC step in this process as it is helpful in determining the specificity of amplification. Additional details are provided in Section 2.1.1.8.

Following qPCR and QA/QC steps, Applied Biosystems Sequence Detection System software is used to determine standard calibration curve relationships and estimate gene and transcript abundance in the samples. After carefully accounting for dilutions made throughout the process, the amount of luciferase genes and transcripts quantified is compared to the amount added, and the percent loss is applied as a correction factor when quantifying *etnC*, *etnE*, *mmo*X, *pmoA*, *bvcA*, and *vcrA* gene and transcript abundance. Finally, gene expression is shown in terms of the transcript per gene ratio.

Additional qPCR information, including primer concentrations, template concentrations, qPCR linear range, qPCR efficiency range of the standard curves, and Y-intercepts need to be recorded in accordance with Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines for the purpose of quality control (Bustin, *et al.*, 2009).

2.1.1.6 qPCR standard construction

We use an absolute quantification qPCR approach to estimate the gene copy numbers in the DNA or cDNA sample, which means that each qPCR experiment needs to include a standard curve. A qPCR standard curve establishes a linear relationship between the Ct values and log of input nucleic acid concentration. For our qPCR experiments, we use PCR products amplified from pure cultures as standards. We chose to use PCR products larger than the target amplicon rather than the amplicon cloned into a plasmid because we found in earlier experiments this

resulted in improved PCR efficiencies (Jin & Mattes, 2010). Specifically, PCR product amplified from luciferase DNA with primer set ref-STF/R is used as standard for luciferase mRNA qPCR. For *etn*C and *etn*E qPCR, PCR products amplified from JS614 (ATCC AF498452) genomic DNA with RTC-f/r and RTE-f/r are used as standards. For *mmo*X and *pmo*A qPCR, standards are PCR products amplified from *Methylococcus capsulatus* (ATCC 33009) genomic DNA with mmoX-std2f/r and from *Methylocystis* sp. strain Rockwell (ATCC 49242) genomic DNA with pmoA-std1f/r, respectively. For *bvcA* and *vcrA* qPCR, standards were linearized pCR2.1 TOPO-TA vectors containing *bvcA* and *vcrA* gene insertions (Ritalahti, *et al.*, 2006). The information about primer sets that are used to amplify PCR products for use in standards is shown in Table 1. The qPCR primers that we use are shown in Table 2.

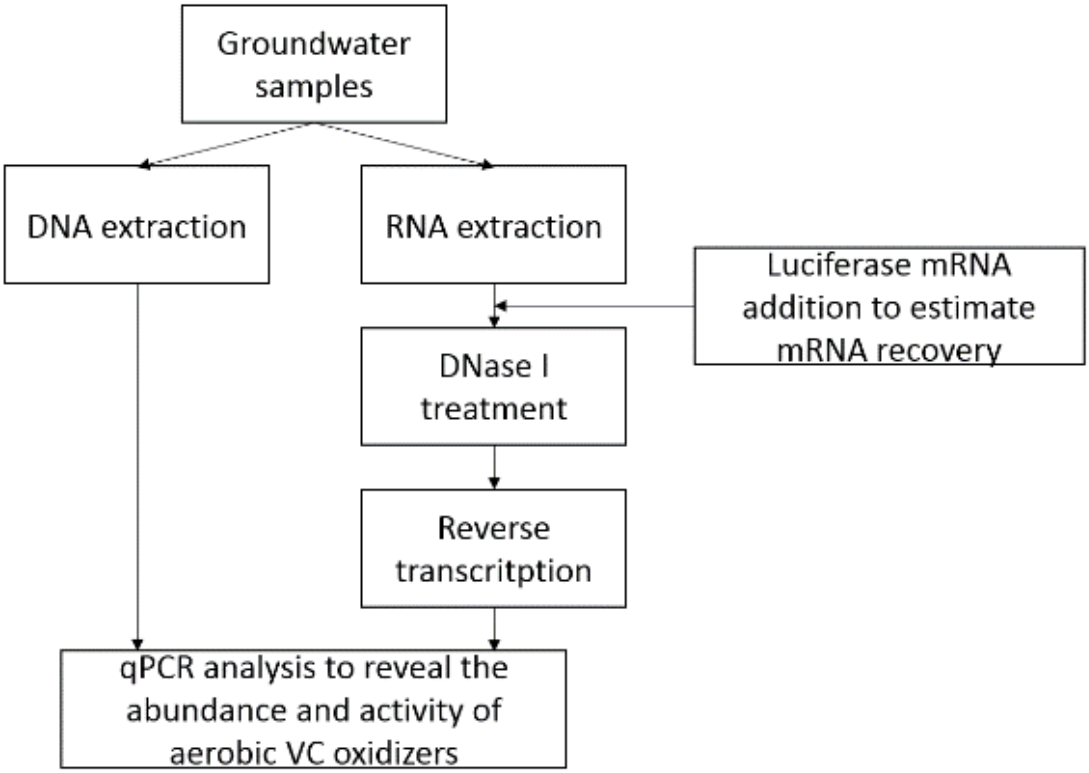

Figure 3. Overall schematic diagram of the nucleic acid extraction workflow.

Table 1. Primer sets used for the amplification of standards. Note: plasmids were used as standards for *bvcA* and *vcrA*.

Target gene	Primer name	Sequences		Expected product size	Source
luciferase mRNA	ref-STF	5'-CCAGGGATTTCAGTCGATGT-3'		1014 bp	(Liang, *et al.*, 2014)
	ref-STR	5'-TTTTCCGTCATCGTCTTTCC-3'			
*etn*C	JS614-etnCF	5'-GCGATGGAGAATGAGAAGGA-3'		1138 bp	(Jin & Mattes, 2010)
	JS614-etnCR	5'-TCCAGTCACAACCCTCACTG-3'			
*etn*E	CoM-F1L	5'-AACTACCCSAAYCCSCGCTGGTACGAC-3'		891 bp	(Mattes, *et al.*, 2005)
	CoM-R2E	5'-GTCGGCAGTTTCGGTGATCGTGCTCTTGAC-3'			
*mmo*X	mmoX-std2f	5'- AGGCAGTCAAGGACGAAAGG-3'		1123 bp	this study
	mmoX-std2r	5'-ATCTGGCCGTTGTACTCGTG-3'			
pmoA	pmoA-std1f	5'-TCGGTCCGTTCAACTCCG-3'		703 bp	this study
	pmoA-std1r	5'-GAATACCAACGGCCCATGAA-3'			

2.1.1.7 Platforms used for qPCR

The platforms (96 well and 384 well plate) that we utilize for qPCR and their capabilities are described below and illustrated in Fig. 4. The choice of platform depends on the number of target genes and the number of samples to be processed. Both 96-well plate and 384-well plate qPCR are performed with an ABI 7900 HT Fast Real-Time PCR System.

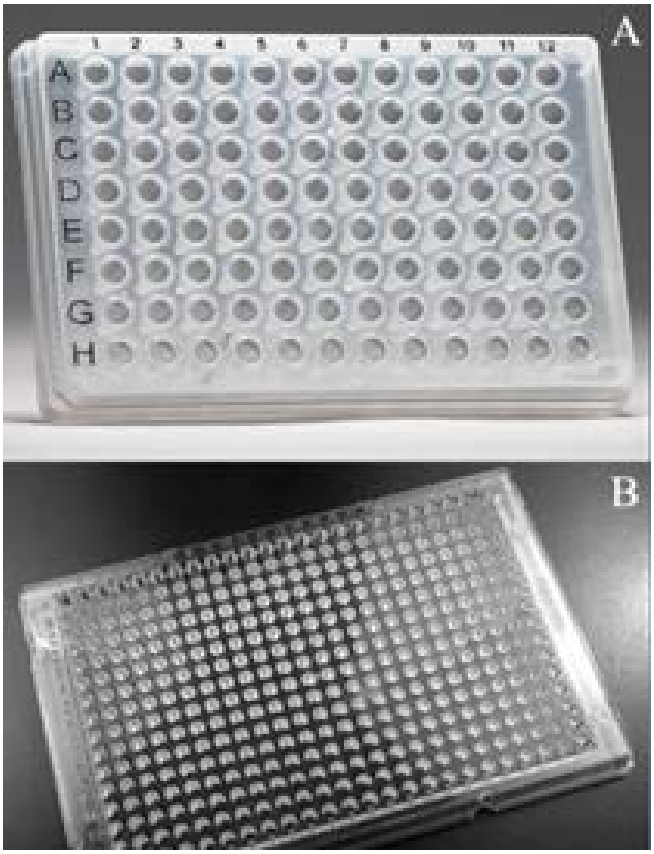

Figure 4. A) 96-well and B) 384-well qPCR plates

96-well plate

The 96-well plate (8 rows x 12 columns) is the smallest platform available for qPCR/RT-qPCR. We often use this platform when a small number of samples is being tested. Our 96 well plate qPCR workflow usually includes one set of standards, which comprises 6 dilutions of a known amount of one target gene (with each dilution in triplicate). Th. For each sample, qPCR is usually performed in replicate. For each monitoring well, DNA

and RNA samples are usually taken in triplicate. The 96-well plate to accommodate 5 monitoring wells at most for the test of one gene of interest. One no template control (NTC) is required for each plate for QA/QC.

384-well plate.

The 384-well plate is similar to the 96-well plate, but is an array of wells 16 rows x 24 columns. A 384-well plate can accommodate DNA, cDNA, and RNA samples from 24 monitoring wells (assuming triplicate filters are taken for DNA and RNA extraction for each monitoring well, respectively) at the same time for the test of one gene of interest. If two genes of interest are analyzed on one plate, nucleic acids from 11 monitoring wells can be included on one 384-well plate.

2.1.1.8 Calculating genes and/or transcripts per liter of groundwater

Equation used for gene abundance calculation per liter of groundwater (GW):

$$\frac{genes}{L\ GW} = \frac{genes\ per\ qPCR}{ng\ DNA\ used\ for\ qPCR} \times \frac{ng\ DNA\ after\ extraction}{volume\ of\ groundwater\ sampled}$$

Equation used for transcript abundance calculation per liter of GW:

$$\frac{transcripts}{L\ GW} = \frac{transcripts\ per\ qPCR}{fraction\ RNA\ recovery} \times \frac{cDNA\ volume\ after\ R.T.}{cDNA\ volume\ used\ for\ qPCR} \times \frac{RNA\ vol\ after\ RNA\ clean\ up}{RNA\ volume\ used\ for\ R.T.} \times \frac{2\ (factor\ corrected\ for\ single\ strand)}{volume\ of\ groundwater\ sampled}$$

2.1.1.9 Quality assurance/quality control of nucleic acid extraction and qPCR

A successful DNA extract is one that contains amplifiable DNA, with concentrations above the Qubit™ dsDNA HS assay kit detection limit (0.05 ng/µl). An RNA extract is deemed successful if, after reverse transcription and qPCR with the ref-f/r primer set, it yields a luciferase gene PCR product. The integrity of DNA and RNA extracts should always be confirmed prior to conducting qPCR analysis.

The specificity of qPCR primers (RTC-f/r, RTE-f/r, mmoX-536f/898r, pmoA472-A189f/mb661r) should be routinely checked by observing the qPCR product melting curves of each sample. For example, the melting curves of RTE-f/r, mmoX-536f/898r and pmoA472-A189f/mb661r showed similar pattern in groundwater samples as in the standards (Figure 5), which indicates that those primer sets are amplifying their specific targets..

For the RTC-f/r primer set, different groundwater DNA/cDNA samples were found to generate different melting curves, some of which are different from the ones generated by standard DNA. For example, in samples from NAS Oceana and NSB Kings Bay, multiple peaks were observed

of melting curves, indicating possible primer dimer formation or non-specific amplification (Figure 5). A clone library was then constructed to investigate the sequences from NAS Oceana and NSB Kings Bay DNA samples amplified with RTC primer set. In most cases, the peaks seen at low melting temperatures can be attributed to primer dimers being formed at low template concentrations. However, we found that the RTC primer set could also amplify other soluble di-iron monooxygenase alpha subunit (SDIMO) genes. SDIMOs are key enzymes in the bacterial oxidation of hydrocarbons. The AkMO and sMMO enzymes targeted here are SDIMOs (Coleman, *et al.*, 2006). Because many SDIMOs can oxidize VC by co-metabolism, the presence of SDIMO also represent the oxidation potential for VC. In contrast, for Parris Island samples, RTC primers generated similar melting curves for both groundwater samples and standards, indicating their good specificity for Parris Island samples at the higher *etnC* concentrations observed there.

If the RTC primer set does not perform well, the MRTC primer set provides another option to amplify *etn*C. MRTC is a mixture of unique, non-degenerate gene-specific primers that based on the database of 38 *etn*C sequences targeting the same priming sites as the RTC primers (Jin & Mattes, 2011)(Table 2). The MRTC primer set possibly minimizes the mismatches with template sequences and thus introduces less primer bias than the RTC primer set. Despite this, we chose to utilize the RTC primer set for the samples analyzed in the demonstration for consistency.

Strict quality control procedures are employed to ensure the accuracy of qPCR technology. For each qPCR experiment, one standard curve is included with a dynamic linear range usually between 30 - 3×10^6 or 3×10^7 gene copies per reaction. A linear regression analysis is used to evaluate the linearity of the standard curve and the amplification efficiency.

PCR efficiency (E, %) is calculated based on the slope of standard curve as follows:

$$E = (10^{(-1/slope)} -1)*100\%$$

A standard curve is considered to be acceptable when the R^2 of the linear regression is larger than 0.99, and the PCR amplification efficiency is between 90% and 110%.

No template (DI water) controls (NTCs) should be used with each qPCR primer set on each qPCR plate. This control ensures that cross contamination of samples has been not occcured (at least within the water and reagents used for PCR). In NTCs, the target gene should either be not detected or detected only as primer dimers (as assessed by dissociation curve analysis). RNA sample aliquots (corresponding to each reversed-transcribed sample) should also be included on the same qPCR plate to test for DNA contamination if cDNA samples are to be analyzed. If amplification levels observed in any RNA sample are similar to its corresponding cDNA sample, the cDNA sample should be removed from subsequent analysis.

For DNA and cDNA samples, replicate or triplicate qPCR is performed for the same sample, the Ct difference or standard deviation should be less than 0.3. Samples with target gene abundances that are less than the lower quantification limit (usually 30 gene copies per qPCR reaction) are labeled accordingly. The gene abundance estimation of those samples is considered not as accurate since it is outside the linear range of the standard curve.

For each qPCR, dissociation curves are used to assess amplification specificity. This facilities identification of potential false-positive SYBR green qPCR results. Dissociation curves of samples are compared with standards to rule out non-specific amplification or dimer formation. Agarose gel electrophoresis can be used to confirm the size of PCR product. When necessary, cloning and sequencing should be performed to verify the specificity of PCR products. We recommend that the specificity of qPCR products be verified with clone library analysis prior to embarking on extensive qPCR analysis of samples from each site.

According to this analysis, the RTC primers have a tendency to form primer dimers, particularly when the target gene is at low abundance. Primer dimer formation by both the methanotroph functional gene primer sets could occur when target gene abundance is low. Occasional primer dimer formation was noted with Bvc925f/1017r.

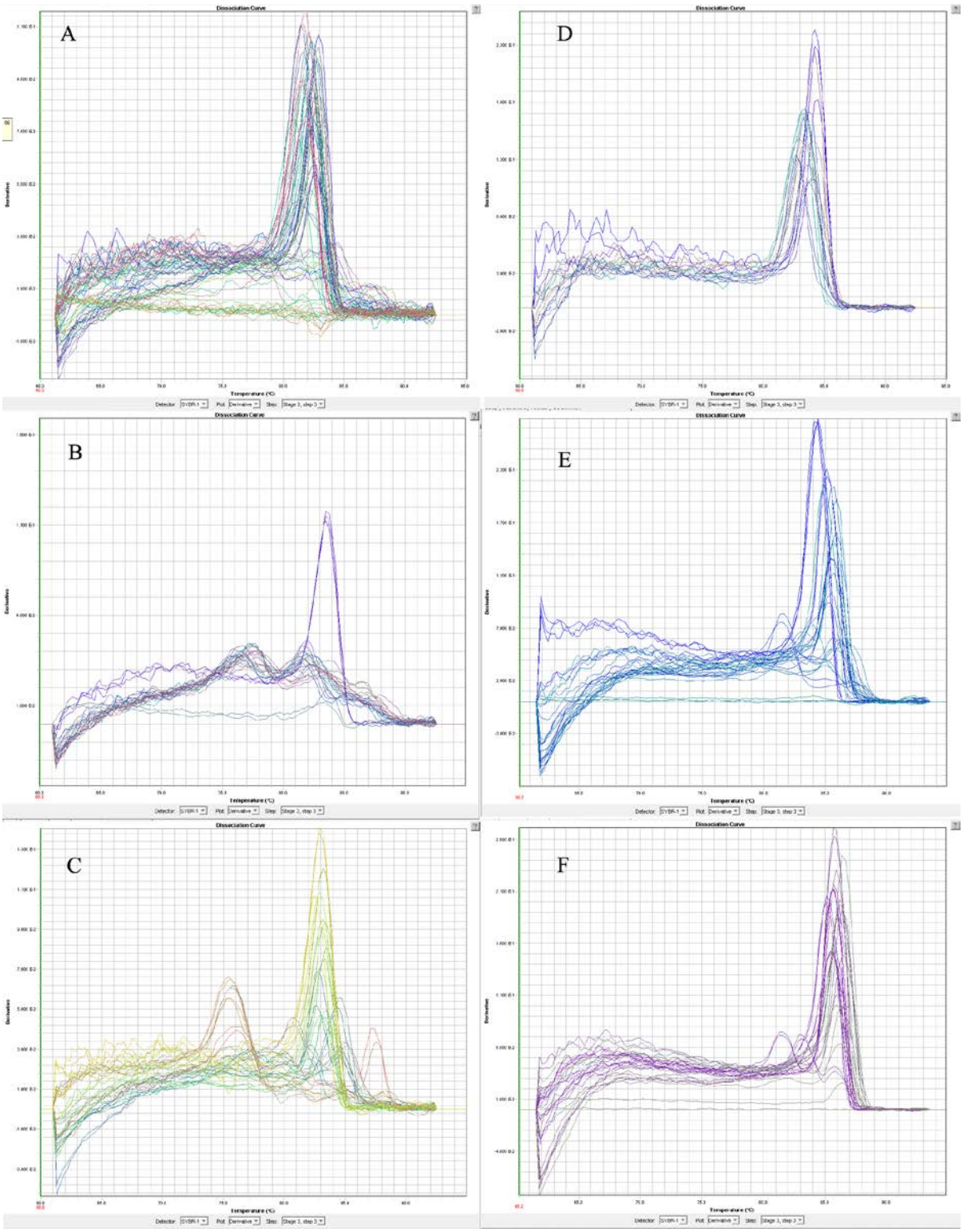

Figure 5. Melting curves of primer sets A) RTC-f/r amplifying DNA samples from MCRD Parris Island, B) RTC-f/r amplifying DNA samples from NAS Oceana, C) RTC-f/r amplifying DNA samples from NSB Kings Bay, D) RTE-f/r amplifying DNA samples from MCRD Parris Island, E) mmoX-536f/898r amplifying DNA samples from MCRD Parris Island, and F) pmoA472-A189f/mb661r amplifying DNA samples from MCRD Parris Island.

Table 2. Primer sets used for qPCR experiments

Target gene	Primer name	Sequences	Expected product size	Source
luciferase mRNA	ref-f	5'-TACAACACCCCAACATCTTCGA-3'	150 bp	(Johnson, *et al.*, 2005)
	ref-r	5'-GGAAGTTCACCGGCGTCAT-3'		
*etn*C	RTC-f	5'- ACCCTGGTCGGTGTKSTYTC-3'	106 bp	(Jin & Mattes, 2010)
	RTC-r	5'- TCATGTAMGAGCCGACGAAGTC-3'		
*etn*C	MRTC-f	5'-ACACTCGTCGGCGTTGTTTC-3' 5'-ACCCTGGTCGGTGTGCTCTC-3' 5'-ACGCTGGTCGGTGTTCTTTC-3' 5'-GCTCTGGTCGGCGTTCTTTC-3' 5'-ACTCTGGTCGGCGTTCTTTC-3' 5'-ACCTTGGTTGGTGTGCTTTC-3' 5'-ACCCTGGTCGGTGTGGTCTC-3' 5'-ACCCTGGTCGGCGTGGTCTC-3'	106 bp	(Jin & Mattes, 2011)
	MRTC-r	5'-TCATGTACGAGCCGACGAAGTC-3' 5'-TCATGTAAGAGCCGACGAAGCC-3' 5'-TCATGTAAGAGCCGACGAAGTC-3' 5'-TCATGTAGGAGCCGACGAAGTC-3' 5'-TCATGTAAGAACCGACGAAGTC-3' 5'-TCATGTACGAACCGACGAAGTC-3'		
*etn*E	RTE-f	5'-CAGAAYGGCTGYGACATYATCCA-3'	151 bp	(Jin & Mattes, 2010)
	RTE-r	5'-CSGGYGTRCCCGAGTAGTTWCC-3'		
*mmo*X	mmoX536f	5'-CGCTGTGGAAGGGCATGAAGC G-3'	542 bp	(McDonald, *et al.*, 1995, Fuse, *et al.*, 1998)
	mmoX898r	5'-GCTCGACCTTGAACTTGGAGC C-3'		
pmoA	pmoA472-A189f	5'-GGNGACTGGGACTTCTGG-3'	283 bp	(Holmes, *et al.*, 1995, Kolb, *et al.*, 2003)
	pmoA472-mb661r	5'-CCGGMGCAACGTCYTTAC C-3'		
*bvc*A	Bvc925F	5'-AAAAGCACTTGGCTATCAAGGAC-3'	92 bp	(Ritalahti, *et al.*, 2006)
	Bvc1017R	5'-CCAAAAGCACCACCAGGTC-3'		
*vcr*A	Vcr1022F	5'-CGGGCGGATGCACTATTTT-3'	71 bp	(Ritalahti, *et al.*, 2006)
	Vcr1093R	5'-GAATAGTCCGTGCCCTTCCTC-3'		

Table 3. Primer concentration and template mass for qPCR set up.

Target gene	primer concentration, μM	template
luciferase mRNA	0.1	2 ul cDNA
etnC	0.8	10 ng DNA or 2 ul cDNA
etnE	0.8	10 ng DNA or 2 ul cDNA
mmoX	0.3	10 ng DNA or 2 ul cDNA
pmoA	0.3	10 ng DNA or 2ul cDNA
bvcA	0.3	10 ng DNA or 2ul cDNA
vcrA	0.3	10 ng DNA or 2ul cDNA

2.2 PRACTICAL IMPLICATIONS OF THE TECHNOLOGY

Although decreases in VC concentrations are observed on the fringes of VC plumes at several sites, there are no published accounts that provide any link between VC concentrations and VC-oxidizing populations measured with molecular tools or otherwise. During this project, field demonstration of these tools in a variety of different groundwater environments (e.g. different VC concentrations, different hydrogeological conditions, natural attenuation vs. biostimulation scenarios) has now established relationships between VC-oxidizer functional gene abundance and expression with VC concentration as well as other geochemical parameters (Liang*, et al.*, 2017b). This is further described in Section 3.0 below. The data collected and guidance provided as a result of this project could therefore provide site managers the ability to document VC oxidation as a viable pathway responsible for reducing VC concentrations at their site. These crucial lines of evidence could speed up regulatory acceptance of MNA and ultimately reduce lifecycle costs for clean-up.

Therefore, it is strongly suggested that these new tools for assessing the presence and activity of VC oxidation pathways be included as part of a molecular diagnostics toolbox for site managers and remediation practitioners interested in documenting VC bioremediation in the field.

2.3 ADVANTAGES AND LIMITATIONS OF THE TECHNOLOGY

The RT-qPCR/qPCR technology for etheneotrophs is an innovative and rapid means of revealing information about the potential for *in situ* microbial aerobic VC oxidation at contaminated sites. This information is useful in evaluating and monitoring the natural attenuation processes and help inform remediation decisions and strategy development. Conventional microcosm studies are laborious, time-consuming and relatively expensive, and in some cases might not represent *in situ* conditions. Contaminant analyses are useful in understanding the contaminant concentration and distribution at the site but could not provide information about the microbial degradation potential at present and in the future.

qPCR/RT-qPCR technology estimates the gene and transcript abundances in the environment and provides more accurate and valuable information than traditional PCR (and RT-PCR), which is not quantitative. It also goes a step further than typical qPCR approaches as RNA is analyzed, and thus provides a measure of gene expression as well as abundance. RT-qPCR is more sensitive and precise in detecting the microbial VC-oxidizers in the groundwater than hybridization-based techniques such as fluorescence in situ hybridization (FISH). We have strict QA/QC procedures to ensure the sensitivity and precision of this technology. In our case, the lower limit of quantification (LOQ) is approximately 500 genes per liter of groundwater and 1000 transcripts per liter of groundwater. This level of sensitivity is especially advantageous in investigating the VC oxidizers at low concentrations.

RT-qPCR/qPCR technology is more time efficient compared to conventional microcosm methods. Depending on the sample amount, RT-qPCR/qPCR analysis from a sampling event containing 18 Sterivex filter samples takes 1-2 weeks. This is in contrast to a typical microcosm study, which can take several months to complete.

A potential disadvantage of RT-qPCR is that it only targets the known functional genes and transcripts associated with aerobic VC oxidation. Any as yet undiscovered VC oxidation pathways that are not targeted by the current primer sets are inherently overlooked, which will result in underestimation of VC oxidation potential and activity. For this reason, expanding current primer sets to include novel VC oxidation biomarkers should be considered a possibility in the future. As we cannot predict when new VC oxidation biomarkers will be discovered, we expect that expanded primer sets will be developed by either work funded elsewhere or in additional proposals to SERDP and/or ESTCP.

It is also prudent to constantly evaluate existing primer sets for performance with environmental samples. It is possible that variability in target gene composition and abundance from site to site could lead to variation in qPCR/RT-qPCR performance. As biomarker gene sequences from the environment are added to the database, revisions to the primer sets could help improve performance. However, it is important to note that the primer sets we employ that target functional genes in etheneotrophs (i.e. *etnC* and *etnE*) utilize the most current complement of genes in the database.

Groundwater samples from monitoring wells are advantageous in that they are easily collected, and provide a composite sample of the microbial community within the radius of influence of the well. It is likely that the distribution of VC-oxidizers is heterogeneous and that they will be found in regions where VC (diffusing from deeper anaerobic regions of the groundwater) and oxygen (diffusing in from the unsaturated zone and rainwater recharge of the aquifer) co-occur. Groundwater sampling alone could be biased towards microbes that are planktonic or easily detached from surfaces. Neglecting microbial VC-oxidizers attached to the sediment may lead to bias in evaluating the *in situ* VC oxidation potential. In addition, the spatial variability of VC-oxidizers in the subsurface would provide an improved understanding of VC oxidation potential and could inform site management decisions concerning MNA. Aquifer sediment sampling is grab sampling – it provides a single point snapshot of the microbial community. Sediment sampling is thus more difficult, more variable, and it requires a large quantity of sediment samples to ensure representative data (thus more expensive).

3.0 INTERPRETATING qPCR DATA AT VC-CONTAMINATED SITES

3.1 Overview

The data collected during project ER-201425 consisted of 95 groundwater samples from six different VC contaminated sites. The VC plumes studied featured VC concentrations ranging from dilute (<10 ug/L) to very high (20-50 mg/L). Functional genes from ethenotrophs and methanotrophs and anaerobic VC-dechlorinators were frequently present (99%) and expressed (59%) in the same groundwater samples the majority of which featured low DO and ORP levels (Liang, *et al.*, 2017b). This initially was an unexpected result because etheneotrophs and methanotrophs are considered obligately aerobic while VC-dechlorinating *Dehalococcoides mccartyi* are considered strictly anaerobic and very sensitive to oxygen (Amos, *et al.*, 2008, Mattes, *et al.*, 2010). We further investigated the possibility that aerobic VC-oxidizing bacteria were contributing significantly to VC biodegradation at these sites. This included estimating bulk VC attenuation rates at sites and determining if there are correlations between qPCR data and the rates, investigating relationships between

3.2 Contributions of VC-degrading bacteria to the bulk VC attenuation rate

First, we investigated whether the abundance and expression of VC biodegradation biomarker genes were correlated with the bulk VC attenuation rates estimated at the 6 sites. In addition we distinguished between the VC plumes at Parris Island that exist (both northern and southern plumes)

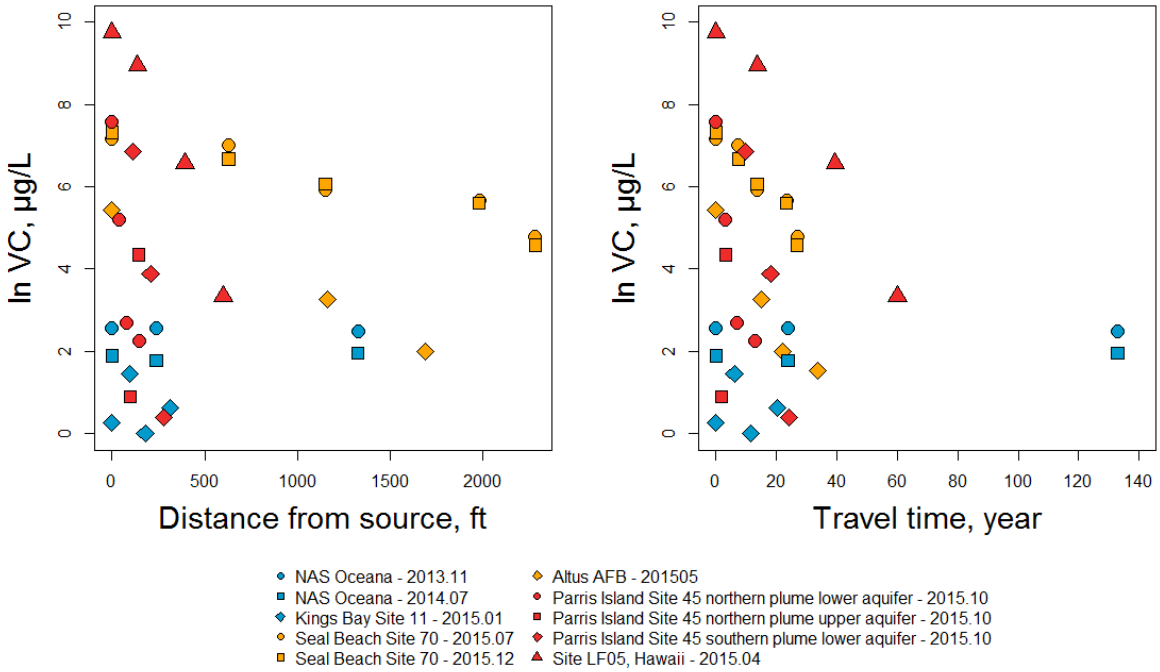

Figure 6. Plot depicting VC vs. distance (left panel) and VC vs. travel time (right panel) in six different VC plumes, some of which have multiple time points.

Using site maps and monitoring well data we developed plume transects and plotted VC concentration vs. distance according to the direction of groundwater flow. Using reported hydrogeological data (hydraulic gradient, porosity, hydraulic conductivity) we estimated seepage velocities along transects so that VC concentration vs. distance plots can be expressed as VC concentration vs. travel time (Fig. 6). Bulk VC attenuation rates are estimated from these plots by plotting ln VC vs. travel time and performing a linear regression to obtain the slope. The results of this analysis are shown in Table 4.

We encountered significant uncertainties associated with estimating the seepage velocity, which is a crucial parameter in determining bulk VC attenuation rate. At different parts of different sites and different time points, seepage velocity could vary substantially. In some cases, the calculated bulk VC attenuation rate was not statistically significant (i.e. probability that the slope is not zero is not within the 95% confidence interval). This can result from a lack of data (not enough wells), a very low rate to begin with, or strong variability in the data leading to poor linear relationships. As a result confident quantitative relationships between bulk VC attenuation rates and functional gene abundance and expression could not be made. However, we were confident enough in differences between some estimated rates such that we placed them into either a low rate or high rate category. Two sites were placed in the low rate category and the remaining in the high rate category. We then proceeded to analyze the samples with a categorical regression of the categorized bulk VC attenuation rates against functional gene and transcript abundances of VC biodegradation biomarker genes. The results of this analysis, shown in Table 5, reveal that both etheneotroph functional gene (*etnC* and *etnE)* abundances are significantly correlated with categorical bulk VC attenuation rate. This relationship was also seen for VC reductive dehalogenase gene *vcrA* and the sum of VC reductive dehalogenase genes *bvcA* and *vcrA*. In contrast, methanotroph functional genes *pmoA* and *mmoX* were not correlated with categorical bulk VC attenuation rates.

This analysis appears to validate the concept that aerobic VC oxidizing bacteria (etheneotrophs) are contributing to the bulk rate of VC attenuation along with VC reducing bacteria at these sites. Conducting this analysis at other VC contaminated sites (using the qPCR technology) could provide important lines of evidence for MNA of VC. However, employing this approach is recommended only if groundwater seepage velocities along a plume transect can be determined with confidence. Additionally, attempting to develop quantitative relationships between a bulk (site-wide) VC attenuation rate and functional gene/transcript abundances requires that the functional gene abundance and expression also be averaged site-wide. Doing so is not ideal because VC concentration must by definition vary over a plume transect in order to estimate a bulk VC attenuation rate. We have determined there are relationships between VC concentration and the abundance and expression of VC biodegradation biomarkers genes as described in the next section. Therefore, we conclude that more accurate approaches to estimating VC biodegradation rates in the field need to be developed. This could include the use of surrogate substrates along with in situ push pull tests. With better estimates of VC biodegradation rates, we should be able to obtain better correlations between VC biodegradation gene abundance/expression with these rates.

Table 4. Bulk VC attenuation rate estimate and rate categories. p values < 0.05 are in bold. Low rate: bulk VC attenuation rates < 0.005 per year. High rates: bulk VC attenuation rate >0.08 per year.

Site	Remediation approach	Time	Bulk VC attenuation rate, yr^{-1}	R^2	p Value	Rate Category
VA Site 2C	Oxygen releasing compound injection	2013.11	0.0006	0.972	0.518	Low
		2014.07	-0.0009	0.472	0.108	Low
GA Site 11	Pump and treat; in situ chemical oxidation	2015.01	0.0037	0.003	0.950	Low
CA Site 70	Enhanced anaerobic bioremediation with biobarriers	2015.07	0.0849	0.909	0.012	High
		2015.12	0.0958	0.891	0.006	High
OK Site SS-17	Mulch biowalls	2015.05	0.1210	0.942	0.030	High
HI Site LF05	Enhanced anaerobic bioremediation - bioreactor	2015.04	0.1057	0.969	0.016	High
SC Site 45* (north plume lower aquifer)	Enhanced anaerobic bioremediation – emulsified vegetable oil injection	2015.10	0.4350	0.932	0.008	High
		2016.07	0.3752	0.879	0.019	High
SC Site 45* (north plume upper aquifer)		2016.07	1.0858	0.344	0.299	High
		2015.10	1.0409	0.23	0.414	High
SC Site 45* (south plume lower aquifer)	In situ chemical oxidation	2015.10	0.5182	0.764	0.126	High
		2016.07	0.2836	0.840	0.028	High

*At SC site 45, a northern and southern VC plume exist within a surficial aquifer which has upper and lower hydrogeologic units. Bulk VC attenuation rates were estimated in each of these cases.

Table 5. Average gene and transcript abundances within slow and high rate group categories as assessed by categorical multilevel regression. p values <0.05, which indicate a statistically significant difference between two groups, are in bold.

	Rate category	gene		transcript	
		$\log_{10}(x+1)$	p value	$\log_{10}(x+1)$	p value
etnC	low rate	4.58		4.39	
	high rate	6.49	**0.040**	3.91	0.743
etnE	low rate	3.49		1.35	
	high rate	5.73	**0.036**	3.80	**0.026**
mmoX	low rate	5.61		2.79	
	high rate	4.79	0.454	1.81	0.474
pmoA	low rate	6.93		5.62	
	high rate	6.23	0.593	3.76	0.202
bvcA	low rate	2.41		1.21	
	high rate	3.28	0.389	2.62	0.349
vcrA	low rate	0.45		0.00	
	high rate	3.54	**0.030**	3.96	**0.035**
mmoX+pmoA	low rate	6.96		5.66	
	high rate	6.32	0.605	3.91	0.206
bvcA+vcrA	low rate	2.60		1.12	
	high rate	4.36	**0.024**	4.66	**0.002**

3.3 Relationships between VC concentrations and the abundance and expression of VC biodegradation genes

Although uncertainties associated with confidently estimating bulk VC attenuation rates did not facilitate a strong quantitative analysis of relationships between VC attenuation rates and VC biodegradation gene abundance and expression, VC concentrations (which are also important in determining rates) can be more confidently measured in environmental samples. VC concentrations were measured in 95 groundwater samples along with abundance and expression of VC biodegradation genes from etheneotrophs, methanotroph and anaerobic VC-dechlorinators. Relationships according Spearman's rank correlation analysis and linear regression analysis were performed. We took the hierarchical data structure into account (i.e. multiple samples from the same monitoring wells as well as multiple samples from sites) with a multi-level modeling approach. Strong positive relationships using both Spearman's correlation analysis (Liang, *et al.*, 2017b) and multilevel modeling were observed between VC concentrations and the abundance and expression of functional genes from etheneotrophs and anaerobic VC-dechlorinators (Fig. 7). However, this relationship with VC concentration was not observed with methanotroph functional genes (Fig. 7). This is an important observation with respect to etheneotrophs, and indicates the abundance of etheneotrophs is greater when VC concentrations are greater irrespective of the dissolved oxygen concentration.

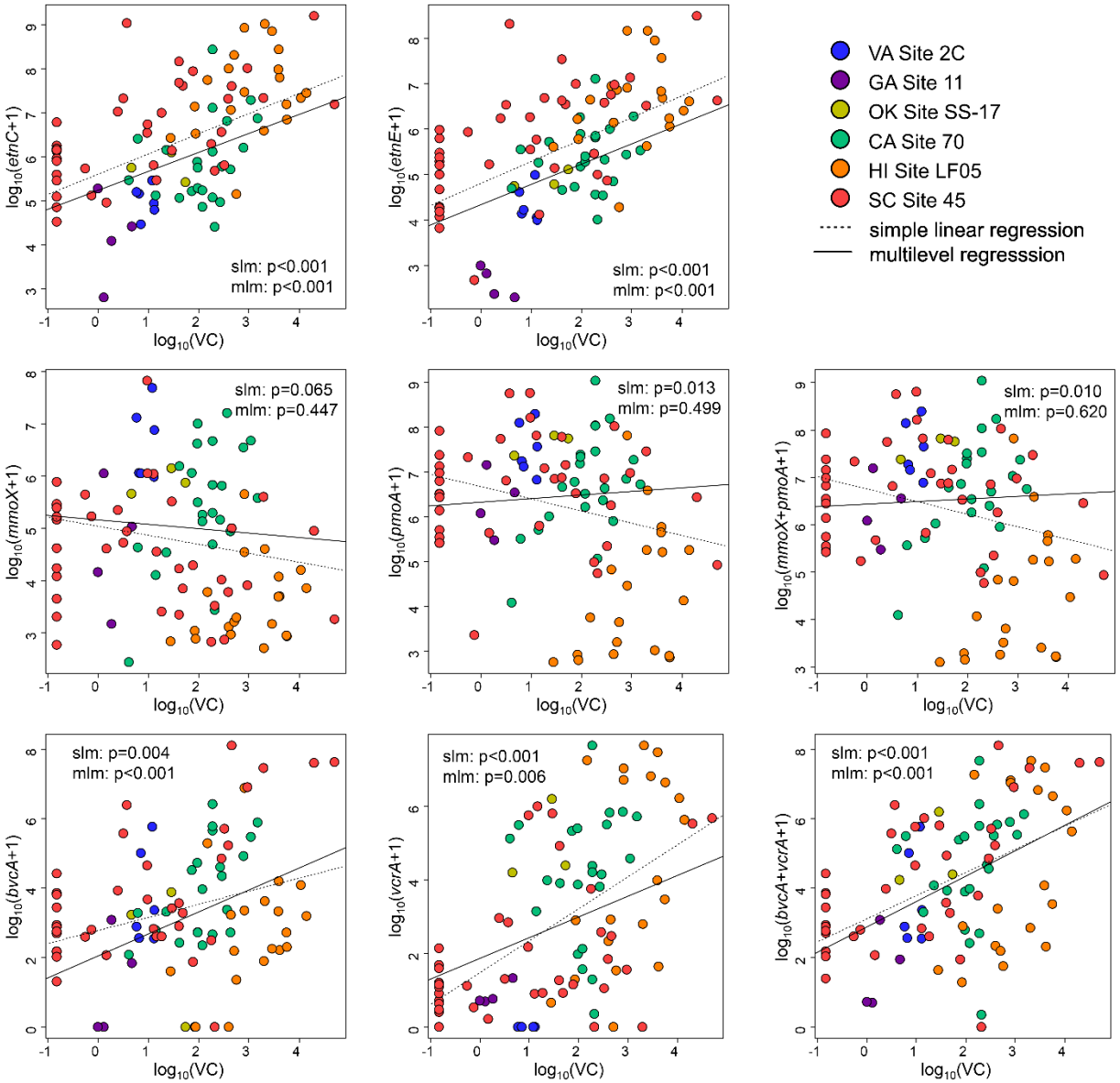

Figure 7. Relationships among functional genes and VC concentrations as assessed by multilevel regression (mlr, solid line) and simple linear regression (slr, dotted line). p values for slr and mlr are indicated for each functional gene (Liang, *et al.*, 2017b).

As stated earlier, we also found a high level of co-occurrence of etheneotroph functional genes and VC reductive dehalogenase genes in groundwater samples. ***Therefore, we conclude from this analysis that etheneotrophs have a strong potential to contribute to VC biodegradation in groundwater when VC concentrations are high and in areas of the aquifer that may be considered anaerobic. It is recommended that qPCR analyses for aerobic VC oxidizers always be conducted concurrently with those for anaerobic VC dechlorinators during long term groundwater monitoring, irrespective of DO concentrations, so as to build lines of evidence for natural attenuation of VC for the entire site.***

3.4 Spatial patterns of VC biodegradation gene abundance and geochemical parameters with depth in sediment samples

Although the simultaneous presence and expression of genes from etheneotrophs and anaerobic VC-dechlorinators in groundwater samples is a novel and important observation that has implications for bioremediation of VC, the spatial co-occurrence of aerobic and anaerobic VC degrading bacteria remained inconclusive. Plausible explanations for this observations are that aquifer material within the influence of monitoring wells contain aerobic and anaerobic microenvironments in close proximity to each other or that mixing of aerobic and anaerobic groundwater zones occurred during sampling. Analysis of aquifer sediment samples was therefore conducted to provide more precise spatial proximity and distribution of aerobic and anaerobic VC-degrading populations in contaminated aquifers Aquifer sediments were collected from four locations in the northern VC plume at Parris Island site 45 with the cryo-coring technique (refer to SERDP ER-201587 Final report for technical details on the cryo-coring technique). Sediment samples were analyzed for chlorinated ethenes (including cDCE and VC), ethene, and methane. Geochemical results for core 1, located near the PCE source zone are shown in Fig. 8. The surficial aquifer at Parris Island site 45 has an upper unit composed on silt and a lower unit composed of silty sand. Inspection of Fig. 8 reveals that dissolved chlorinated ethenes are migrating in both the upper and lower surficial aquifers, and that reductive dechlorination of VC to ethene is also occurring in both units.

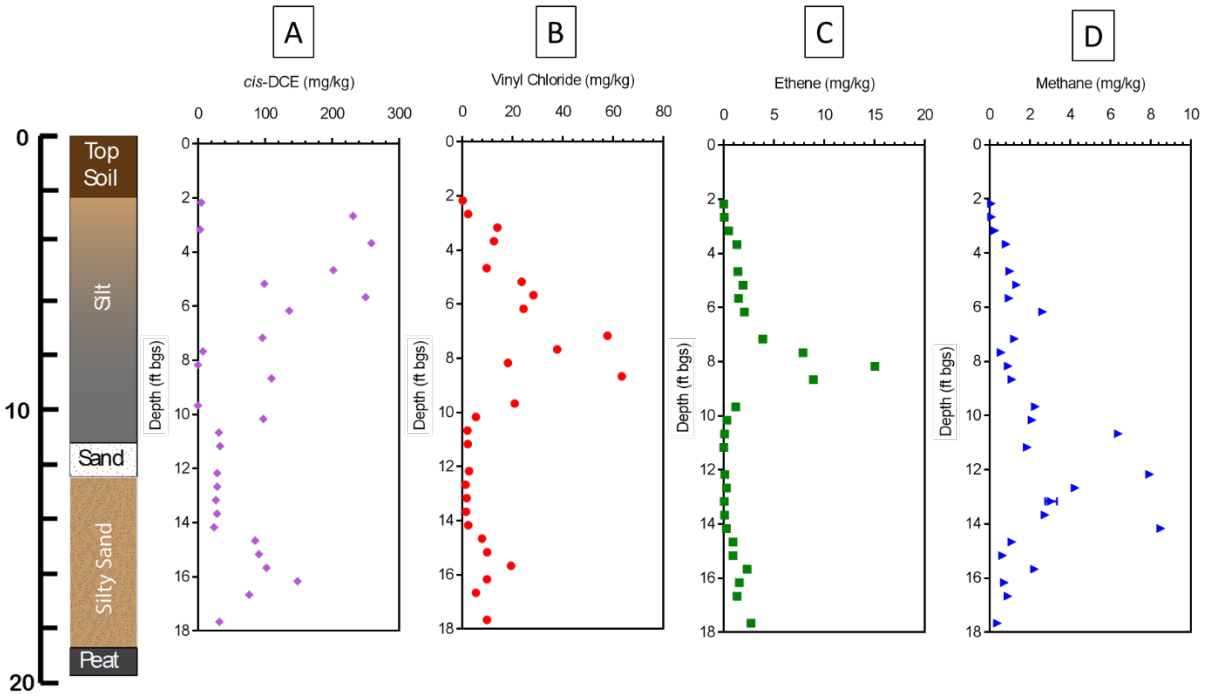

Figure 8. Spatial resolution of A) cDCE, B) VC, C) ethene, and D) methane in an aquifer sediment core, frozen in place with liquid nitrogen, obtained from Parris Island Site 45. The graphic on the left shows the soil characteristics of the upper (silt) and lower (silty sand) surficial aquifers.

DNA was extracted from relatively small aquifer sediment samples (~0.25 g) (also within the same 1" cryo-core sections) with the DNeasy PowerSoil kit (Qiagen, Germantown, MD) according to manufacturer's instructions, and qPCR was performed for six functional genes (*etnC*, *etnE*, *mmoX*, *pmo*A, *bvcA*, and *vcrA*), as well as total 16S rRNA genes. The results of this analysis for core 1, is shown in Fig. 9 below.

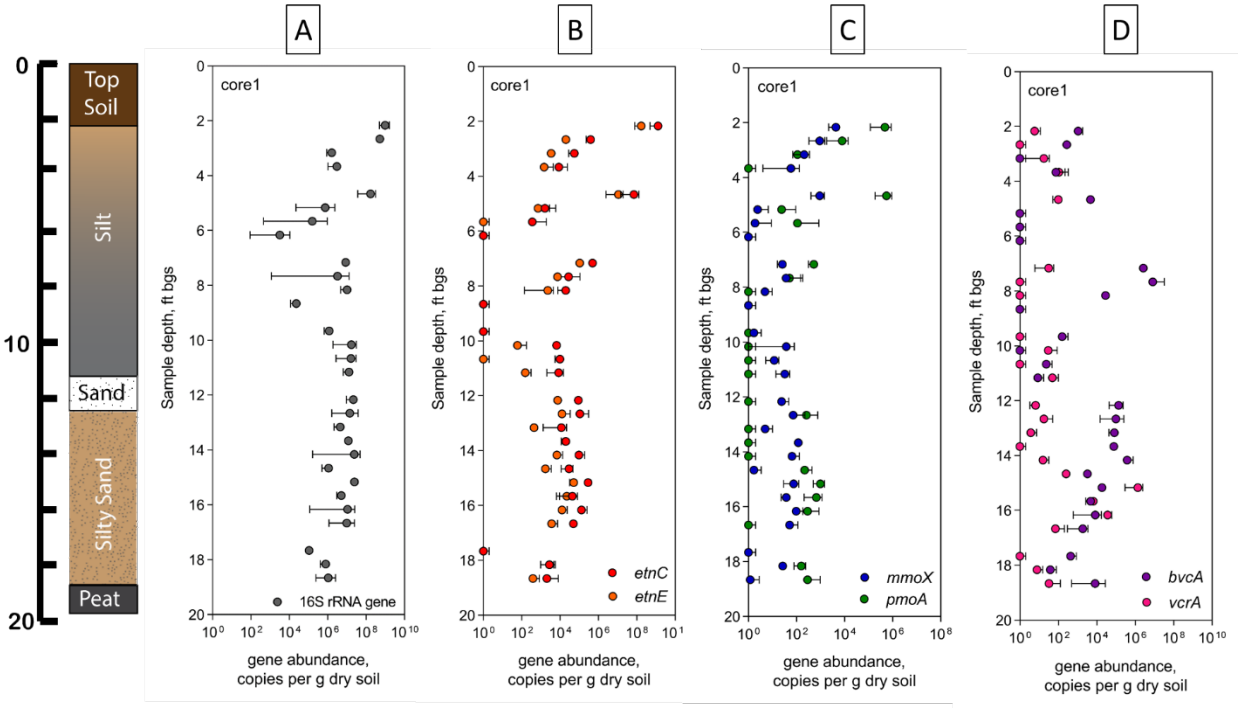

Figure 9. Spatial resolution of A) total 16S rRNA genes, B) *etnC* and *etnE* (etheneotroph functional genes), C) *mmoX* and *pmoA* (methanotroph functional genes), and D) *bvcA* and *vcrA* (VC reductive dehalogenase genes) in an aquifer sediment core, frozen in place with liquid nitrogen, obtained from Parris Island Site 45. The graphic on the left shows the soil characteristics of the upper (silt) and lower (silty sand) surficial aquifers.

The qPCR analysis shows that indeed, functional genes from etheneotrophs and anaerobic VC-dechlorinators coexist within small (~0.25 g) sediment samples as deep as 18 feet below ground surface in this particular sediment core. ***This suggests that there is the potential for essentially simultaneous aerobic and anaerobic VC biodegradation at this site, even at a substantial depth.*** The source of DO at depth is currently unknown and poorly understood. However, as expected, the abundance of etheneotroph and methanotroph functional genes are highest closer to the ground surface, presumably because there is the strongest oxygen gradient here near the shallow groundwater table (~2-3 feet bgs).

4.0 Lessons learned

The following are offered as lessons learned from the controlled evaluation:

1. The RT-qPCR/qPCR technology for etheneotrophs is an innovative and rapid means of revealing information about the potential for in situ microbial aerobic VC oxidation at contaminated sites.

2. Of the 95 samples analyzed during this effort, functional genes from etheneotrophs and methanotrophs and anaerobic VC-dechlorinators were frequently present (99%) and expressed (59%) in the same groundwater samples the majority of which featured low DO and ORP levels.

3. Although confident quantitative relationships between bulk VC attenuation rates and functional gene abundance and expression could not be made, data analysis shown in Table 5, revealed that both etheneotroph functional gene (*etnC* and *etnE)* abundances are significantly correlated with categorical bulk VC attenuation rate. This relationship was also seen for VC reductive dehalogenase gene *vcrA* and the sum of VC reductive dehalogenase genes *bvcA* and *vcrA*. In contrast, methanotroph functional genes *pmoA* and *mmoX* were not correlated with categorical bulk VC attenuation rates.

4. Strong positive relationships using both Spearman's correlation analysis (Liang et al., 2017b) and multilevel modeling were observed between VC concentrations and the abundance and expression of functional genes from etheneotrophs and anaerobic VC-dechlorinators (Fig. 7). However, this relationship with VC concentration was not observed with methanotroph functional genes (Fig. 7). This is an important observation with respect to etheneotrophs, and indicates the abundance of etheneotrophs is greater when VC concentrations are greater irrespective of the dissolved oxygen concentration. Functional gene abundance for etheneotrophs will be proportional to VC concentration irrespective of measured DO concentrations in the groundwater.

5. During the project, we also found a high level of co-occurrence of etheneotroph functional genes and VC reductive dehalogenase genes in groundwater samples. Therefore, we concluded from this analysis that etheneotrophs have a strong potential to contribute to VC biodegradation in groundwater when VC concentrations are high and in areas of the aquifer that may be considered anaerobic. Therefore, it is recommended that qPCR analyses for aerobic VC oxidizers always be conducted concurrently with those for anaerobic VC dechlorinators during long term groundwater monitoring, irrespective of DO concentrations, so as to build lines of evidence for natural attenuation of VC for the entire site.

The spatial resolution of A) total 16S rRNA genes, B) *etnC* and *etnE* (etheneotroph functional genes), C) *mmoX* and *pmoA* (methanotroph functional genes), and D) *bvcA* and *vcrA* (VC reductive dehalogenase genes) in an aquifer sediment core, frozen in place with liquid nitrogen, obtained from Parris Island Site 45 showed that indeed, functional genes from etheneotrophs and anaerobic VC-dechlorinators coexist within small (~0.25 g) sediment samples as deep as 18 feet below ground surface in this particular sediment core. This suggests that there is the potential for

essentially simultaneous aerobic and anaerobic VC biodegradation at this site, even at a substantial depth.

4.1 Statistical analyses

We recommend that when performing statistical analyses of multiple groundwater samples from different sites and different time points from single monitoring wells that a multi-level modeling approach be used to account for the hierarchy in the data. We found that this improved the accuracy of the resulting linear regression analyses (Liang, *et al.*, 2017b). To perform multi-level modeling we used a statistical program called R (Ihaka & Gentleman, 1996). The function lmer() in the lme4 package was used, considering a monitoring well as the first hierarchical level and site as the second hierarchical level (Snijders, 2011). For regression analysis, functional gene/transcript values and chlorinated ethene concentrations were log transformed to more closely follow a normal distribution than raw values.

Non-detects in geochemical and microbial datasets could introduce bias into regression analyses. Data points with non-detectable transcript abundances were removed from the analysis to minimize their influence on regression models. Non-detects for geochemical parameters (i.e. VC) were replaced by 0.5 × the detection limit to facilitate log transformations and minimize the impact of censored data on regression analyses. Model residuals were plotted and Akaike's Information Criterion (AIC) scores (Akaike, 1992) were calculated to facilitate a quantitative comparison between multilevel regression and simple linear regression models.

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