EXECUTIVE SUMMARY

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

ESTCP Project ER-201425

FEBRUARY 2019

Distribution Statement A
This document has been cleared for public release

Page Intentionally Left Blank

This report was prepared under contract to the Department of Defense Environmental Security Technology Certification Program (ESTCP). The publication of this report does not indicate endorsement by the Department of Defense, nor should the contents be construed as reflecting the official policy or position of the Department of Defense. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the Department of Defense.

Page Intentionally Left Blank

EXECUTIVE SUMMARY

Project: ER-201425

TABLE OF CONTENTS

Page

1.0 INTRODUCTION & OBJECTIVES 1

2.0 TECHNOLOGY DESCRIPTION 3

3.0 PERFORMANCE ASSESSMENT 5

- 3.1 ANALYSIS OF TEMPORAL RELATIONSHIPS IN GROUNDWATER SAMPLES 5
- 3.2 ANALYSIS OF SPATIAL RELATIONSHIPS IN GROUNDWATER SAMPLES 5
- 3.3 ANALYSIS OF SPATIAL RELATIONSHIPS IN CRYO-CORES 7
- 3.4 ANALYSIS OF RELATIONSHIPS BETWEEN FUNCTIONAL GENES/ TRANSCRIPTS AND BULK VC ATTENUATION RATES 8
- 3.5 ANALYSIS OF RELATIONSHIPS BETWEEN FUNCTIONAL GENES/ TRANSCRIPTS AND BULK VC ATTENUATION RATES 9
- 3.6 ANALYSIS OF RELATIONSHIPS BETWEEN ETNE ABUNDANCE AND VC BIODEGRADATION RATES 10

4.0 COST ASSESSMENT 13

5.0 IMPLEMENTATION ISSUES 15

LIST OF FIGURES

Page

Figure E1. Relationships Among Functional Genes and VC Concentrations as Assessed by Multilevel Regression (mlr, solid line) and Simple Linear Regression (slr, dotted line). 6

Figure E2. Spatial Resolution of A) cDCE, B) VC, C) Ethene, and D) Methane in Sediment Core 1, 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..... 7

Figure E3. 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..... 8

Figure E4. Raw VC Depletion Rate vs etnE Concentration in VC-grown Nocardioides sp. Strain JS614 (Black Circles) as Compared to VC-oxidizing Enrichment Cultures from Pearl Harbor (PH; Red Squares) and Naval Air Station North Island (NI; blue triangles). 11

LIST OF TABLES

Page

Table E1. Average Gene and Transcript Abundances within Slow and High Rate Group Categories as Assessed by Categorical Multilevel Regression..... 10

Page Intentionally Left Blank

1.0 INTRODUCTION & OBJECTIVES

Vinyl Chloride (VC) plumes are present at many DoD sites, and there are significant costs associated with their cleanup (e.g., VC plumes are often dilute, and a large volume of VC-contaminated water must be treated). Microbial VC oxidation could be occurring at many of these contaminated sites and contribute significantly to VC natural attenuation. However, there are few options available to site managers for documenting these aerobic processes and the effects they may be having at their site. The overall objective of this project is to demonstrate and validate quantitative, real-time polymerase chain reaction (PCR) (qPCR) and reverse transcription (RT)-qPCR technologies for enumerating the abundance and expression of key functional genes from two important groups of VC-oxidizing bacteria (the etheneotrophs and methanotrophs) at several VC-contaminated Department of Defense (DoD) sites. Over the course of the project, we built a biological data set that describes how the abundance and functionality of etheneotrophs and methanotrophs change both spatially and temporally at several locations in one or more VC plumes. We analyzed this data set with respect to the bulk rate of VC attenuation observed across plume transects over the same time period. This demonstration has shown that application of environmental molecular diagnostic tools such as qPCR during long-term monitoring of contaminated groundwater yields useful and cost-effective information for site managers interested in better understanding the potential for aerobic natural attenuation of VC at their sites.

Page Intentionally Left Blank

2.0 TECHNOLOGY DESCRIPTION

Under SERDP project ER-1683, the team developed a qPCR method that yields rapid information concerning presence, abundance, and functionality of VC-oxidizing etheneotrophs. This method employs degenerate oligonucleotide primers that target the functional genes *etnC* and *etnE*, both of which are known to be involved in the aerobic VC and ethene biodegradation pathways of etheneotrophs. The gene *etnC* encodes the alpha subunit of alkene monooxygenase (AkMO). The gene *etnE* encodes the epoxyalkane:coenzyme M transferase (EaCoMT). AkMO converts VC and ethene to epoxide intermediates, while EaCoMT detoxifies and/or transforms these epoxides into compounds that can enter central metabolic pathways. For comparison to etheneotroph functional genes, four additional functional genes are quantified. This includes genes encoding particular and soluble methane monooxygenase alpha subunits in methanotrophs (*pmoA* and *mmoX*, respectively), and genes encoding VC reductive dehalogenases *vcrA* and *bvcA*. Protocols for groundwater sampling, DNA and mRNA extraction, use of internal nucleic acid controls, and subsequent qPCR analysis were developed as part of the technology. This novel technology is innovative because it can now be used to reveal the abundance and functionality of etheneotrophs at VC-contaminated sites. By considering qPCR data alongside geochemical and contaminant data, as well as the site-wide VC attenuation rate, this technology could provide important lines of evidence that aerobic VC biodegradation is a significant contributor to overall VC natural attenuation processes at contaminated sites.

Page Intentionally Left Blank

3.0 PERFORMANCE ASSESSMENT

For this demonstration we developed three qualitative and 11 quantitative performance objectives. The qualitative performance objectives addressed ease of technology, implementation in the field, usefulness of data collected, and regulatory acceptance. Of the 11 quantitative performance objectives, seven assessed specific qPCR performance metrics. These were time-efficiency, cost-effectiveness, accuracy, precision, reproducibility, comparability of different qPCR primer sets, and sensitivity. The remaining four quantitative performance objectives aimed to determine temporal and spatial relationships between qPCR data and geochemical and contaminant parameters (e.g., VC concentrations), and correlations between qPCR data and VC attenuation/ degradation rates.

During the demonstration we collected 95 distinct groundwater samples from VC plumes located at 6 different contaminated DoD sites (including NAS Oceana SWMU 2C and Parris Island Site 45). Both DNA and RNA were extracted from these samples. RNA was concerted to complementary DNA (cDNA) by RT. The resulting DNA and cDNA were subjected to qPCR estimation of *etnC*, *etnE*, *pmoA, mmoX, bvcA*, and *vcrA* gene and transcript abundance. An array of geochemical and contaminant parameters was also obtained from the same groundwater samples. A statistical analysis was performed to investigate potential spatial and temporal relationships among gene and transcript data and the contaminant and geochemical parameters. The qPCR, geochemical and contaminant data, along with the results of the statistical analyses were used to assess the remaining four quantitative performance objectives as follows.

3.1 ANALYSIS OF TEMPORAL RELATIONSHIPS IN GROUNDWATER SAMPLES

The first of these four performance objectives addressed temporal relationships between functional gene and transcript abundances. The assessment was performed using data from a dilute VC plume located at NAS Oceana. The abundance and expression of functional genes from VC-oxidizing bacteria was low. It was also difficult to obtain enough data points for statistical analysis due to the small size of our gene abundance database. Despite this we observed a significant increase in *mmoX* abundance with time in all three wells. In the case of monitoring well MW25, *mmoX* abundance was also negatively correlated with VC concentration, as would be expected if methanotrophs were controlling the rate of VC attenuation at this site. This indicates that although difficult in dilute VC plumes, it is possible to use the qPCR technology to observe statistically significant temporal relationships between geochemical and contaminant parameters and functional genes, thus we deemed the success criteria met for this performance objective.

3.2 ANALYSIS OF SPATIAL RELATIONSHIPS IN GROUNDWATER SAMPLES

Application of qPCR technology to the 95 groundwater samples from several VC plumes indicated that *etnC*, *etnE*, *pmoA, mmoX, bvcA*, and *vcrA* were quantified (DNA) in 99% and expressed (cDNA) in 59% of the samples. Statistical analysis (i.e., multi-level modeling) revealed a statistically significant correlation between etheneotroph functional genes and transcripts and VC concentrations (**Figure E1**). VC reductive dehalogenase genes *bvcA* and *vcrA* also displayed statistically significant correlations with VC concentrations. Conversely, there was no relationship between methanotroph functional gene and transcript abundance and VC concentrations (**Figure E1**).

The relationship etheneotroph functional genes and transcripts and VC concentrations can be used to roughly predict that the higher the VC concentration in groundwater, the higher the etheneotroph abundance and activity will be. This was somewhat surprising as areas of higher VC centration also tended to have little or no dissolved oxygen (DO). However, either there was no relationship or negative correlations were observed between etheneotroph functional gene/transcript abundance and DO and ORP measurements. These results are difficult to interpret but suggest that DO and ORP values do not hold any predictive value with respect to etheneotroph abundance or activity in the environment.

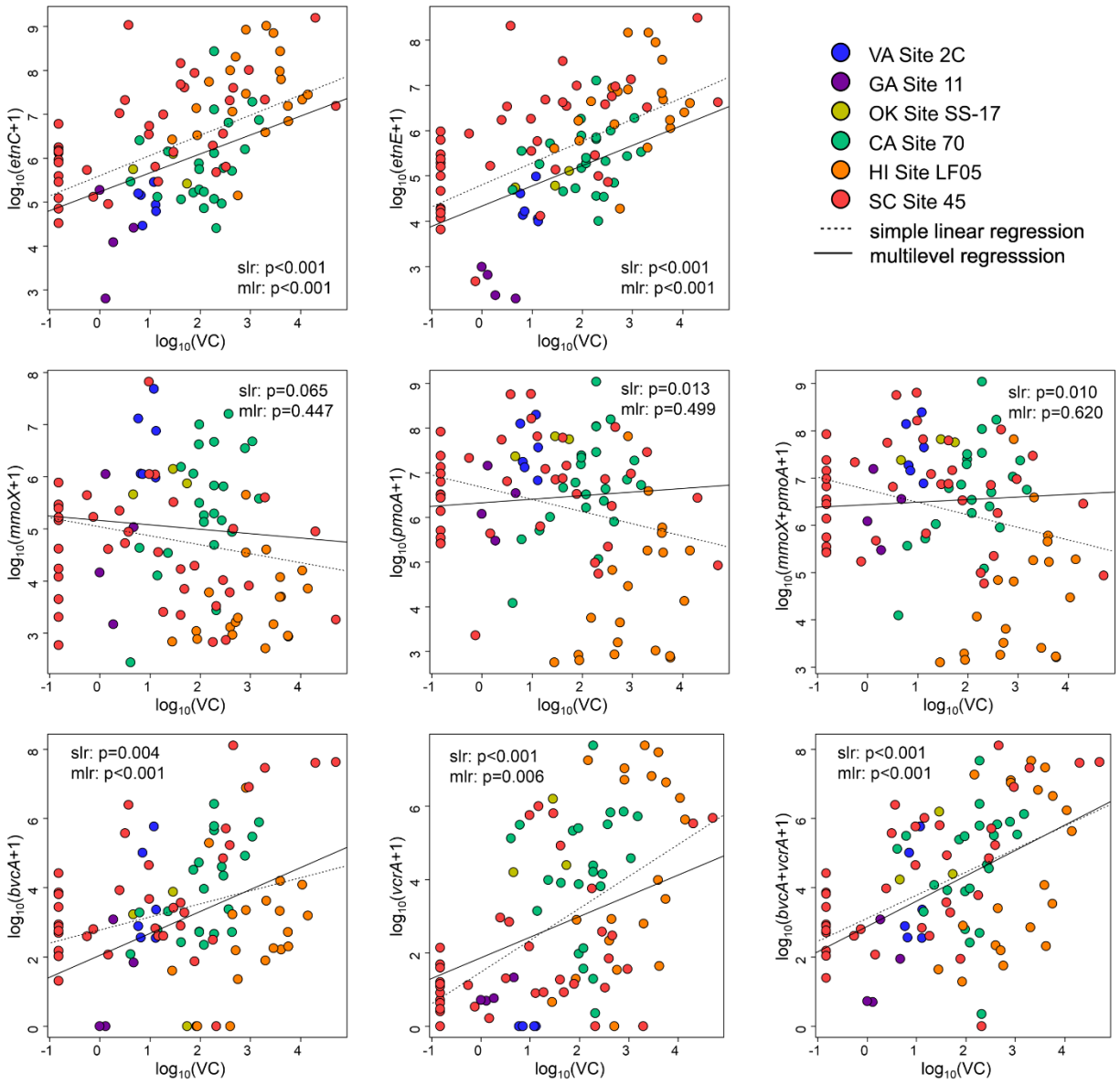

Figure E1. Relationships Among Functional Genes and VC Concentrations as Assessed by Multilevel Regression (mlr, solid line) and Simple Linear Regression (slr, dotted line).

p values for slr and mlr are indicated for each functional gene.

3.3 ANALYSIS OF SPATIAL RELATIONSHIPS IN CRYO-CORES

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. However, the co-occurrence of aerobic and anaerobic VC-degrading bacteria in the subsurface environment remained inconclusive following our initial assessment of this performance objective. It could be that aquifer material within the influence of monitoring wells contains 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. Aquifer sediments were collected from four borehole locations in the northern VC plume at Parris Island site 45 with a cryo-coring procedure. The surficial aquifer at Parris Island site 45 has an upper unit composed of silt and a lower unit composed of silty sand. These are termed the upper surficial aquifer and the lower surficial aquifer (a diagram of the two hydrogeologic units is shown in **Figure E2**).

Initial geochemical results (cDCE, VC, ethene and methane concentrations) for core 1, located near the PCE source zone, are shown in **Figure E2**. The data indicates 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 regions of the aquifer.

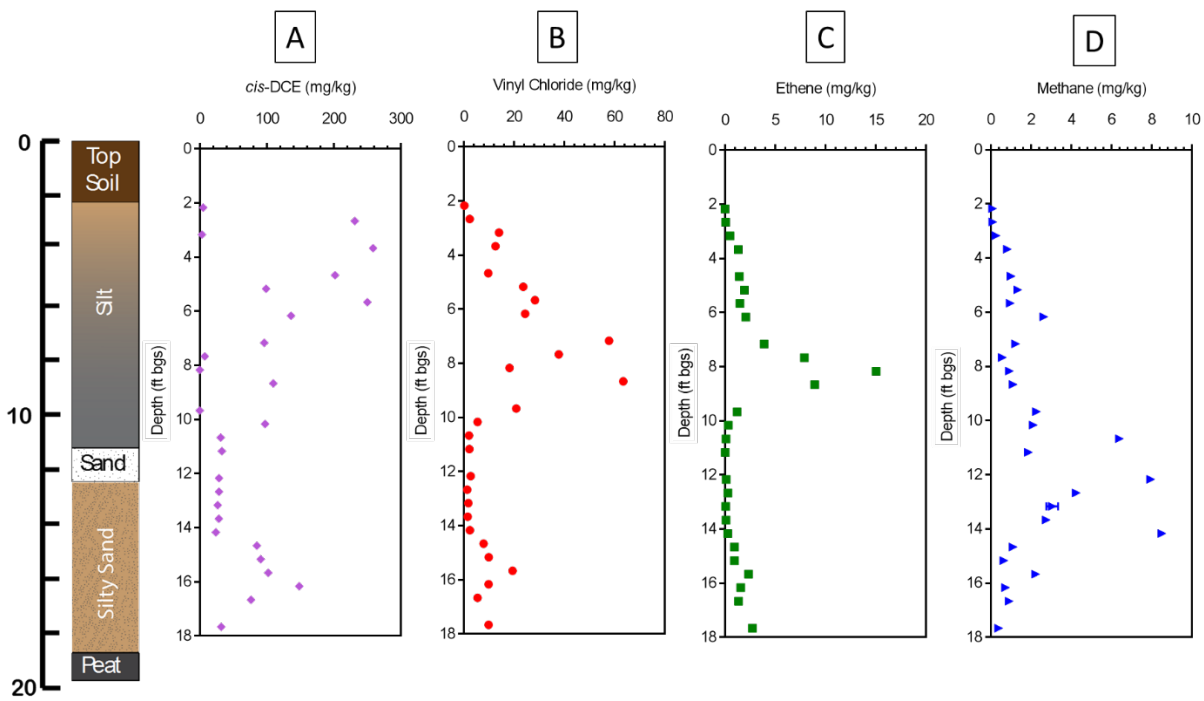

Figure E2. Spatial Resolution of A) cDCE, B) VC, C) Ethene, and D) Methane in Sediment Core 1, Frozen in Place with Liquid Nitrogen, Obtained from Parris Island Site 45. The Graphic on the Left Shows the Soil Characteristics of the Upper (Silt) and Lower (Silty Sand) Surficial Aquifers.

DNA was extracted from relatively small aquifer sediment samples (~0.25 g) (also within the same 1" cryo-core sections) 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 **Figure E3** below. 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).

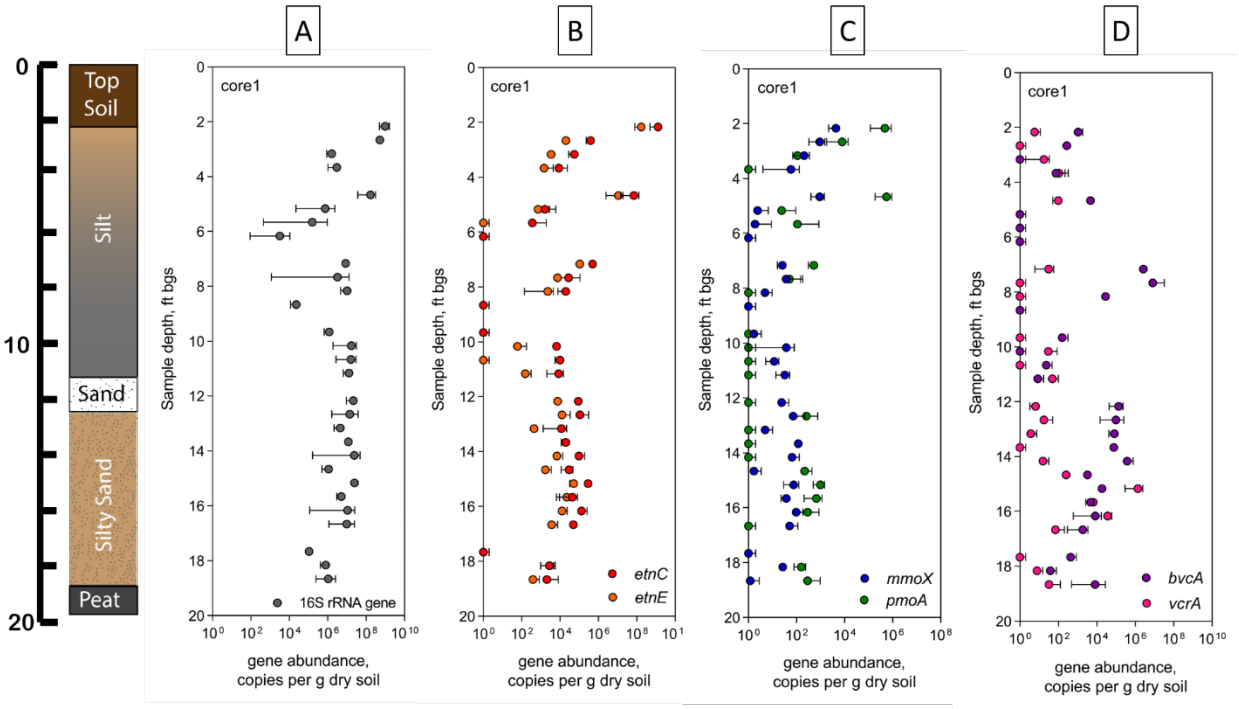

Figure E3. Spatial Resolution of A) Total 16S rRNA Genes, B) *etnC* and *etnE* (Etheneotroph Functional Genes), C) *mmoX* and *pmoA* (Methanotroph Functional Genes), and D) *bvcA* and *vcrA* (VC Reductive Dehalogenase Genes) in an Aquifer Sediment Core, Frozen in Place with Liquid Nitrogen, Obtained from Parris Island Site 45. The Graphic on the Left Shows the Soil Characteristics of the Upper (Silt) and Lower (Silty Sand) Surficial Aquifers.

3.4 ANALYSIS OF RELATIONSHIPS BETWEEN FUNCTIONAL GENES/ TRANSCRIPTS AND BULK VC ATTENUATION RATES

The positive relationship between VC concentration and etheneotrophs noted in the previous performance objective assessment suggests that there will also be a relationship between etheneotroph and activity and VC degradation rates, as estimated over a plume transect. This is because as the VC plume moves further from the source it will be subject to attenuation processes, such as biodegradation.

Using site data (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 for the transects so that VC concentration vs. distance plots were expressed as VC concentration vs. travel time. Bulk VC attenuation rates were estimated from these plots by linear regression. However, because of uncertainties in reported values of seepage velocity, hydraulic gradient, porosity, and hydraulic conductivity, we were not confident in the absolute values of the estimated bulk VC attenuations. Therefore, to determine the relationship between functional genes/transcripts and bulk VC attenuation rates, we used a categorical regression approach. Following estimation of the bulk VC attenuation rate over a transect at a site, each site was assigned to one of two categorical rate groups: high attenuation or low attenuation. Sites in the low attenuation group include NSB Kings Bay site 11 and NAS Oceana SWMU 2C, Altus AFB biowalls and Seal Beach Site 70. Sites included in the high attenuation group included Parris Island site 45 (northern and southern plumes) and Joint Base Pearl Harbor Hickam site LF05. For the categorical regression analysis, a multilevel modeling approach was also used to take into account the effect of intercorrelations between samples from the same sites and the same monitoring wells sampled on different dates. Regression analysis was performed to test the hypothesis that functional gene or transcript abundances are different among the different rate groupings (null hypothesis is that there is no difference).

3.5 ANALYSIS OF RELATIONSHIPS BETWEEN FUNCTIONAL GENES/ TRANSCRIPTS AND BULK VC ATTENUATION RATES

We found that log transformed etheneotroph functional gene (*etnC* and *etnE*) abundances in high rate group were significantly higher than those in the slow rate group, while no significant difference was found for methanotroph functional genes among different groups (**Table E1)**. For reference, log transformed *vcrA* abundances were also significantly higher in the high rate group as compared to the low rate group (although *bvcA* was not). The significantly higher abundances of etheneotroph functional genes and anaerobic VC reductive dehalogenase genes in the high rate group (as compared to the low rate group) suggests that VC degradation was categorically faster when there were higher abundances of etheneotrophs and anaerobic VC-degraders together in the same groundwater sample. It is possible that under field conditions, VC may support the growth of etheneotrophs and anaerobic VC degraders, and etheneotrophs and anaerobic VC dechlorinators both contributed significantly to VC degradation in the contaminated groundwater.

Table E1. 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**

For transcripts, significant differences were observed between *etnE* transcript abundances of the high rate group and slow rate group, suggesting that etheneotrophs were more active at sites where more rapid VC attenuation was observed. The *vcrA* transcript abundance in the high rate group was also significantly higher than the low rate group (**Table E1**).

In summary, the results of correlation analysis between functional gene/transcript abundances and bulk VC attenuation rates suggest that etheneotrophs and anaerobic VC-degraders were more abundant and active at sites with faster bulk VC attenuation rate, and they could be both important factors contributing to VC degradation in groundwater. Therefore, we believe that the results of this performance assessment indicate a success.

3.6 ANALYSIS OF RELATIONSHIPS BETWEEN ETNE ABUNDANCE AND VC BIODEGRADATION RATES

Because of uncertainties in estimating bulk VC attenuation rates in the field, we sought to look at relationships between *etnE* abundance and actual VC biodegradation rates in aerobic VC-degrading laboratory cultures. This required demonstrating that qPCR for the *etnE* gene can be used as a specific surrogate for active etheneotroph biomass in the enrichment cultures, and that the abundance of *etnE* is correlated to the rate of VC biodegradation. This was demonstrated with pure cultures of VC-assimilating *Nocardioides* strain JS614, grown on VC We also extracted DNA from the cultures after the end of the experiment and measured *etnE* abundance with qPCR. The relationship between the VC biodegradation rate and *etnE* abundance is shown in **Figure E4**. We observed a strong linear relationship (R^2=0.98) between the biomass concentration (as *etnE*) and the maximum VC biodegradation rate by strain JS614.

This data supports the idea that the rate of VC oxidation in these cultures is proportional to etheneotroph functional gene concentration, and that VC oxidation rates can be adequately described by Michaelis-Menton enzyme kinetics when functional gene concentrations are used as the biomass concentration. Thus, the success criteria for performance objective "Determine relationships between etheneotroph functional genes and the VC biodegradation rate in VC-oxidizing laboratory enrichment cultures" appears to be met, at least on a preliminary basis.

We hypothesize that if etheneotrophs are contributing significantly to the rate of VC oxidation in enrichment cultures that we will observe a similar linear relationship between the raw VC biodegradation rate and *etnE* abundance. Subsequently we compared measured aerobic VC-biodegradation rates in VC-oxidizing enrichment cultures from PH and NI (in duplicate) under the same pH and temperature conditions as the JS614 experiments. These data points are included in **Figure E4**. The data indicate that relationships between *etnE* gene abundance and VC biodegradation rate in enrichment cultures are different than that for a pure culture of JS614, even at the same pH and temperature. A possible explanation for the observed differences is active etheneotroph biomass. In other words, there may have been lower active etheneotroph biomass in the PH culture and higher active biomass in the NI culture, as compared to the JS614 culture. Because we only measured DNA in this experiment, we do not know the expression level of *etnE* or the activity of the EtnE product in this case. We also know that the activity of EtnE is variable even among different pure cultures of etheneotrophs (Coleman & Spain, 2003a, Mattes, *et al.*, 2005). Further study of the relationship between *etnE* expression (or transcript per gene ratio) as well as the correlation of *etnC* gene and transcript abundance and VC biodegradation rates is warranted.

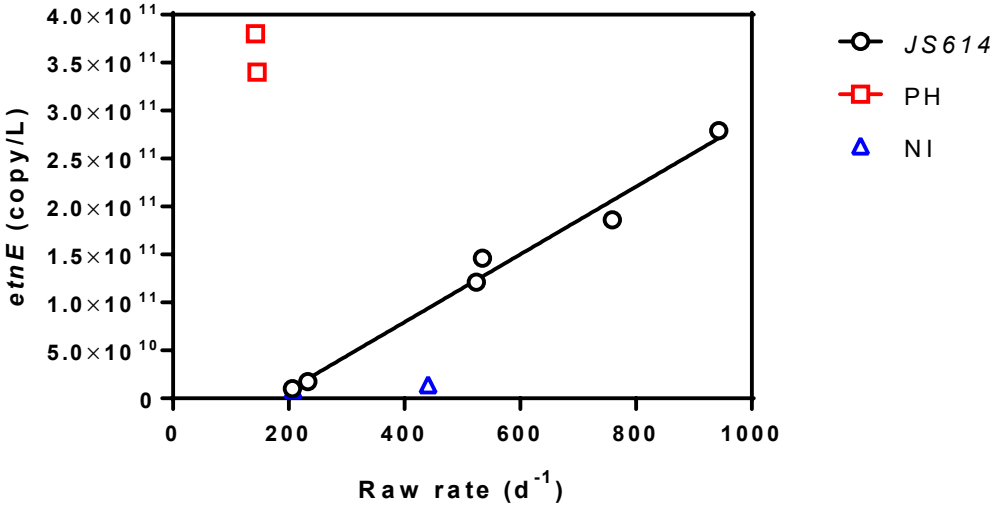

Figure E4. Raw VC Depletion Rate vs etnE Concentration in VC-grown Nocardioides sp. Strain JS614 (Black Circles) as Compared to VC-oxidizing Enrichment Cultures from Pearl Harbor (PH; Red Squares) and Naval Air Station North Island (NI; blue triangles).

For the JS614 experiments, six microcosms were prepared with varying amounts of active JS614 biomass. Substrate depletion curves were fit to the Michaelis-Menton kinetic model and the maximum VC biodegradation rae was estimated. DNA was extracted from the microcosms at the end of the experiment, and etnE was quantified by qPCR.

Page Intentionally Left Blank

4.0 COST ASSESSMENT

There are two major elements of the cost model for implementing the qPCR technology into existing monitoring plans: sampling cost and qPCR/RT-qPCR costs. The only cost drivers that were considered was the number of samples and replicates required, as this will impact the total cost of implementing the technology as part of a long-term monitoring strategy. Typically, we take duplicate samples for DNA and RNA to assess sampling variability.

Overall, our analysis indicates that labor and materials costs for qPCR/RT-qPCR were $530 per monitoring well. This does not include the sampling costs in the field. We estimate that the time required to set-up and take two 1 L filter samples for DNA and two 3L filter samples for RNA was on average about 2 hours per monitoring well. Although one person could sample wells alone, it was often helpful to have another helping at times. So, a conservative estimate is 3 hours per well ($90). This brings the total costs per monitoring well to $620.

We do not include the costs for flights, hotel, food, rental car, equipment rental in this analysis. When we were sampling groundwater at Parris Island Site 45, there were additional costs for disposal of investigation derived wastes (IDW) incurred that perhaps should be taken into account as part of the overall cost per monitoring well. The groundwater IDW collection, storage, analysis, and disposal costs for one sampling event in 2015 were $2,800 for 17 monitoring wells (or ~ $165 per monitoring well). However, we were not charged for IDW costs for obtaining samples for any of the other sites when working alongside a contractor. This leads us to conclude that the extra groundwater filtered for qPCR analysis would not likely contribute very much extra to the overall IDW costs that we were forced to incur separately at Parris Island.

Page Intentionally Left Blank

5.0 IMPLEMENTATION ISSUES

Implementation of qPCR technology for etheneotrophs at chlorinated ethene contaminated sites is already feasible in real-world situations, as many long-term groundwater monitoring programs currently prescribe DNA extraction and qPCR analysis for anaerobic chlorinated ethene degrading bacterial biomarkers. However, RNA is not typically used in qPCR practice, as DNA is more readily obtained and easier to work with than RNA. It may be possible to obtain meaningful relationships between attenuation (or biodegradation rates) and qPCR data obtained with DNA. DNA is not necessarily a reliable indicator of active biomass, qPCR analysis of RNA may yield stronger correlations with rates than DNA. When using RNA, technology performance is dependent on the efficiency of RNA extraction. We recommend that when RNA is desired, that at least 3L of each groundwater sample should be filtered, if possible, to maximize RNA yields.