ESTCP
Cost and Performance Report

(ER-200518)

Application of Nucleic Acid-Based Tools for Monitoring Monitored Natural Attenuation (MNA), Biostimulation and Bioaugmentation at Chlorinated Solvent Sites

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ACRONYMS AND ABBREVIATIONS

AFB	Air Force Base
AOC	area of concern
C_t	threshold cycle
COC	contaminant of concern
CSM	conceptual site model
cis-DCE	*cis*-1,2-dichloroethene
DCE	dichloroethene
Dhc	*Dehalococcoides*
DNA	deoxyribonucleic acid
DNQ	detectable but not quantifiable
DO	dissolved oxygen
DoD	Department of Defense
ESTCP	Environmental Security Technology Certification Program
EVO	emulsified vegetable oil
ITRC	Interstate Technology & Regulatory Council
MCL	maximum contaminant level
MBT	molecular biological tool
MLP/VAB	Mobile Launch Platform/Vehicle Assembly Building
MNA	monitored natural attenuation
mRNA	messenger ribonucleic acid
NAS	Naval Air Station
NASA	National Aeronautics and Space Administration
NAVFAC	Naval Facilities Engineering Command
NAWC	Naval Air Warfare Center
ND	not detected
PCE	tetrachloroethene
PCR	polymerase chain reaction
qPCR	quantitative real-time polymerase chain reaction
RDase	reductive dechlorinase
RNA	ribonucleic acid
RPD	relative percent difference
RPM	remedial project managers
SERDP	Strategic Environmental Research and Development Program
SLC	Space Launch Complex
SOP	standard operating procedure

SWMU	Solid Waste Management Unit
TCE	trichloroethene
TEAP	terminal electron accepting process
trans-DCE	*trans*-1,2-dichloroethene
USEPA	U.S. Environmental Protection Agency
VC	vinyl chloride
VFA	volatile fatty acid
VOC	volatile organic compound

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1.0 EXECUTIVE SUMMARY

1.1 BACKGROUND

Successful anaerobic bioremediation at chlorinated solvent sites relies on the presence of bacteria, such as *Dehalococcoides* (*Dhc*), capable of organohalide respiration (i.e., respiratory reductive dechlorination or "[de]chlororespiration"). Nucleic acid-based assays like the quantitative real-time polymerase chain reaction (qPCR) technique detect and enumerate *Dhc* in soil or groundwater samples by targeting *Dhc*-specific biomarker genes, including the 16S rRNA gene and the *tceA*, *bvcA*, and *vcrA* reductive dechlorinase (RDase) genes implicated in chlorinated ethene reductive dechlorination. The results of nucleic acid-based tests, like the qPCR approach, are expected to assist site managers and practitioners to identify sites where implementation of long-term monitored natural attenuation (MNA) will be effective; where biostimulation will achieve complete dechlorination without DCE/VC "stall"; and where bioaugmentation is required.

1.2 OBJECTIVES

This project's goals included: (1) demonstrating correlations between dechlorination of chlorinated ethenes and the presence and abundance of *Dhc* biomarker genes; (2) defining limitations of the deoxyribonucleic acid (DNA) biomarker-based approach and specifying conditions where qPCR assay offers or fails to provide meaningful information; and (3) developing a guidance protocol for practitioners to apply this tool.

1.3 TECHNOLOGY DESCRIPTION

The project was conducted in two phases. In the first phase, a standard operating procedure (SOP) was developed for collecting groundwater samples. To avoid problems associated with contemporary procedures that rely on shipment of large volumes of contaminated groundwater, on-site biomass collection using Sterivex cartridges for *Dhc* biomarker quantification was developed and validated. In the second phase, this SOP was used to collect groundwater samples from a selection of chlorinated ethene-impacted sites, including sites undergoing MNA and enhanced bioremediation (biostimulation and/or bioaugmentation).

1.4 RESULTS

Data were evaluated using qualitative and quantitative (e.g., Spearman correlations) methods. As a result of this effort, the following performance objectives were met:

Validation of Use of RDase Gene Targets

To date, the four functional genes *pceA* (presumably encoding a tetrachloroethene [PCE]-to-trichloroethene [TCE] RDase), *tceA* (encoding a TCE-to-vinyl chloride [VC] RDase), and *bvcA* and *vcrA* (both encoding VC-to-ethene RDases) have been identified in chlorinated ethene-dechlorinating *Dhc* strains. At each site included in this study, groundwater samples were collected for qPCR analysis of the RDase gene targets *tceA*, *bvcA*, and/or *vcrA*. The gene copy numbers were correlated to concentrations of PCE, TCE, dechlorination intermediates (*cis*-DCE and VC), and/or ethene, the nontoxic dechlorination end product, as well as contaminant/product

ratios using the Spearman Correlation approach. Strong Spearman Correlations (greater than 0.66) were obtained consistently using *vcrA* as a predictor of ethene production. The *vcrA* and *bvcA* genes are both implicated in VC-to-ethene reductive dechlorination. Selection of an appropriate functional gene target (or targets) will be governed by site-specific conditions and objectives; however, based on the results of this study, the quantitative analysis of *Dhc* 16S rRNA genes and the VC RDase genes *vcrA* and *bvcA* at chlorinated solvent sites is anticipated to provide useful, reliable information describing complete reductive dechlorination to ethene.

RDase gene copy number correlations to daughter product concentration ratios or concentrations of individual dechlorination intermediates provided site-specific information about the relationship between these variables but were not consistent from site to site.

Identification of Minimum Number of Dhc Gene Copies indicative of ethene formation— Groundwater samples were collected from all sites for *Dhc* 16S rRNA gene and/or RDase gene analysis and results were correlated to ethene concentrations in the sample. Strong Spearman correlations (greater than 0.66) were observed when *Dhc* cell titers or *vcrA* gene copies exceeded 10^6 to 10^7 per liter of groundwater.

Correlation of Dhc Cell Titers to Dechlorination Rates—Groundwater samples were collected from the National Aeronautics and Space Administration (NASA) Mobile Launch Platform/Vehicle Assembly Building (MLP/VAB) site for *Dhc* 16S rRNA gene and RDase gene analysis. First-order dechlorination rates were calculated from chlorinated ethene data collected from wells inside the plume. The first-order dechlorination rates were correlated to *Dhc* and *vcrA* abundance using the Spearman Correlation. Strong correlations were established between TCE, *cis*-DCE and VC dechlorination rates and *Dhc* cell titers, while medium correlations were observed between VC dechlorination rates and *vcrA* gene copy numbers. The analysis was limited by the use of only three monitoring well locations for rate calculations.

Influence of Alternative TEAPs on Dhc Abundance—Geochemical data for identifying terminal electron accepting processes (TEAPs) were obtained for monitoring locations where *Dhc* analyses were conducted. These data were reviewed qualitatively, since mixed TEAPs are typically observed in contaminated aquifers. *Dhc* cell titers above the detection limit of 10^3 gene copies per liter were generally observed when conditions were reducing (anaerobic), as reflected in dissolved oxygen (DO) concentrations of less than 0.5 mg/L or redox potentials below -75 mV.

Evaluation of False Positive and False Negative Dhc Detections—Biomarker loss during sample handling may result in false negative results. This issue was addressed by improving sampling and handling procedures to obtain *Dhc* biomarker recoveries of greater than 90%. False positive results were eliminated by optimized qPCR protocols and appropriate controls. Further, the simultaneous quantification of *Dhc* 16S rRNA gene and RDase gene targets in undiluted and 10-fold diluted samples enabled the detection of PCR irregularities, including the presence of PCR inhibitors.

Evaluation of Sample Collection Methods—The groundwater sampling procedure was optimized and applied throughout this project to ensure sample consistency (i.e., minimize the

effect of sampling procedures on the results) and quality (i.e., avoid biomarker loss). A study comparing off-site (in the lab) to on-site (in the field) groundwater filtration and biomass collection indicated that the *Dhc* yield of field-filtered samples exceeded 90% with high precision.

Evaluation of Analytical Sensitivity—A reliable limit for *Dhc* 16S rRNA or RDase gene detection is 10^3 cells (i.e., gene copies) per L of groundwater. The quantification limit (i.e., the minimum gene target number that can be reliably quantified) is about five-fold greater than the method detection limit. Greater sensitivity is not needed, as reductive dechlorination is not observed in the field at gene copy abundances below 10^3 per L.

Evaluate Analytical Sample Reproducibility—The qPCR technique is highly reproducible. All qPCR data were generated with at least two replicate DNA extractions, each analyzed for at least two dilutions in triplicate qPCR runs. Differences between replicate samples analyzed in terms of DNA yields and biomarker gene quantification using the same biomass collection method were less than two-fold.

Ease of Using On-Site Filtration Methods—Groundwater sampling methods included attaching a Sterivex cartridge to low-flow discharge tubing, measuring the discharge volume during sampling, and packaging the cartridge. This method added 15 to 30 minutes to the time needed to sample a monitoring well for volatile organic compounds (VOCs).

A Guidance Protocol was developed that includes an SOP for groundwater sampling, as well as guidelines for sampling locations, sampling frequency, and data interpretation. Flowcharts are provided in the Guidance Protocol for use of *Dhc* biomarker gene data to support decision making at sites where MNA is being evaluated, to predict sites where biostimulation will be successful, and to identify sites where bioaugmentation is required.

1.5 IMPLEMENTATION ISSUES

The Guidance Protocol entitled Use of Nucleic Acid-Based Tools for Site Assessment and Monitoring Bioremediation at Chlorinated Solvent Sites will provide site RPMs and contractors across DoD the ability to implement engineered bioremediation and to support decision making regarding MNA and enhanced bioremediation. With the increased knowledge and understanding of the reductive dechlorination process, along with improved and rigorously tested assessment and monitoring tools, as well as appropriate guidance documents, site managers and regulators will have the means to convincingly argue that MNA and/or enhanced treatment are viable, cost-effective approaches for source zone remediation and plume control to achieve long-lasting risk reduction.

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2.0 INTRODUCTION

2.1 BACKGROUND

The chlorinated solvents tetrachloroethene (PCE), trichloroethene (TCE), and their anaerobic dechlorination intermediates (daughter products) *cis*-1,2-dichloroethene (*cis*-DCE), *trans*-1,2-dichloroethene (*trans*-DCE), 1,1-DCE, and vinyl chloride (VC) are prevalent groundwater contaminants at many Department of Defense (DoD) sites. PCE and TCE are resistant to metabolic degradation under aerobic conditions but can be reductively dechlorinated stepwise to lesser chlorinated ethenes under anaerobic conditions. DCEs and VC can be completely dechlorinated to ethene, and sometimes transformed to ethane, by anaerobic microorganisms. Alternatively, these compounds can be mineralized to carbon dioxide and inorganic chloride under aerobic conditions (Coleman et al., 2002; Singh et al., 2004) and possibly anoxic conditions (Bradley and Chapelle, 1998).

Laboratory findings and field studies indicate that reductive dechlorination can be an effective process for transforming chlorinated ethenes under anaerobic conditions (Löffler et al., 2003). However, at many PCE/TCE contaminated sites, MNA or injection of organic substrates (i.e., electron donor) to stimulate the reductive dechlorination process (i.e., biostimulation) leads to the accumulation of *cis*-DCE and VC with limited or no ethene formation. The accumulation of VC is of particular concern because VC is classified as a human carcinogen. Incomplete dechlorination lengthens remediation times and increases costs before site closure and/or redevelopment of DoD property can be achieved.

Complete reduction of chlorinated ethenes to the environmentally benign products ethene (or ethane) and inorganic chloride is required to achieve detoxification and successful anaerobic remediation of chlorinated ethenes. In addition to biostimulation, bioaugmentation with consortia containing dechlorinating *Dhc* bacteria has been implemented to address incomplete dechlorination and accumulation of toxic intermediates (Ellis et al., 2000; Major et al., 2002; Lendvay et al., 2003; Scheutz et al., 2008). In order to ensure successful application of both biostimulation and bioaugmentation, nucleic acid-based tools were designed (Löffler et al., 2000; Hendrickson et al., 2002; He et al., 2003 a,b; Müller et al., 2004; Krajmalnik-Brown et al., 2004; Ritalahti et al., 2006; Holmes et al., 2006; Smits et al., 2004) for qualitative and quantitative assessment of the dechlorinating bacterial community. Biomarker identification and the refinement of procedures and tools are ongoing activities in laboratories worldwide.

Although some of the available nucleic acid-based tools have been rigorously tested in laboratory settings and are commercially available (e.g., www.microbe.org, www.siremlab.com), the beneficial use of these approaches had not been established in field studies. For example, little was known about the minimum number of bacterial (i.e., *Dhc*) cells needed per volume of groundwater for sustained reductive dechlorination activity. A sufficient database providing quantitative information on key dechlorinating microbes (i.e., *Dhc*), geochemistry, and dechlorination activity was not available for making generalized or site-specific recommendations. Further, no standardized groundwater sampling procedures were applied, *Dhc* biomarker loss during sample handling, shipping to the analytical laboratory, and storage were not known, and guidance documents for the application of nucleic acid-based tools and interpretation of the results were not available. To promote a more widespread application of

molecular biological tools (MBTs) and enhance implementation of bioremediation technologies at chlorinated solvent sites, standardized protocols are needed. With validated protocols in place, quality and uniformity of test results can be ensured, which in turn will allow comparisons of data obtained from different sites and generated in different laboratories.

The guidance developed in this study is expected to provide remediation project managers with the background to understand the value of MBT application, to judge what information the MBT approach can and cannot provide, and to interpret MBT data. In other words, the Guidance Protocol promotes a more widespread application of MBTs and results in significant cost reductions and reduced project timelines. The remediation project manager end user will be provided with additional relevant information to interpret site conditions to select:

- Sites where implementation of long-term MNA will be effective
- Sites where biostimulation will achieve complete dechlorination without dichloroethene (DCE)/VC "stall"
- Sites where bioaugmentation is required, ultimately shortening remediation times
- Identify sites where the conditions (e.g., low pH, insufficient supply of electron donor, unfavorable geochemical conditions) are limiting biodegradation activity.

By clearly understanding how site geochemistry and the presence and abundance of key microbes (i.e., *Dhc*) affect contaminant detoxification, investments in the technology could focus on those sites amenable to bioremediation, and a more efficient and rapid transition from system design to full-scale remediation is expected. This could save months to years on a given remediation project timeline, would achieve more rapid site closures, and save DoD resources that can be invested elsewhere.

2.2 OBJECTIVES OF THE DEMONSTRATION

During this demonstration, we evaluated and validated the use of nucleic acid-based tools for site assessment and bioremediation process monitoring at chlorinated solvent sites undergoing MNA, biostimulation, or bioaugmentation treatment. Use of these tools is anticipated to reduce remediation costs by supporting identification of sites amenable to MNA; predicting sites where biostimulation can be successfully implemented; identifying sites where bioaugmentation is required early in the design process; and recognizing sites where the reductive dechlorination process cannot be productively implemented. The specific project objectives included:

- Evaluating groundwater sampling methods that collect planktonic (i.e., unattached) cells on membrane filters on site to avoid shipping of groundwater to the analytical laboratory
- Applying nucleic acid-based tools to assess the distribution and abundance of *Dhc* biomarker genes at 12 sites at different stages of bioremediation treatment
- Integrating the MBT information with data typically collected (e.g., contaminant concentration data, geochemical data) at bioremediation sites to develop

correlations between reductions in contaminant concentrations and the abundance of specific *Dhc* biomarker genes

- Evaluating if qPCR data are useful predictors for the feasibility of MNA, biostimulation, or bioaugmentation as productive cleanup remedies at a given site contaminated with chlorinated ethenes
- Developing a guidance document for application of nucleic acid-based qPCR tools at chlorinated solvent sites where MNA, biostimulation, or bioaugmentation are being considered or have been implemented
- Identifying the limitations of the qPCR approach for the analysis of groundwater samples and specifying the site conditions where this tool can/cannot provide useful information.

The approach was demonstrated at selected DoD sites that are contaminated with chlorinated ethenes and where MNA, biostimulation, or bioaugmentation treatments have been implemented. Groundwater samples were collected during routine monitoring efforts by the respective site responsible parties. Samples were forwarded to the Georgia Institute of Technology for qPCR analysis.

2.3 REGULATORY DRIVERS

The U.S. Environmental Protection Agency (USEPA) maximum contaminant level (MCL) for PCE and TCE in drinking water is 5 µg/L. This concentration is considerably lower than the concentrations present in groundwater at many DoD sites. The MCLs for *cis*-DCE and VC are 70 µg/L and 2 µg/L, respectively. MNA and enhanced bioremediation have been shown to be cost-effective technologies for remediating chlorinated ethene-contaminated sites. Therefore, this demonstration sought to improve the selection, design, and implementation of MNA and bioremediation treatment to achieve cleanup goals and site closures. Importantly, the findings communicated in the Guidance Protocol will assist regulators to better understand and judge the meaning of qPCR data as a relevant component for predicting contaminant concentrations and future plume behavior.

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3.0 TECHNOLOGY

The following sections provide an overview of technology history and application (Section 3.1), a description of technology development (Section 3.2), and a description of the potential advantages and limitations of the technology (Section 3.3).

3.1 TECHNOLOGY DESCRIPTION

Discoveries over the past decade significantly advanced our understanding of microbial processes that contribute to the fate of chlorinated ethenes in contaminated subsurface environments. Although not all processes contributing to chlorinated ethene detoxification are fully understood, there is conclusive evidence that reductive dechlorination plays a major role in anaerobic aquifers where MNA, biostimulation, or bioaugmentation are implemented. The complete dechlorination of PCE to ethene is a multistep process and is most effectively carried out by more than one microbial population (reviewed in Major et al., 2003; Löffler et al., 2003; Smidt and de Vos, 2004).

Several bacterial groups are involved in partial reductive dechlorination of PCE and TCE to DCEs (e.g., *Dehalobacter, Desulfitobacterium, Desulfuromonas, Geobacter*, and *Sufurospirillum* species), but *Dhc* are the key players involved in complete reductive dechlorination and detoxification (i.e., ethene formation) (Löffler et al., 2003, Smidt and de Vos, 2004). Since complete reductive dechlorination is firmly linked to *Dhc* bacteria, evidence for *Dhc* presence and abundance will guide the decision making process on treatment options. The current knowledge of the detoxification process (i.e., the link between ethene formation and the presence of *Dhc*) justifies that site assessment and bioremediation monitoring focuses on members of this bacterial group.

qPCR techniques have been developed to quantify target microbes (i.e., *Dhc*) (He et al., 2003a,b; Smits et al., 2004; Ritalahti et al., 2006). The qPCR approach offers sensitive detection combined with quantitative information. Thus, qPCR is useful to monitor the effects of treatment on the size of the dechlorinating *Dhc* population (i.e., the amount of catalyst present in the contaminated aquifer). qPCR has several advantages over traditional endpoint PCR. qPCR is faster and highly sensitive (>five copies per reaction), requires no post-PCR steps (e.g., agarose gels), minimizes the risk of cross contamination, and multiplex assays are feasible. Multiplex assays allow the quantification of up to four targets in a single assay tube, thus reducing chemical and labor costs; however, multiplex assays require careful testing and optimization to avoid interferences of the multiple primers and fluorescent probes in the reaction mix. Nevertheless qPCR quantifies DNA (and possibly ribonucleic acid [RNA]) targets precisely and reproducibly because it relies on threshold cycle (Ct) values determined during the exponential phase of PCR rather than endpoints (e.g., competitive quantitative polymerase chain reaction [PCR] (Cupples et al. 2003)).

To date, four functional genes (e.g., *pceA*, *tceA*, *bvcA*, and *vcrA*) involved in chloroethene reductive dechlorination have been identified in *Dhc* strains. The *pceA* gene encodes a PCE-to-TCE RDase in *Dhc ethenogenes* strain 195, and *tceA* gene is responsible for TCE-to-VC reductive dechlorination in *Dhc ethenogenes* strain 195 and *Dhc* sp. strain FL2. The *vcrA* and *bvcA* genes are implicated in VC-to-ethene reductive dechlorination. *Dhc* sp. strain GT and

strain VS carry the *vcrA* gene, and *Dhc* sp. strain BAV1 carries the *bvcA* gene (Krajmalnik-Brown et al., 2004; Müller et al., 2004; Sung et al., 2006). Although the current knowledge of *Dhc* RDase genes involved in chlorinated ethenes reductive dechlorination is incomplete, and many more RDase genes await discovery (Ritalahti et al. 2006, 2010), the combined quantitative assessment of *Dhc* 16S rRNA genes and the *tceA*, *bvcA*, and *vcrA* RDase genes are a basis to establish correlations between *Dhc* biomarker presence and complete dechlorination in groundwater samples from contaminated field sites. The known *Dhc* strains harbor a single copy of the aforementioned biomarker genes indicating that that the number of target genes enumerated with qPCR equals the number of *Dhc* cells in the sample.

3.2 ADVANTAGES AND LIMITATIONS OF THE TECHNOLOGY

The use of MBTs has contributed greatly to our understanding of microbial detoxification processes and our ability to exploit naturally occurring bacteria to biodegrade DoD-relevant contaminants (SERDP and ESTCP, 2005; Stroo et al., 2006). qPCR has emerged as the MBT of choice for site assessment and bioremediation monitoring. The advantages of qPCR include: (1) the technique provides quantitative information of the target gene(s) and organism(s), and hence, is a powerful tool to establish cause-and-effect relationships between treatment and contaminant detoxification; (2) it provides excellent sensitivity and detects as few as five cells per reaction tube; (3) it is relatively inexpensive and broadly available; (4) and it is available from commercial laboratories and numerous academic laboratories.

Inherent limitations that present a challenge to the application of MBTs for the assessment of environmental samples (Stroo et al., 2006) include (1) non-uniform distribution of microbes in the subsurface; (2) insufficient sampling technologies to retrieve representative samples from the subsurface; (3) insufficient knowledge of key biomarkers; and (4) possible presence of inhibitors (e.g., humic acids) that interfere with nucleic acid extraction procedures and PCR amplification. Understanding and quantifying the impacts of these limitations on qPCR analysis of *Dhc* biomarker genes were beyond the scope of this project; however, because each of these issues can affect qPCR data and interpretation, efforts were expended to minimize the impacts of these factors.

Another key issue involves sample collection procedures and sample handling, which can significantly impact the results (Casey, 2006), and hence, affect data analysis and interpretation. Team members explored sampling and sample handling procedures, and these results have been incorporated into this study to the extent possible. The sampling approach selected for this project provides a conservative approach to avoid false positive results and minimize the effects of sample collection and handling procedures on the qPCR results. The sampling and shipping procedures used in this project were developed to minimize the biases introduced during sampling, and sample handling, shipping, and storage.

4.0 PERFORMANCE OBJECTIVES

The purpose of this demonstration was to evaluate and refine the use of nucleic acid-based tools to assess chlorinated solvent bioremediation using MNA, biostimulation alone, or biostimulation combined with bioaugmentation. It is expected that the use of these tools will lead to informed remediation decisions, reduced remediation times at lower costs and will enhance the efficiency of full-scale applications towards site closure and increase confidence in the application of MBTs for bioremediation projects.

The performance objectives, provided in Table 1, are described in detail below:

- To establish qualitative and quantitative criteria correlating *Dhc* target gene abundance with reductive dechlorination and contaminant concentrations
- To determine the minimum number of *Dhc* gene copies (i.e., cell titers) to observe reductive dechlorination to ethene
- To correlate *Dhc* 16S rRNA gene and RDase gene abundances with contaminant (e.g., PCE, TCE) dechlorination rates
- To correlate RDase gene abundance with the dominant TEAP suggested by groundwater geochemical data
- To identify conditions that can generate false positive and/or false negative results.

Table 1. qPCR performance objectives.

Type of Objective	Performance Objective	Success Criteria	Results
Quantitative	Validate use of RDase gene targets	Correlations of functional target genes (e.g., *tceA*, *bvcA*, *vcrA*) with evidence for reductive dechlorination (e.g., change in contaminant concentration ratios)	Positive Spearman correlation >0.34
Quantitative	Identify minimum number of *Dhc* gene copies to achieve detoxification	Minimum number of target *Dhc* 16S rRNA gene or functional gene copies observed with complete dechlorination (e.g., ethene formation)	Ethene formation always observed with *Dhc* $>10^7$ cells/L
Quantitative	Evaluate correlation between *Dhc* cell titers and dechlorination rates	Correlations of *Dhc* biomarkers gene targets with contaminant dechlorination rates	Positive Spearman correlation >0.34
Quantitative	Evaluate effects of contaminant concentrations on *Dhc* abundance	Correlation of *Dhc* biomarker gene copies with contaminant (e.g., PCE, TCE) concentrations	Weak Spearman correlations dechlorination daughter products

Table 1. qPCR performance objectives. (continued)

Type of Objective	Performance Objective	Success Criteria	Results
Quantitative	Evaluate optimum sample collection method	On-site filtered or off-site laboratory-filtered groundwater samples with *Dhc* biomarker gene copies within 50% RPD	Yield of on-site, field-filtered samples > 90%
Quantitative	Evaluate analytical sensitivity	Measure *Dhc* biomarker gene copies at 10^4 gene copies/L	Detection and quantification limits of 10^3 and 10^4 cells/L, respectively
Quantitative	Evaluate reproducibility of analytical procedure	Does the analysis of replicates yield results within 50% RPD?	qPCR of duplicate extractions within 50% RPD (most environmental samples); <10% for standards
Qualitative	Evaluate influence of dominant TEAP on *Dhc* abundance	Identify TEAP trends associated with *Dhc* biomarker gene copy numbers	Anaerobic conditions needed for observing *Dhc* biomarker genes
Qualitative	Evaluate likelihood of false positive/ negative detections	Identification of false positive and false negative detections of *Dhc* biomarker genes and its impact on decision making	Simultaneous quantification of phylogenetic and functional biomarker genes eliminates false positives. Analytical sensitivity reduces false negatives.
Qualitative	Evaluate implementability of on-site biomass collection	Feedback from field personnel on ease and feasibility of on-site groundwater filtration and biomass collection	Sterivex cartridges easy to use in the field

Notes:
TEAP – terminal electron accepting process
RPD – relative percent difference

5.0 SITE DESCRIPTIONS

Numerous DoD sites were identified as potential demonstration sites. The goal was to demonstrate the value of the qPCR approach at chlorinated ethene-contaminated sites where MNA, biostimulation and/or bioaugmentation had been implemented. Each potential site was pre-screened for inclusion in the demonstration and then evaluated based on a detailed set of the following criteria. First, only those sites that had undergone a detailed site characterization including hydrogeologic and geochemical characterization and source and plume delineation were considered. Furthermore, only those sites that had been sampled and monitored in accordance with EPA guidance for MNA sites (EPA, 1998) and enhanced bioremediation guidance (Parsons, 2004) were considered. Only sites with PCE or TCE concentrations greater than 100 μg/L were considered to ensure the potential for efficient biological degradation (i.e., the dechlorinating bacteria require the chlorinated contaminants as growth substrates [Cupples et al., 2003]). However, sites with documented accumulation of DCE and/or VC (DCE-VC "stall") with respect to these parent compounds were also included to determine the impact of *Dhc* 16S rRNA gene and RDase gene abundance on the degradation of these compounds. Additional screening criteria for sites considered for MNA evaluation in this study included evidence of reducing conditions favorable for reductive dechlorination in accordance with EPA guidelines (EPA, 1998). For sites slated for the implementation of enhanced bioremediation, anaerobic conditions were not a prerequisite because reducing conditions would be achieved with the addition of electron donors.

The selected sites represent a broad spectrum of chlorinated ethene-contaminated aquifers with various geologic and hydrogeologic conditions. A summary of site descriptions is provided in Table 2.

Table 2. Summary of site descriptions.

Parameter	OU1, Anniston Army Depot	SWMU 21 NAS Dallas, TX	MLP/VAB, NASA Cape Canaveral	Site 8, Space Launch Complex (SLC-4) East Vandenberg AFB, CA	Site 13/14, Space Launch Complex (SLC-4) East Vandenberg AFB, CA	AOC A, NSA Mid-South
Bioremediation type	MNA	MNA	Biostimulation	Biostimulation	Biostimulation	Biostimulation
Baseline parent compound	3.6-27 million lb. TCE mass, predominantly free product	TCE up to 290 μg/L	TCE up to 50,000 μg/L	TCE up to 2000 μg/L	TCE up to 850 μg/L	TCE up to 1400 μg/L
Baseline degradation products	DCE and VC present	*cis*-DCE up to 22 μg/L VC <0.3 μg/L	Ethene production up to 395 μg/L	*cis*-DCE up to ~50 μg/L. No VC.	*cis*-DCE up to ~230 μg/L, VC detected, no ethene production observed	*cis*-DCE up to ~200 μg/L. Limited VC.
Redox conditions	Anaerobic	Aerobic conditions	Anaerobic conditions	Semi-aerobic and anaerobic conditions present	Semi-aerobic and anaerobic conditions present	Anaerobic
Geology/hydrogeology	Three hydrostratigraphic units. From top: unconsolidated clay, weathered bedrock, and unweathered bedrock	Fine-grained fill and alluvial deposits. Weathered shale at ~24 ft bgs.	Sandy materials with marine clay at ~96 ft bgs. Water table at ~ 5 ft bgs. Hydraulic conductivity of sands ~10^{-6}-10^{-4} cm/sec	2-8 ft layer of sandy material underlain with shale bedrock	Hydraulic connection between lake and site groundwater. Paleochannel identified.	Fluvial deposits, which are hydraulically connected with the Cockfield aquifer via an erosional scarp.

Table 2. Summary of site descriptions. (continued)

Parameter	Site 59, NAS Cecil Field	OU 24 NAS North Island, CA	Magazine 1 Area, Fort Dix	Plume B, Bachman Road	Milledgeville	Former NAWC Trenton
Bioremediation type	Bioaugmentation	Bioaugmentation	Bioaugmentation	Bioaugmentation	Bioaugmentation	Bioaugmentation
Baseline parent compound	TCE >2000 µg/L	Less than 5 µg/L	TCE up to 2000 µg/L	PCE >100,000 µg/L	TCE up to 10,000 µg/L	TCE up to 88,000 µg/L
Baseline degradation products	Limited DCE and VC detections	DCE up to 1100 µg/L, VC up to 660 µg/L	DCE up to 1200 µg/L	*cis*-DCE (110,000 µg/L) and VC (8900µg/L)	*cis*-DCE up to 300 µg/L VC up to 1 µg/L	*cis*-DCE up to 52,000 µg/L, VC up to 21,000 µg/L
Redox conditions	Anaerobic	Anaerobic	Anaerobic	Anaerobic	Anaerobic	Anaerobic
Geology/hydrogeology	Fine sands with increasing clay content with depth. Intervals of sandy clay and clayey sand present above bedrock.	Shallow aquifer consisting of dredged sediments from San Diego Bay. Fresh water-salt water interface at ~35 ft bgs.	Predominantly Kirkwood Formation materials in study area	Glacial outwash sand with silty lenses. Clay at ~24 ft bgs. Average groundwater flow ~0.5 ft/day	Heterogeneous patterns of sands, silts, and clays to ~20 ft bgs, underlain by weathered bedrock	Heterogeneous fractured rock aquifer with spatially varied hydraulic properties

Notes:
MNA – monitored natural attenuation
cis-DCE – cis-1,2-dichloroethene
VC – vinyl chloride
µg/L – micrograms per liter
mg/L – milligrams per liter
DO – dissolved oxygen
ft bgs – feet below ground surface
SWMU – Solid Waste Management Unit
AFB – Air Force Base
SLC – Space Launch Complex
AOC – area of concern
NAS – Naval Air Station

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6.0 TEST DESIGN

This section provides the detailed description of the testing conducted during the demonstrations.

6.1 CONCEPTUAL EXPERIMENTAL DESIGN

The performance objectives, provided in Table 1, include criteria such as:

- Correlation of *Dhc* RDase gene targets with reductive dechlorination activity, and the number of *Dhc* 16S rRNA gene copies (i.e., cells)
- Correlations of RDase gene copies with contaminant concentrations
- Correlation of *Dhc* biomarker gene abundance with the dominant TEAP (i.e., redox condition)
- Correlation of RDase biomarker genes with stable or shrinking contaminant plumes
- Identification of false positive and false negative qPCR results and their impact on decision making processes. This effort also evaluated sample collection methods and determined the sensitivity and reproducibility of the analytical procedures.

To evaluate the performance objectives of this demonstration, samples were collected from the field sites described in Section 5. The resulting chemical, geochemical, and microbial data are presented in this section and were also compiled in a central database. Various data evaluation methods (e.g., statistical correlations) were conducted to determine whether the success criteria for each performance objective were achieved. The data interpretation is presented and discussed in Section 7.

6.2 BASELINE CHARACTERIZATION

For the purposes of this study, baseline characterization is defined as site characterization prior to implementation of enhanced bioremediation. No baseline characterization was conducted by the ER-0518 team prior to sampling because baseline characterization is not required prior to implementing the use of MBTs at a site. However, baseline characterization activities were conducted by the individual site managers, and data resulting from those activities, which were used to build the conceptual site model (CSM), are summarized in Table 2 and incorporated herein.

6.3 TREATABILITY OR LABORATORY STUDY RESULTS

There were no treatability or laboratory confirmation studies conducted as part of this demonstration as the qPCR technology is mature and does not require a proof-of-principle effort. Considerable efforts were expended to compare groundwater sampling and on-site versus off-site biomass collection procedures. All samples were collected from the test sites and analyzed in the laboratory utilizing qPCR methodology.

6.4 FIELD TESTING

This project was conducted in a phased approach. The phases outlined in this section have been designed to meet the objectives as described in Section 2.2.

6.4.1 Phase I: Sample Preservation and Handling

This evaluation yielded an effective and efficient methodology for groundwater sampling and biomass collection in the field (i.e., on site). The improved technology combines several advantages over contemporary procedures by (1) minimizing biomarker loss due to sample handling (e.g., producing false negative results) and (2) generating other benefits such as reducing overall sampling and analytical costs. Cost savings by on-site filtration are realized through lower shipping costs and reduced extraction and handling efforts in the laboratory. Two preservation methods were tested: (1) collecting groundwater samples in a container, preserving it at 4°C and shipping it to the laboratory (i.e., traditional method) and (2) on-site filtering of the same volume of groundwater and shipment of the filter cartridges to the laboratory at 4°C.

6.4.2 Phase II: Quantification of Biomarkers and Chemical Constituents

In the second phase of the project, qPCR was used to assess the distribution and abundance of *Dhc* biomarker genes at selected sites, which were in various stages of bioremediation treatment (e.g., MNA, biostimulation/bioaugmentation treatment). In addition, contaminants of concern (COCs), including chlorinated ethenes and other VOCs, were analyzed based on project specific requirements. At each site, standard sampling methods were followed and traditional MNA/geochemistry parameters were collected.

6.4.3 Data Compilation

The scope of project ER-200518 was to analyze groundwater samples collected at sites undergoing different bioremediation treatment and to demonstrate the value of quantitative *Dhc* biomarker gene analysis. This project relied on non-ESTCP resources to obtain samples for qPCR analysis. Following sample processing and quantification of *Dhc* biomarker genes, the results were transferred to a central database. Likewise, to the extent practical and available, the information from the site managers, including VOC and sampling/geochemical parameters, were transferred from the site managers to the ER-200518 project team and into the central database. The central database was maintained by Tetra Tech.

Generally, site samples were collected from a well in the source area, two to three wells inside the plume near source and downgradient from the source, a well upgradient of the source/plume, and/or a well downgradient of the plume. At sites with active bioaugmentation/biostimulation tests occurring, samples were collected from wells inside the test area and at least one well outside the test area whenever possible.

6.5 SAMPLING METHODS

Field sample collection, preservation, and handling methods were evaluated and validated during this project. Groundwater samples were collected in amber, 1-L glass bottles and shipped on bagged ice in a cooler (i.e., 4°C) to the analytical laboratory where the biomass was collected via

vacuum filtration on membrane filters (e.g., MO BIO Ultraclean Water DNA kit, Carlsbad, CA) for subsequent DNA extraction and *Dhc* biomarker quantification. In the laboratory, a Masterflex peristaltic pump and easy load drive head (Cat. # 7518-10) were used with Masterflex peroxide-cured silicone tubing (L/S 16) (Cole Parmer) for laboratory filtration.

The on-site field filtration method includes the collection of suspended particles (i.e., microbial cells) from groundwater samples with the use of a single-use Sterivex™-GP filter cartridge. (Millipore Cat# SVGPL10RC). This ready-to-use cartridge consists of a hydrophilic polyethersulfone membrane (0.2-µm pore size) in a 1.7 cm diameter and 6.7 cm length Eastar copolyester housing. The filter was affixed to the effluent end of the discharge sample tubing and once a sufficient and quantified amount of groundwater passed through the filter, the filter was removed, packaged, and shipped according to standardized procedures. Sterile Sterivex™-GP cartridges were connected via Luer Lock adapters to tubing. Male and Female Luer-Lock plugs were used to seal the inlet and outlet of each Sterivex cartridge (Catalog # EW-45503-58 and EW-45500-28, respectively, Cole-Parmer, Vernon Hills, IL.). The Sterivex cartridges were designed for removing particles and microbial cells from large volumes of aqueous solutions, but for this study were used to collect biomass from groundwater for subsequent DNA extraction and *Dhc* biomarker gene quantification.

6.6 SAMPLING RESULTS

Data generated in this project include qPCR results for *Dhc* biomarker genes (i.e., the 16S rRNA gene and the RDase genes *tceA*, *bvcA*, and/or *vcrA*) and total bacterial 16S rRNA genes (based on qPCR with a general primer/probe set). These results were generated and/or compiled for every site included in this demonstration. Other data important in this analysis include contaminants of concern and dechlorination daughter products as well as geochemical data. Whenever these data were available and could be transformed into the database format, they were compiled in the project database. In the event that these data were only available in the original reports from other sources, the data are referenced in this report.

Data about the contaminants of concern (PCE, TCE), dechlorination daughter products (*cis*-DCE, VC, ethene, ethane), and geochemical data including DO, oxidation-reduction potential, carbon dioxide, total organic carbon, nitrate/nitrite, sulfate/sulfide, methane, and alkalinity were collected. These data have been retained in both tabular and graphic formats as well as in a project database. Based on the interpretation of these data, a guidance protocol has been generated for remedial project managers (RPMs) and field practitioners on the application of qPCR for the assessment of *Dhc* biomarker genes.

A central database was developed containing groundwater monitoring and geochemical and microbiological data. The database was designed to allow queries that support the development of correlations between field-observed dechlorination activities, geochemistry, and the presence and abundance of *Dhc* biomarker genes. The database contains a collection of historical data and current data obtained during this study for many of the sites included in this demonstration. Information generated by the ESTCP project team including qPCR data were included in the database.

6.7 PROTOCOL DEVELOPMENT

Based on the results of this effort, selection criteria were derived to assist in the selection and application of MNA, biostimulation, and/or bioaugmentation at chlorinated solvents sites. Such guidance, developed during the course of the project, is documented under a separate document titled "Guidance Protocol: Application of Nucleic Acid-based Tools for Monitoring MNA, Biostimulation or Bioaugmentation at Chlorinated Solvent Sites."

The purpose of this document is to provide guidance to RPMs and field practitioners on the application of MBTs, specifically, nucleic-acid based tools for evaluating MNA, biostimulation, and bioaugmentation, at chlorinated solvent sites. This protocol summarizes the current state of the practice of these tools and is intended to provide a technically sound and practical approach for using these tools. This guidance document provides recommendations regarding sampling approaches and data evaluation criteria for use in remedial decision making.

The protocol includes background information to provide RPMs with basic understanding of the reductive dechlorination process and the bacteria of interest. A description of qPCR analysis and guidance with data interpretation is included. Most importantly, MBT application to MNA evaluation and decision making on bioaugmentation are presented in flowcharts. SOPs for groundwater sampling are presented.

7.0 PERFORMANCE ASSESSMENT

This section provides a summary of data analysis in support of the assessment of performance objectives. The performance criteria were categorized into qualitative and quantitative criteria as shown in Table 1. These criteria constitute the performance objectives of this demonstration, which were developed from the criteria listed in Table 1, and have been linked to the objectives of the demonstration defined in Section 2.2. Quantitative metrics have a numerical value or precise determination. Conversely, the qualitative metrics do not have a numerically or otherwise precise result (e.g., a positive correlation of target genes with a dominant TEAP).

In addition, the Spearman's rank correlation coefficient (a measure of statistical dependence between two variables) was calculated for the data from the following sites: Anniston Army Depot OU1, NASA Cape Canaveral, NAS North Island OU24, Milledgeville, and Naval Air Warfare Center (NAWC) Trenton sites. The Spearman correlation is a non-parametric correlation, which was used since the distributions of the data were unknown. If the data for the sites referenced above contained more than six data pairs, an evaluation of whether a statistical correlation existed was performed, but if there were less than six data pairs, only a general evaluation of the correlation could be performed. The general evaluation for the Spearman correlation is based on the following:

- Less than or equal to |0.33| indicates a low correlation
- Between |0.34| and |0.66| indicates a medium correlation
- Greater than or equal to |0.67| indicates a strong correlation.

The results of the statistical analyses have been incorporated in the performance assessment discussions below, as appropriate. Performance assessment results are described in the following subsections; a subsection is provided for each demonstration performance criterion.

7.1 VALIDATION OF RDASE TARGET GENES

Correlations of *Dhc* RDase biomarker genes (e.g., *tceA*, *bvcA*, and/or *vcrA*) with daughter product to parent compound concentration ratios (e.g., [*cis*-DCE, VC]/TCE; [VC, ethene]/*cis*-DCE) and combined VOC concentrations (e.g., TCE, *cis*-DCE, VC) were used to evaluate the predictive use of qPCR data on in situ reductive dechlorination performance. The confirmation metric for this performance objective was the achievement of a Spearman correlation of greater than |0.33|.

Correlations between dechlorination product ratios and *tceA* and *bvcA* gene abundances were evaluated in data sets collected from the Milledgeville site, and correlations with the *vcrA* gene were evaluated in data sets collected from NAS North Island OU24, NASA Cape Canaveral, and the Milledgeville site. A summary of the *Dhc* RDase gene evaluations is provided below.

The Spearman correlation between the *tceA* gene and the daughter to parent compound concentration ratio of (*cis*-DCE, VC)/TCE was weak (less than or equal to |0.33|) for the Milledgeville data. Similarly, the Spearman correlation between the *tceA* gene and the

individual contaminant concentrations (i.e., TCE and *cis*-DCE) was weak (less than or equal to |0.33|) for Milledgeville site data sets.
The Spearman correlation was greater than |0.33| for *vcrA* to the daughter to parent compound concentration ratio of (VC, ethene)/TCE for NAS North Island, NASA Cape Canaveral and the Milledgeville site. A statistical correlation coefficient (r) greater than $r_{critical}$ was found between the ratio of *vcrA* to the daughter to parent compound concentration ratio of (VC, ethene)/TCE for the data from the NASA Cape Canaveral site. No correlation was observed between *bvcA* and the daughter to parent compound concentration ratio of (VC, ethene)/*cis*-DCE.

For the Milledgeville site data, a Spearman correlation greater than |0.33| was observed between *bvcA* and VC, and a statistical correlation (r greater than $r_{critical}$) was obtained for this correlation. A weak correlation was observed between *bvcA* and *cis*-DCE for the Milledgeville site data. A Spearman correlation of greater than |0.33| was obtained between *vcrA* and *cis*-DCE and VC for NAS North Island OU24, NASA Cape Canaveral, and the Milledgeville site. A statistical correlation (r greater than $r_{critical}$) was obtained between *vcrA* and *cis*-DCE for the Milledgeville site data.

These results suggest that correlations between the *Dhc* RDase genes and ratios of dechlorination product/parent compound and/or the individual contaminant concentrations are inconsistent between sites. Therefore, the selection of an appropriate suite of functional gene targets will be governed by site-specific conditions and data objectives.

The *Dhc* 16S rRNA gene and the *tceA*, *bvcA* and *vcrA* genes were included in the analyses of samples from most sites. With the expected identification of additional biomarker genes for the reductive dechlorination process, the analysis of select biomarker genes that provide the key information for the contaminants of interest at a given site should be envisioned because the analysis of all possible biomarker genes may not yield additional information for decision-making.

7.2 IDENTIFICATION OF MINIMUM NUMBER OF *Dhc* TARGET GENE COPIES

An assessment of *Dhc* 16S rRNA gene and RDase target gene copies was conducted to establish minimum abundances in support of complete reductive dechlorination (e.g., ethene formation). In addition to data compiled from the study sites, information in support of this performance objective was gathered from bioremediation efforts at several additional sites, which was available to the project team.

Dhc 16S rRNA gene and/or RDase gene targets below 10^4 to 10^5 gene copies per liter have typically been associated with sub-optimal conditions to support and sustain effective reductive dechlorination rates and detoxification (Dennis, 2010, personal communication; Ritalahti et al., 2010). At sites where *Dhc* 16S rRNA and/or RDase gene targets have been detected at greater than 10^6 to 10^7 gene copies per liter, appreciable dechlorination rates and ethene formation have been reported (this study; Lu, 2006). However, the presence of a certain abundance of *Dhc* 16S rRNA gene and/or RDase gene targets is not necessarily an indicator of complete reductive dechlorination. A study by van der Zaan et al. (2009) showed that the presence of VC RDase genes did not always relate to VC dechlorination, but an order of magnitude or more increase

above baseline values in VC RDase gene abundance in response to treatment (e.g., biostimulation) correlated well with VC dechlorination activity.

Following biostimulation and/or bioaugmentation, *Dhc* 16S rRNA gene targets were detected at or above 107 gene copies per liter, and ethene production was noted at the Milledgeville, NASA MLP/VAB, and NAWC Trenton study sites. Samples collected as part of this study were grouped into four categories by *Dhc* cell abundances: greater than 10^6 cells/L, 10^3 to 10^6 cells/L, detected but not quantifiable (DNQ), and not detected (ND). All sites had wells with *Dhc* abundances in the 10^3 to 10^6 cells/L range, and three sites each had wells with *Dhc* $>10^6$ cells/L, DNQ and ND. Among the 25 wells where ethene was detected at concentrations up to 75 ppb, 21 had detectable or quantifiable *Dhc*. Six wells had $>10^6$ *Dhc* cells/L, but two of them tested negative for the known VC RDase genes *bvcA* and *vcrA* suggesting that other as yet unidentified genes encode VC RDases. The known *Dhc* are strict organohalide respiring bacteria and presumably strains carrying *vcrA* or *bvcA* are responsible for VC reductive dechlorination to ethene. High abundances of *Dhc* 16S rRNA genes significantly exceeding the number of *Dhc* cells carrying *bvcA* and *vcrA* at sites producing ethene suggest that the unknown VC RDase genes are encoded on *Dhc* genomes. In two wells, *Dhc* 16S rRNA and all three *Dhc* RDase biomarker genes were present at titers exceeding 10^6 cells/L but no ethene was detected. In one of these wells, total chlorinated ethene concentrations were in the low ppb range and ethene concentrations may have been too low for detection. At the other well, temporal concentration measurements suggested polychlorinated ethene reductive dechlorination progressed and VC was consumed but no ethene was detected.

Detoxification of VC without measureable ethene has been reported (Bradley and Chapelle, 2000). A general correlation has been found between the presence of *Dhc* and ethene generation (Hendrickson et al., 2002; Major et al., 2002; Imfeld et al., 2008; Abe et al., 2009; van der Zaan et al., 2009). Frequently, ethene formation serves as a benchmark for successful reductive dechlorination (i.e., detoxification), but recent observations suggest that the lack of ethene formation should be interpreted cautiously because implementation of the anaerobic reductive dechlorination process can achieve cleanup goals without measureable ethene.

Several processes including anaerobic VC and/or ethene oxidation may explain detoxification without ethene formation, and alternative degradation pathways should be explored (Bradley and Chapelle, 2000; Gossett, 2010). Ethene was observed in just one-third of the wells (11 out of 32) with *Dhc* abundances between 103 and 106 cells/L. Only three of the 11 ethene-producing wells had detectable *tceA*, one had *bvcA*, and *vcrA* was absent, supporting the notion that additional *Dhc* VC RDases exist. Higher ethene concentrations correlated with higher *Dhc* cell titers. The minimum number of *Dhc* cells that predict ethene production is 10^6 cells/L. Supporting this conclusion are the results of a recent study that compared 24 wells at six sites and found that active dechlorination of DCEs and VC occurred with $>10^7$ *Dhc* cells cells/L (Lu et al., 2006). In wells with $<10^4$ or DNQ *Dhc* cells L^{-1}, ethene concentrations were below 2 ppb (6 out of 7 wells) or ethene was not detected at all (18 out of 18 wells). In 11 of the 59 wells evaluated, *Dhc* were not detected, and in seven of those ethene was not detected; however, in four of the wells, ethene was observed in low concentrations (<2 ppb) even though none of the known *Dhc* RDase biomarker genes were present, and the contaminants PCE, TCE, and *cis*-DCE were not being reduced to VC.

Table 3 shows ranges of observed *Dhc* cell titers and their associated activity. These results may be used by practitioners as rules of thumb when interpreting *Dhc* data at chlorinated solvent sites.

Table 3. Observed *Dhc* and associated dechlorination activity.

***Dehalococcoides* 16S rRNA gene copies per L**	**Interpretation**
$<10^4$	Low *Dhc*, efficient dechlorination and ethene production unlikely
$10^4 – 10^6$	Moderate *Dhc*, which may or may not be associated with observable dechlorination and ethene formation
$>10^6$	High *Dhc*, which is often associated with high rates of dechlorination and ethene production

7.3 CORRELATION OF *Dhc* TARGET GENE COPY NUMBERS WITH CONTAMINANT DECHLORINATION RATES

Correlations of average *Dhc* 16S rRNA gene copy numbers and *vcrA* gene copy numbers with TCE, *cis*-DCE, or VC dechlorination rates were used to evaluate the predictive use of qPCR data on reductive dechlorination. The performance metric for this performance objective was the achievement of a positive Spearman correlation of greater than |0.33|.

The calculation of dechlorination rates was only performed for the NASA Cape Canaveral site since this site had data from multiple monitoring wells that were collected frequently (bi-monthly) over several years. Dechlorination rates were calculated assuming first-order reaction kinetics and were evaluated for TCE, *cis*-DCE, and VC, utilizing data from three site monitoring wells. Since rates could only be calculated from three monitoring wells, the data set contained less than six data points, and thorough statistical analyses were not possible.

Spearman correlations between *Dhc* 16S rRNA gene copies and *vcrA* RDase gene copies with TCE, *cis*-DCE, and VC dechlorination rates were found to be greater than |0.33|. The Spearman correlation between the *Dhc* 16S rRNA gene copies and TCE, *cis*-DCE, and VC dechlorination rates were all strong (greater than |0.67|), while the Spearman correlation between the *vcrA* gene copies and TCE, *cis*-DCE, and VC dechlorination rates were all medium (between |0.34| and |0.66|.

These results suggest a correlation between the 16S rRNA gene copies, the *vcrA* gene copies, and the observed dechlorination rates; however, only three wells were included in the analysis, which precluded a robust statistical testing. Further evaluation of the correlation between *Dhc* 16S rRNA gene copies and individual RDase (e.g., *vcrA*) gene copies with dechlorination rates is recommended to establish a metric to evaluate reductive dechlorination.

7.4 CORRELATION OF CONTAMINANT CONCENTRATIONS ON *Dhc* POPULATION SIZE

Correlations of *Dhc* 16S rRNA gene copy abundances with TCE, *cis*-DCE, or VC concentrations and with daughter-to-parent compound (e.g., [*cis*-DCE, VC]/TCE; [VC, ethene]/*cis*-DCE) concentration ratios were used to evaluate the predictive use of qPCR data on reductive

dechlorination activity. The metric used for this performance objective were positive Spearman correlation coefficients of greater than |0.33|.

Spearman correlations between the *Dhc* 16S rRNA gene copy number and a daughter-to-parent compound concentration ratio of (*cis*-DCE, VC)/TCE were found to be greater than |0.33| for the Anniston and NASA Cape Canaveral sites. This correlation was either not performed or resulted in a weak correlation for the NAS North Island OU24, NAWC Trenton, and Milledgeville sites, probably due to low concentrations of TCE present at these sites.

Spearman correlations between the *Dhc* 16S rRNA gene copy number and the daughter-to-parent compound concentration ratio of (VC, ethene)/*cis*-DCE were found to be greater than |0.33| for all sites evaluated. A statistical correlation (r greater than $r_{critical}$) was observed between the *Dhc* 16S rRNA gene copy number and the daughter to parent compound concentration ratio of (VC, ethene)/*cis*-DCE for the data from NAWC Trenton site.

Spearman correlations between the *Dhc* 16S rRNA gene copy number and contaminant concentrations (e.g., TCE, *cis*-DCE, or VC) greater than |0.33| were observed for all sites. A statistical correlation was observed between the *Dhc* 16S rRNA gene and TCE and *cis*-DCE concentrations for data from the NAWC Trenton site.

These results suggest that there is no correlation between the *Dhc* 16S rRNA gene abundance and the contaminant concentrations or the daughter-to-parent compound (e.g., [cis-DCE, VC]/TCE; [VC, ethene]/*cis*-DCE) concentration ratios. The limitation of the Spearman correlation analysis was the low number of data sets included in the analysis; however, further evaluation with additional data sets is warranted. Data should be obtained from a larger number of suitable sites to establish or reject correlations between *Dhc* 16S rRNA gene abundance data, contaminant concentrations and the daughter-to-parent compound concentration ratios as measures for reductive dechlorination performance. SiREM and Microbial Insights have compiled larger data sets from their customers' sites. Such data sets could be evaluated using the Spearman approach to corroborate correlations between *Dhc* biomarker gene abundances and dechlorination performance.

7.5 INFLUENCE OF TEAP ON *Dhc* ABUNDANCE

A qualitative evaluation of groundwater geochemistry and its influence on *Dhc* biomarker gene abundances was conducted. Biodegradation of chemical groundwater constituents have been associated with particular geochemical conditions. For example, PCE and TCE are resistant to metabolic degradation under aerobic conditions but can be reductively dechlorinated stepwise to less chlorinated ethenes under reducing conditions in the absence of oxygen. DCEs and VC can be reductively dechlorinated to ethene, and sometimes to ethane, by anaerobic microorganisms, or they can be mineralized to carbon dioxide and inorganic chloride under aerobic conditions (Coleman et al., 2002; Singh et al., 2004; Gossett, 2010).

The findings of recent studies suggested that *Dhc* strains containing *tceA* are more tolerant of oxidizing conditions, whereas *Dhc* strains containing *vcrA* or *bvcA* are more susceptible to redox fluctuations (van der Zaan et al., 2009; Amos et al., 2008; Fletcher et al., 2010). Studies by van der Zaan et al. (2009) showed a strong negative correlation between the abundance of *Dhc* 16S

rRNA genes and the *vcrA* gene to increasing sulfate concentrations, but found a positive correlation between *Dhc* 16S rRNA gene and *vcrA* gene abundances to high methane concentrations. Apparently, sulfate, or the reduced product sulfide, do not favor VC-dechlorinating *Dhc* populations whereas methanogenic conditions support VC reduction.

Field and analytical data collected for this demonstration support the findings of these investigations and indicate that lower redox conditions representative were generally favorable for reductive dechlorination of chlorinated ethenes. For example, following biostimulation and bioaugmentation at the Bachman Road demonstration site, increases in *Dhc* biomarker gene copies were noted concurrent with reductions in TCE and sulfate concentrations as well as increases in dissolved methane concentrations.

7.6 IDENTIFICATION OF FALSE POSITIVES/NEGATIVE qPCR DATA

A comparison of different membrane filter materials and DNA extraction methods showed that false negative results can be reduced through consistent and appropriate sample handling and adherence to SOPs. Adopting an on-site filtration approach combined with DNA extraction with the MO BIO Powersoil Kit reduced false negative results (Ritalahti et al., 2010). A key issue is the volume of groundwater collected for biomass collection. As a rule of thumb, reproducible results were obtained when volumes containing $>10^4$ total *Dhc* target gene copies were collected. The careful design and thorough testing of qPCR parameters and the application of a TaqMan approach (rather than SYBR Green chemistry for monitoring target gene amplification) eliminated false negative results. It is important to note that different qPCR protocols can yield accurate data, but it is crucial that each analytical laboratory establish rigorous SOPs to avoid false positive and false negative qPCR results.

7.7 IMPLEMENTABILITY OF GROUNDWATER SAMPLING AND BIOMASS COLLECTION

In initial laboratory studies with groundwater amended with known amounts of *Dhc* target cells, Sterivex cartridges yielded one-third of the total DNA and 9-18% of the total *Dhc* biomarker gene copies compared with vacuum filtration. Subsequent method optimization increased DNA yields to 94 ± 38% of those obtained with the vacuum filtration method. A comparative analysis of on-site and off-site biomass collection procedures, performed with groundwater from 59 wells at nine chlorinated ethene-contaminated sites, corroborated the applicability of the Sterivex cartridge for *Dhc* biomarker quantification in groundwater. On-site biomass collection with Sterivex cartridges avoids problems associated with shipping groundwater and has broad applicability for biomarker monitoring in aqueous samples. From most wells included in this demonstration, Sterivex cartridges and groundwater for off-site (i.e., in the analytical laboratory) biomass collection were available for direct comparison of on-site and off-site procedures.

To provide additional evidence that the Sterivex cartridges have advantages over the traditional methodology, two defined laboratory experiments were conducted with the PCE-to-ethene-dechlorinating consortia BDI and KB-1. In separate experiments, groundwater, which did not contain *Dhc* biomarkers, and artificial groundwater samples were augmented with defined amounts of consortium BDI and consortium KB-1, respectively. In the laboratory, the biomass was collected from triplicate, augmented groundwater samples. The data corroborated the

observations with the field samples, and it was concluded that the on-site Sterivex filtration approach is a viable and superior alternative for groundwater sampling and biomass collection for subsequent qPCR analysis (Ritalahti et al., 2010).

The detailed findings of the method development and application of the on-site biomass collection approach using commercial Sterivex cartridges have been published in the peer-reviewed literature (Ritalahti et al., 2010).

7.8 ANALYTICAL SENSITIVITY

The sensitivity of the PCR method for quantification of *Dhc* biomarker genes was evaluated. Like all analytical procedures, qPCR has a detection limit and a minimum number of target gene copies (i.e., template DNA) is required in the qPCR reaction tube to generate measurable fluorescence increase during the light cycler run. For detection, >5 biomarker gene copies must be distributed into each of the three replicate reaction tubes. For reliable quantification, >20 *Dhc* biomarker gene copies should be present in the reaction tube. In other words, with a 100 mL groundwater sample, the qPCR assays can enumerate *Dhc* biomarker genes at abundances >2 x 104 L^{-1}, even in samples with high bacterial background (e.g., bacterial 16S rRNA gene copy abundances of 10^{12} per L, or a seven orders of magnitude difference). Quantification uses standard curves prepared with dilutions of known amounts of plasmid DNA that contains the target genes. The dynamic range spans concentrations over several orders of magnitude and linear standard curves over eight orders of magnitude are utilized for environmental monitoring (Ritalahti et al., 2006). The quantification limits for individual genes with the TaqMan approach vary somewhat with the primers and probe combinations used, but accurate quantification is typically achieved when >100 target gene copies are present per reaction tube.

The presence of PCR inhibitors can affect *Dhc* biomarker gene detection and quantification. Consequences of the presence of inhibitors are false negative results (i.e., *Dhc* biomarker genes are present but were not detected or accurately quantified). The presence of PCR inhibitor is always a concern and substantial efforts have been devoted to remove such inhibitors during DNA extraction. Unfortunately, additional purification steps that efficiently remove inhibitors lead to reduced DNA and biomarker gene recoveries. Even more troublesome is the fact that no universal purification procedure for removal of inhibitors from all sample types exists, and DNA extraction procedures must be optimized for different samples, or the compromise is accepted with the understanding that additional analysis may be warranted for some site materials. To recognize PCR inhibition, undiluted, 1:10 and 1:100 diluted template DNA samples were assayed with qPCR. Nonexponential fluorescence signal increase, or other than a 10-fold difference in target enumeration in the dilutions of template DNA indicated inhibition, and those samples were not included in this analysis. This procedure adds to total number of qPCR assays (and hence increases cost); however, assaying template DNA dilutions reliably detected PCR inhibition and also helped identifying tubes that yielded erroneous results due to pipetting errors. Further, the results from the dilution tubes added robustness to statistical analyses and increased confidence in the qPCR data.

7.9 ANALYTICAL SAMPLE REPRODUCIBILITY

An assessment of data reproducibility was conducted to evaluate potential impacts on the outcome of the MBT results and their interpretations. A thorough comparative analysis using defined laboratory samples and site groundwater demonstrated that the Sterivex approach is suitable for reproducible collection of microbial biomass. When combined with commercially available DNA extraction kits, the DNA preparations yielded highly reproducible qPCR data for the *Dhc* biomarker genes. Analysis of replicate samples comparing on-site Sterivex filtration with off-site Sterivex filtration methods demonstrated that cartridge handling, shipping, and storage did not affect qPCR enumeration of *Dhc* biomarker genes. Differences between replicate samples analyzed in terms of DNA yields and biomarker gene quantification using the same biomass collection method were less than two-fold. All qPCR data were generated with at least two replicate DNA extractions, each analyzed for at least two dilutions in triplicate qPCR runs.

7.10 RECOMMENDATIONS FOR THE APPLICATION OF MBTS AT VINYL CHLORIDE-CONTAMINATED SITES

With the currently available knowledge about *Dhc* and *Dhc* RDase genes involved in VC reductive dechlorination, the following conclusions can be drawn.

- *vcrA* and the *bvcA* encode for RDases that dechlorinate VC to ethene. Both genes, *vcrA* and *bvcA*, have only been found on the genomes of *Dhc*, and no other microbes harboring these genes are known. Therefore, the presence and abundance of *Dhc* carrying *vcrA* or *bvcA* are linked to VC-to-ethene dechlorination.
- At some sites with VC as the major chlorinated ethene, the total number of *Dhc* cells exceeds the sum of *Dhc* cells carrying *vcrA* and *bvcA*. This finding indicates that additional, not yet identified VC RDase genes harbored on *Dhc* genomes exist. Nevertheless, in the vast majority of wells where VC dechlorination to ethene occurs, *Dhc* carrying the *vcrA* or *bvcA* genes are present.
- Data from very few sites suggest that VC-to-ethene dechlorination occurs in the presence of *Dhc* but *vcrA* or *bvcA* were not detected. These are exceptions and research teams would be very interested to receive samples from such sites.
- If *Dhc* are abundant (i.e., >10e5 cells per liter) at sites where chlorinated ethenes are the predominant contaminants, it is very likely that these *Dhc* strains are using one or more chlorinated ethene as electron acceptor.
- If VC is the predominant contaminant, and qPCR data suggest a high abundance of *Dhc* 16S rRNA genes, it is very likely that these *Dhc* strains respire VC. Correlating the abundance of *Dhc* 16S rRNA genes with the abundances of the *vcrA* and the *bvcA* gene provides additional confidence that VC-to-ethene dechlorination occurs.
- The argument can be made that the presence of the *Dhc* 16S rRNA gene alone is sufficient to infer that *Dhc* strains are responsible for VC reductive dechlorination at VC-contaminated sites, and additional analyses targeting individual RDase

genes will not provide additional information. This conclusion is based on the assumption that *Dhc* require a halogenated compound (e.g., VC) for growth. However, this assumption is only valid if VC is the only halogenated compound from which *Dhc* can derive energy. At most sites, higher chlorinated ethenes and other chlorinated compounds (i.e., co-contaminants) are present that may support a sizable *Dhc* population. Therefore, *Dhc* 16S rRNA genes, *vcrA* and *bvcA* should be quantitatively monitored.

- For site assessment and to predict if indigenous *Dhc* strains with the ability to respire VC, the *Dhc* 16S rRNA genes and both the *vcrA* and the *bvcA* should be enumerated.
- Following bioaugmentation with the consortia currently in use, *bvcA* will not be abundant at most sites; however, site monitoring, especially following the initial phase of PCE/TCE dechlorination should quantify the *Dhc* 16S rRNA genes and both the *vcrA* and the *bvcA* genes.
- Currently, only three *Dhc* biomarker genes are available for monitoring chlorinated ethenes reductive dechlorination. For the analytical laboratory, the efforts to analyze two or three target genes are not significantly different. While experts may be able to guide practitioners to reduce the number of samples tested for all three target genes, the cost savings will be marginal. Customized qPCR assays can be envisioned that target only those RDase genes that provide information that influences decision making. However, such customized assays will only make sense when a larger number of biomarker genes that inform about the process of interest are available.

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8.0 COST ASSESSMENT

8.1 SAMPLE COLLECTION AND ANALYSIS COST ASSESSMENT

The costs for a typical chlorinated solvent site involving bioremediation usually include capital costs and the subsequent monitoring costs. Use of the MBTs will result in incremental additional costs (i.e., the costs for qPCR analysis) that are small in comparison to the total project costs; however, the return on investment is significant, as reflected by the improvement in site assessment and remediation performance. These benefits could lead to shortened remediation time frames (early site closures) and reductions in the associated overall remediation costs (see Section 8.2).

Costs for the use of qPCR were tracked throughout the demonstration using a management information system, which allows detailed tracking of material, labor, travel, and subcontractor costs by major project milestones. A summary of the cost breakdown is presented in Table 4. The cost items in the table include groundwater sampling and laboratory analyses for *Dhc* biomarker genes. As shown in Table 4, the additional cost for qPCR analysis is currently $400 to $485 per sample (including sampling and labor).

Table 4. Cost summary.

Cost Category	Subcategory	Details
Start-up costs		Not applicable
Capital costs		Not applicable
Operating costs	Consumables, supplies (membrane filter, tubing, shipping)	Approximately $15 per sample[1]
	Operator labor	Approximately $75 per sample
	Equipment maintenance and calibration	Approximately $10 per sample depending on procedures[1]
	Purge water disposal	Approximately $0 to $10 per sample depending on procedures[1]
	Laboratory analysis	$300 to $375 per sample[2]
Indirect environmental costs		Not applicable
Demobilization		Not applicable

Note:
1 These costs are already incurred with traditional groundwater sampling.
2 Costs of on-site field filtering are included in the cost of sample analysis.

The Phase I sampling results suggest that cost reductions can be achieved due to savings associated with on-site biomass collection using the Sterivex cartridges. The major cost component for use of qPCR is for laboratory analysis at the current cost of $300 to $375 per sample. However, these costs are expected to decrease due to technological advances and the increasing demand for nucleic acid-based analyses (i.e., more vendors will offer these services). Nevertheless, the greatest cost savings realized by this technology are through improved decision making in remedial design and implementation of pilot test and full-scale remedies of MNA, biostimulation, and bioaugmentation.

8.2 COST MODEL

To estimate the reduction of project costs that could result from MBT use, a cost model was developed to allow estimation and comparison of the costs associated with three remediation scenarios, which achieve project objectives under different conditions.

The cost estimation was based on modified bioaugmentation implementation costs for Site 59 at NAS Cecil Field. The following assumptions were made for the cost estimate:

- Thirty monitoring wells will be installed at the site for monitoring purposes for each bioremediation scenario.
- For the scenario with MNA only, the site will be monitored for 20 years. Capital costs for this scenario include regulatory submittals, monitoring, well installation, and baseline sampling and analytical analyses. Monitoring will be conducted quarterly for the first year, semi-annually for the second and third years, and annually from the fourth year forward. Annual operating costs include site visits and documentation, sampling, analytical work (VOCs and other geochemical parameters, and qPCR), and reporting. The costs for 5-year reviews are also included.
- For the biostimulation scenario, two rounds of emulsified vegetable oil (EVO) injections will be conducted in 5 years with the second injection conducted in the third year. The site will be monitored for 5 years. Capital costs for the first injection include regulatory submittals, monitoring well installation, baseline sampling and analytical analyses, and EVO injection via direct push technology. Capital costs for the second injection are assumed to be 10% of that for the first injection. Monitoring will be conducted quarterly during the first year, semi-annually in the second and the third years, and annually from the fourth year forward. Annual operating costs include site visit and documentation, sampling, analytical work (VOCs and geochemical parameters, and qPCR), and reporting. The costs for one 5-year review are also included.
- For the bioaugmentation scenario, one round of EVO and injection of a suitable consortium (e.g., KB-1) will be conducted. The site will be monitored for 2 years. Capital costs include regulatory submittals, monitoring well installation, baseline sampling and chemical analyses, qPCR analysis, and EVO and KB-1 injection via direct push technology. Monitoring will be conducted quarterly during the first year and semi-annually during the second year. The annual operating costs

include site visit and documentation, sampling, analytical work (VOCs, geochemical parameters, and qPCR), and reporting.

- MBT analysis indicates that bioaugmentation is required.

A summary of the cost comparisons for the three scenarios is shown in Table 5. Results of the described estimates suggest that the costs for the MNA scenario are the highest and the costs for implementing bioaugmentation treatment with the use of MBTs are the lowest. The qPCR results show that assisted bioaugmentation can save approximately 15% of the costs in comparison to MNA. Greater cost savings are possible depending on specific site conditions. More benefits of using MBTs are realized through much shorter site longevity and the associated liability issues because early site closures can likely be realized. The developed cost model can assist site managers and other users in decision making processes.

Table 5. Summary of project cost comparison for three remediation scenarios.

Scenario	Estimated Remediation Time Frame (years)	Remediation Specifics	Capital Costs	Long-Term Monitoring and Management Costs	Total Projects Costs
MNA only	20	No active remediation	$414,067	$509,150	$923,217
Biostimulation only	5	Two rounds of EVO injections	$567,339	$327,879	$895,219
Bioaugmentation	2	One round of EVO and KB-1 injection	$609,793	$177,404	$787,197

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9.0 IMPLEMENTATION ISSUES

As a result of this work, the performers have published a peer-reviewed manuscript, three book chapters and a guidance protocol that will aid the future implementation of MBTs at chlorinated solvent sites. In addition, this work has been presented at DoD training sessions and scientific conferences to inform end users of this technology.

No specific regulations pertain to the use of MBTs at chlorinated solvent sites. However, as members of the Interstate Technology & Regulatory Council (ITRC) Environmental Molecular Diagnostics Committee, project team members are drafting guidance and developing training materials to support the use of MBTs.

Sampling supplies are available commercially. MBT analyses are available from commercial laboratories (e.g., SiREM Laboratory, Microbial Insights). Use of Sterivex cartridges eliminates the need for packaging and shipping groundwater. Investigation-derived wastes must be properly disposed, as for all sampling activities at impacted sites. Therefore, avoiding the shipment of groundwater is a major benefit of on-site biomass collection with the Sterivex cartridges.

A Guidance Protocol entitled Use of Nucleic Acid-Based Tools for Site Assessment and Monitoring Bioremediation at Chlorinated Solvent Sites has been drafted as a result of this project. Site RPMs and contractors across DoD will be able to use the Guidance Protocol for implementing engineered bioremediation and to support decision making regarding MNA and enhanced bioremediation. With the increased knowledge and understanding of the reductive dechlorination process, along with improved and rigorously tested assessment and monitoring tools, as well as appropriate guidance documents, site managers and regulators will have the means to convincingly argue that MNA and/or enhanced treatment are viable, cost-effective approaches for source zone remediation and plume control to achieve long-lasting risk reduction.

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