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Validation of Advanced Molecular Biological Tools to Monitor Chlorinated Solvent Bioremediation and Estimate cVOC Degradation Rates

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This demonstration had three specific objectives. The first objective was to demonstrate the utility of quantitative proteomics (qProt) to measure the absolute abundance of Dhc reductive dechlorination biomarker proteins in laboratory-controlled microcosms with various Dhc cell titers. Contaminant concentration and ethene measurements over time were used to determine cis-DCE and VC reductive dechlorination rates. The second objective was to correlate observed degradation rates with Dhc biomarker gene and protein abundances. The successful completion of objectives 1 and 2 lead to a go/no-go decision point before conducting demonstration/validation efforts of the qProt approach at military sites impacted with chlorinated ethenes.

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TABLE OF CONTENTS

EXECUTIVE SUMMARY	vi
1.0 INTRODUCTION.....	1
1.1 BACKGROUND	1
1.2 OBJECTIVES OF THE LABORATORY DEMONSTRATION.....	2
1.3 REGULATORY DRIVERS	2
2.0 TECHNOLOGY	3
2.1 TECHNOLOGY DESCRIPTION	3
2.2 TECHNOLOGY DEVELOPMENT.....	5
2.3 ADVANTAGES AND LIMITATIONS OF THE TECHNOLOGY	9
3.0 PERFORMANCE OBJECTIVES.....	11
4.0 SITE DESCRIPTION.....	14
4.1 NBK Keyport Area 1, Bremerton, WASHINGTON	14
4.2 VAFB SITE SA 288, VANDENBERG, CALIFORNIA	16
4.3 JBLM LANDFILL 2, TACOMA, WASHINGTON.....	19
5.0 TEST DESIGN.....	21
5.1 CONCEPTUAL EXPERIMENTAL DESIGN	21
5.2 BASELINE CHARACTERIZATION ACTIVITIES	22
5.3 DEVELOPMENT OF RDASE PEPTIDE TARGETS	22
5.4 ESTABLISHING MDL AND IDL FOR TARGET PEPTIDES	23
5.5 VALIDATION OF QPROT ASSAY QUANTITATION LIMITS	25
5.6 LABORATORY MICROCOSM TESTING	26
5.6.1 Growth of the SDC-9 Inoculum	26
5.6.2 Microcosm Preparation and Treatments.....	26
5.6.3 Microcosm Sampling Procedure	27
5.7 SAMPLING & ANALYSIS METHODS	30
5.7.1 Analytical Methods: Standard Geochemical cVOC Analyses	30
5.7.2 Analytical Methods: Proteomics	31
5.7.3 Analytical Methods: Quantitative PCR.....	32
5.8 DATA ANALYSIS.....	34
6.0 PERFORMANCE ASSESSMENT.....	36
6.1 DETERMINATION OF RDASE TARGETS WITH SHOTGUN PROTEOMICS.....	36
6.2 METHOD DETECTION AND INSTRUMENT DETECTION LIMIT RESULTS	37
6.2 VALIDATION OF MRM ASSAY QUANTITATION LIMITS RESULTS	38
6.3 MICROCOSM STUDY RESULTS.....	40
6.4 BIOMARKERS AS PREDICTORS OF DECHLORINATION RATES.....	43
6.5 APPLICABILITY OF LABORATORY STUDY RESULTS TO FIELD SITES.....	45
7.0 COST ASSESSMENT	48
7.1 COST MODEL	48
7.2 COST DRIVERS	50
7.3 COST ANALYSIS.....	50
8.0 IMPLEMENTATION ISSUES.....	52
8.1 REGULATORY ACCEPTANCE	52
8.2 LIMITED AVAILABILITY.....	52
8.3 COST COMPARED TO OTHER MONITORING TOOLS.....	53
9.0 REFERENCES.....	54

LIST OF TABLES

TABLE 1-1. SAFE DRINKING WATER ACT MAXIMUM CONTAMINANT LEVELS (MCLs) FOR KEY cVOCs	2
TABLE 2-1. RDASE GENES IDENTIFIED IN SDC-9 METAGENOME	7
TABLE 3-1. DEMONSTRATION PERFORMANCE OBJECTIVES FOR THE MICROCOSM STUDY	11
TABLE 5-1. PARAMETERS OF WATERS XEVO CE EQUATION	24
TABLE 5-2. NUMBER OF MICROCOSM TEST SAMPLES BY ANALYSIS	30
TABLE 5-3. SUMMARY OF STANDARD ANALYTICAL AND RDASE BIOMARKER METHODS.....	30
TABLE 5-4. SUMMARY OF SPECIFIC QPCR ASSAYS RUN FOR MICROCOSM SAMPLES	33
TABLE 6-1. PRM TRANSITIONS OF SELECTED RDASE SDC-9 ENDOGENOUS PEPTIDES	36
TABLE 6-2. MDL FOR PEPTIDES ANALYZED FOR SDC-9 CULTURE	38
TABLE 6-3. RESULTS OF QPROT ASSAY QUANTITATION LIMIT VALIDATION STUDY	39
TABLE 6-4. SUMMARY OF FITTED K_{cisDCE} AND K_{vc} BY MICROCOSM TEST	42
TABLE 6-5. RATE COEFFICIENTS AND BIOMARKER CORRELATIONS	43
TABLE 7-1. COST MODEL FOR PROTEOMICS.....	49
TABLE 7-2. COST COMPARISON OF CONVENTIONAL MBTs (E.G., QPCR) TO THE ADVANCED MBTs.	51

LIST OF FIGURES

FIGURE 2-1. SCHEMATIC OF PROTEOMICS WORKFLOW	4
FIGURE 2-2. RDASE PEPTIDE CONCENTRATIONS VERSUS DEGRADATION RATE CONSTANTS	8
FIGURE 4-1. NBK KEYPORT AREA 1, SITE MAP	15
FIGURE 4-2. VAFB SA288 SITE MAP SHOWING GROUNDWATER cVOC CONCENTRATIONS.....	18
FIGURE 4-3. JBLM LANDFILL 2 TCE CONCENTRATIONS.....	20
FIGURE 5-1. MICROCOSM STUDY CONCEPTUAL DESIGN.....	21
FIGURE 5-2. PHOTOGRAPH OF MICROCOSMS.....	28
FIGURE 5-3. STEPS INVOLVED IN PROTEOMIC ANALYSIS OF MICROCOSM TEST SAMPLES	31
FIGURE 6-1. CONCENTRATIONS OF VOCs, VFAs AND BROMIDE IN MICROCOSMS.	40
FIGURE 6-2. MEASURED AND MODEL-FITTED cVOC CONCENTRATIONS IN MICROCOSMS	41
FIGURE 6-3. RATE COEFFICIENTS VS. BIOMARKER REGRESSION RESULTS.....	44
FIGURE 6-4. BIOMARKER-BASED RATE PREDICTIONS VS. MEASURED RATE COEFFICIENTS	45
FIGURE 6-5. RATE COEFFICIENTS VS. BIOMARKER ABUNDANCES WITH TREATMENTS DISTINGUISHED.	46
FIGURE 6-6. PROTEIN/DHC CELL RATIOS VS. RATE COEFFICIENTS FOR MICROCOSM TESTS.....	47

LIST OF APPENDICES

APPENDIX A – MDL/IDL STUDY RESULTS FOR PROTEOMICS
APPENDIX B – DILUTION STUDY
APPENDIX C – ANALYTICAL SOPS
APPENDIX D – MICROCOSM ANALYTICAL AND BIOMARKER ABUNDANCE DATA
APPENDIX E – KEY POINTS OF CONTACT

LIST OF ACRONYMS

CE	collision energies
CID MS/MS	collision induced dissociation tandem mass spectrometry
<i>cis</i> -DCE	<i>cis</i> -1,2-dichloroethene
CO ₂	carbon dioxide
CTC	cost to complete
cVOC	chlorinated volatile organic compound
<i>Dhc</i>	<i>Dehalococcoides mccartyi</i>
DoD	Department of Defense
EISB	enhanced <i>in situ</i> bioremediation
EPA	Environmental Protection Agency
ESTCP	Environmental Security and Technology Certification Program
IDA	information dependent acquisitions
IDL	instrument detection limit
JBLM	Joint Base Lewis-McChord
LC	liquid chromatography
LC-MS/MS	liquid chromatography tandem mass spectrometry
LOD	level of detection
LOQ	level of quantitation
MBT	molecular biological tool
MCL	maximum contaminant level
MDL	method detection limit
MNA	monitored natural attenuation
MRM	multiple reaction monitoring
MS	mass spectrometry
NBK	Naval Base Kitsap
O&M	operation and maintenance
PRM	parallel reaction monitoring
PCE	tetrachloroethene
PI	Principle Investigator
QA	quality assurance
QC	quality control
qPCR	quantitative polymerase chain reaction
qProt	quantitative proteomics
QTOF-MS	quadrupole time-of-flight tandem mass spectrometer
RDase	reductive dehalogenase
ROD	Record of Decision
RPD	relative percent difference
RPM	remediation project manager

LIST OF ACRONYMS (continued)

RT-qPCR	reverse transcriptase qPCR
TCE	trichloroethene
<i>trans</i> -DCE	<i>trans</i> -1,2-dichloroethene
USACE	United States Army Corps of Engineers
VAFB	Vandenberg Air Force Base
VC	vinyl chloride

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Researchers for this project included Dr. Mandy Michalsen (Principal Investigator, U.S. Army Engineer Research Development Center), Dr. Kate Kucharzyk, Dr. Craig Bartling and Dr. Jayda Meisel (Battelle Memorial Institute), Dr. Paul Hatzinger (Aptim), Dr. John Wilson (Scissortail Environmental Solutions, LLC), Dr. Jonathan Istok (Oregon State University), Fadime Kara Murdoch (University of Tennessee, Knoxville) and Dr. Frank Löffler (University of Tennessee, Knoxville and Oak Ridge National Laboratory, Oak Ridge, TN). Several personnel at Battelle Memorial Institute, including Larry Mullins, Amy Hill and Angela Minard-Smith, were instrumental in assisting with metagenomic and metaproteomic data analysis and interpretation. Dr. Fadime Kara Murdoch from the University of Tennessee, Knoxville participated in analysis and interpretation of qPCR and transcript-related data. Other site personnel that provided significant project support included Charles Condee, Anthony Soto, Sheryl Streger, and Simon Vainberg from Aptim.

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EXECUTIVE SUMMARY

Introduction. Knowledge about the rates of *in situ* contaminant degradation is crucial for optimizing remedial design and supporting site management decisions. Despite progress understanding the factors influencing microbial degradation of chlorinated ethenes, determining rates of microbial contaminant degradation at field sites remains challenging. Molecular biological tool (MBTs) for quantifying *Dehalococcoides mccartyi* (*Dhc*) nucleic biomarkers are available and guide site management decision making; however, these measurements have not been useful to generate good estimates of contaminant degradation rates. Quantification of reductive dehalogenases (RDases) may provide a more direct measure of activity (as these are the actual enzymes/proteins that catalyze biodegradation of chlorinated ethenes), and technological advances in mass spectrometry instrumentation allow the sensitive, quantitative determination of RDase proteins of interest in groundwater. This project explores if RDase gene and protein biomarker abundances, alone or in combination, may be used to estimate degradation rates.

Objectives. This demonstration had three specific objectives. The first objective was to demonstrate the utility of quantitative proteomics (qProt) to measure the absolute abundance of *Dhc* reductive dechlorination biomarker proteins in laboratory-controlled microcosms with various *Dhc* cell titers. Contaminant concentration and ethene measurements over time were used to determine *cis*-DCE and VC reductive dechlorination rates. The second objective was to correlate observed degradation rates with *Dhc* biomarker gene and protein abundances. The successful completion of objectives 1 and 2 lead to a go/no-go decision point before conducting demonstration/validation efforts of the qProt approach at military sites impacted with chlorinated ethenes.

Technology Description. The sensitive and quantitative measurement of proteins in environmental matrices is now possible, and process-specific biomarker proteins such as the *Dhc* RDases TceA, BvcA and VcrA can be measured in groundwater samples. Since the abundances of the catalysts (i.e., the specific RDase enzymes) control the rate of *cis*-DCE and VC reductive dechlorination, the quantitative measurement of these catalysts may be useful for estimating *in situ* degradation rates. Accurate assessment of *in situ* degradation rates often requires *in situ* test design, execution and appropriate data interpretation, which can be costly and time consuming to complete. Demonstration/validation of this qProt tool has significant potential to establish (1) the predictive link between *in situ* RDase enzyme abundances and corresponding *in situ* reductive dechlorination rates at multiple DoD field sites, (2) a framework remediation project managers (RPMs) may use to convert RDase enzyme abundances directly into a rate estimates, and (3) enhanced/expedited site management decisions that can result in substantial cost savings to the DoD and even early site closure.

Performance Assessment. The quantitative and qualitative performance metrics were met through demonstration in defined laboratory microcosm systems prepared using DoD site aquifer materials and the development of a model that predicts cVOC degradation rates based on RDase biomarker abundances. Bioaugmentation with the SDC-9 consortium was used to obtain the desired range of *Dhc* cell abundances and reductive dechlorination rates. Correlation and regression analyses results confirmed that RDase biomarker abundances were significantly and positively correlated with rate coefficients. Regression analysis results were used to test the rate-

predictive power of the RDase biomarker abundances. RDase proteins predicted rate constants k_{cisDCE} and k_{VC} values within one order of magnitude; using RDase proteins and genes combined further improved predictions.

Cost Assessment. Implementation of advanced molecular biological tools (MBTs) such as metagenome sequencing or proteomics, during the long-term monitoring and assessment phase of the project are impacted by multitude of factors such as: the size of the site, proximity of the site to nearby receptors, regulatory requirements, and nature and diversity of contaminant of concern. Although there are currently no regulatory requirements that specifically mandate advanced MBTs be used to assess a site, the data provided by the MBTs are meant to supplement and possibly replace other forms of data that provide lines of evidence that monitored natural attenuation (MNA) is occurring and to estimate a removal rate. Hence, the total sampling and analytical cost is driven by number of sample locations at a site and total number of samples collected (i.e., a greater number of samples equates to a higher cost). It should be noted however that the individual cost per sample may decrease based on a greater number of total samples requiring analyses since the lab work is highly specialized and cost efficiencies generally can be realized for a larger quantity of analyses.

Many of the advanced MBTs such as qProt have only limited commercial availability and/or are available through a university or other research laboratory. As such, application costs remain relatively high. It is expected as these techniques mature, they will become more widely available and the analytical cost per sample will decrease substantially. For comparison purposes, the cost of the metagenomics and metaproteomic analyses based on cost data collected during the commencement of ER-201726 in 2017 were \$300 and \$1,500 per sample, respectively, assuming analysis of a batch of 10 samples. These costs decreased to \$150 and \$1,000 (for cVOCs) when evaluated in 2019. These costs are anticipated to decrease further as the technologies mature.

Implementation Issues. The primary end users of qProt are expected to be DoD site managers, consultants and their contractors. The general concerns of these end users are likely to include the following: (1) regulatory acceptance; (2) insufficient confidence in results and access to specialized laboratories; and (3) technology cost compared to other more conventional monitoring options. Proteomics is a new tool in environmental assessment and one which requires further validation. It is anticipated that, as for many technologies such as qPCR, regulatory acceptance will occur as the technology is field-validated, its benefits over existing approaches (e.g., ability to predict cVOC degradation rates) are realized, and the regulatory community is educated regarding its field application. As noted in the previous section, the issues of limited commercial availability of the technique and relatively high cost are also likely to be improve over time (i.e., more availability and lower cost) as the qProt technology matures.

1.0 INTRODUCTION

1.1 BACKGROUND

The Department of Defense (DoD) is responsible for over 26,000 contaminated groundwater sites with cost to complete (CTC) values estimated at \$12.8 billion (in 2010 dollars) [1]. A majority of these sites are contaminated with chlorinated volatile organic compounds (cVOCs). With over 25% of the remedies in place using enhanced *in situ* bioremediation (EISB) and over 50% of remedies using monitored natural attenuation (MNA) either as a sole remedy or as a final phase after EISB and/or other treatment approaches, a significant portion of the CTC dollars will be spent on EISB and MNA remedy monitoring. For both EISB and MNA, monitoring of a wide range of chemical, geochemical, and microbial parameters is required to demonstrate that biodegradation of cVOCs is occurring and/or progressing as expected. What is currently missing is a monitoring technology that could directly confirm active contaminant degradation and provide *in situ* degradation rate estimates. A direct measure of reductive dechlorination activity and information about degradation rates would be marked improvements for supporting both EISB and MNA approaches for site remediation. Such advances in monitoring strategies are needed to optimize remedy implementation and monitoring, and to develop predictive understanding about the trajectory of a contaminant plume, which will ultimately accelerate site closures.

In groundwater contaminated with chlorinated ethenes, the dominant and productive biodegradation mechanism is typically reductive dechlorination, whereby the parent tetrachloroethene (PCE) and/or trichloroethene (TCE) are sequentially dehalogenated to *cis*-1,2-dichloroethene (*cis*-DCE), vinyl chloride (VC) and finally ethene and/or ethane, which are considered environmentally benign [2]. A number of different dehalogenating bacteria catalyze one or more steps of this process, with *Dehalococcoides mccartyi* (*Dhc*) being the only microbial group known to complete the entire pathway [3]. Assessment of dehalogenating populations at a site is usually based on the enumeration of 16S rRNA genes using quantitative polymerase chain reaction (qPCR) [4]. A number of qPCR assays have been designed to enumerate specific reductive dehalogenase (RDase) genes such as the *Dhc* TCE RDase gene *tceA* and the VC RDase genes *bvcA* and *vcrA* [5-8]. In addition, specific qPCR assays are available to enumerate the 16S rRNA genes of *Dhc* and other dechlorinators. While the number of copies of 16S rRNA genes and RDases can provide useful abundance information, these measures do not necessarily correlate with dechlorination activity. In light of this limitation and in an effort to provide a more robust and specific measurement that directly correlates to degradation rates, a proteomic approach that quantifies specific RDase proteins has been developed. In general, the rate of an enzymatic reaction depends on the concentration of the substrate(s) and enzyme(s) involved; thus, the abundance of an RDase is directly proportional to the rate of dechlorination of the enzyme's substrate (e.g., VC). Such targeted measurements of specific proteins are made possible through technological advances in mass spectrometry and knowledge about keystone RDases involved in the detoxification of chlorinated ethenes. The overarching goal of this project was to validate the utility of quantitative proteomics (qProt), and to demonstrate that the integrated, quantitative analysis of biomarker genes and proteins provides estimates of cVOC degradation rates.

1.2 OBJECTIVES OF THE LABORATORY DEMONSTRATION

The value of molecular biological tools (MBTs) has been demonstrated; however, current tools fall short of providing information about contaminant degradation rates. The overarching goal of this demonstration was to validate a platform combining mature qPCR technology with targeted qProt measurements to generate rate estimates and enhance site-specific bioremediation decision making. The specific objectives were to: (1) demonstrate that proteomics can substantially increase the value of currently accepted MBTs for cVOC biodegradation monitoring, and (2) demonstrate the utility of integrated quantitative nucleic acid- and protein-based biomarker analysis applications to estimate cVOC degradation rates. The ultimate demonstration/validation approach for this technology will be to quantify the predictive relationship between RDase proteins and reductive dechlorination rates at multiple field sites. However, because this qProt technology has not yet been demonstrated for this purpose, the initial demonstration was performed in defined laboratory microcosms established with aquifer materials collected from military sites. A validated approach to assess *in situ* contaminant degradation rates that provides predictive understanding of the longevity of a contaminant plume would be a major advance over the current state-of-the art. The extrapolation of meaningful rate information from MBT data promises more efficient (i.e., lower costs and reduced environmental impact) implementation of EISB, as well as the more frequent implementation of MNA, which will accelerate site closures with substantial cost-savings realized for the DoD.

1.3 REGULATORY DRIVERS

Federal Safe Drinking Water Act Maximum Contaminant Levels (MCLs) for common cVOCs are summarized in Table 1-1. Persistence of cVOCs in groundwater, their prevalence at DoD hazardous waste sites, and their concentrations far in excess of health-based levels drive the need for cost-effective remediation technologies. DoD field sites featured in this demonstration (Section 4.0) all have MCL-based groundwater cleanup objectives.

Table 1-1. Safe Drinking Water Act Maximum Contaminant Levels (MCLs) for Key cVOCs

Compound	MCLs, µg/L*
Tetraloroethene (PCE)	5
Trichlorethene (TCE)	5
<i>cis</i> -Dichloroethene (<i>cis</i> -DCE)	70
<i>trans</i> -Dichloroethene (<i>trans</i> -DCE)	100
Vinyl Chloride (VC)	2
*40 CFR 141.61	

2.0 TECHNOLOGY

2.1 TECHNOLOGY DESCRIPTION

Conventional Molecular Biological Tools (MBTs). The use of MBTs for detection and quantification of biomarkers, especially genes and transcripts, in environmental samples has been rapidly increasing over the last decade. MBTs are used by remediation professionals to aid remedial design, assess remedial performance, and perform long-term monitoring of biodegradation. The goal of MBT application is to measure the abundance of microorganisms of interest and their activities over temporal and spatial scales.

The most widely used MBT for environmental applications is qPCR, which allows absolute abundance measurements of genes or transcripts of interest within a sample. In the case of reductive dechlorination, qPCR assays that specifically quantify 16S rRNA and RDase genes are employed. The nucleic acid-based biomarkers for detoxification at sites impacted with chlorinated ethenes are the *vcrA* and the *bvcA* genes, which both encode VC RDases, as well as *pceA* and *tceA*, which encode PCE RDases, and TCE/DCE RDases, respectively.

The key organisms (i.e., *Dhc*) that aid in detoxification of chlorinated solvents as well as their relevant RDase genes involved in the reductive dechlorination of chlorinated ethenes have been identified, [6, 7, 9] and sensitive qPCR assays for detection and quantification of key biomarker genes have been developed and tested in multiple laboratories [3, 10, 11]. Further, refined protocols for extraction of nucleic acids from groundwater samples are available [8, 12-15]. Thus, qPCR tools that enumerate *Dhc* 16S rRNA genes and RDase genes can provide information about specific cVOC dechlorination steps [3, 6, 10, 16].

To date, efforts have been made to correlate cVOC degradation rates to *Dhc* and/or RDase gene or transcript abundance. The application of Monod-based equations showed that cVOC degradation kinetics can be roughly correlated to *Dhc* cell abundances, as determined with qPCR; however, there were large differences in activity per cell based on qPCR data collected from batch versus column studies [17]. Importantly, these correlations are based on qPCR assays that quantify all *Dhc*-like sequences, not just those responsible for cVOC degradation (i.e., those encoding and expressing RDase genes). In other words, the gene-centric qPCR approach also measures *Dhc* cells that are not contributing to the dechlorination of the target contaminant(s).

Advanced MBTs – Quantitative Proteomics (qProt). In contrast to nucleic acid based MBTs, quantitative proteomics (qProt) involves the identification and quantification of proteins (i.e., enzymes) within a sample. That is, nucleic acid-based tools generate information about potential activity, whereas protein-based measurements generate information about *actual* (i.e., functional) activity.

In general, a shotgun proteomic workflow for protein identification includes protein extraction, digestion with a protease (typically trypsin) to create tryptic peptides, and liquid chromatography tandem mass spectrometry (LC-MS/MS) for peptide separation and generation of mass-resolved spectra (Figure 2-1). Peptide identification involves querying the resulting spectra against a representative protein sequence database using search engines such as Mascot or ProteinPilot [18]. Ideally, these sequence databases are specific to the analyzed samples such that the highest

numbers of proteins can be accurately identified. Once identified using shotgun proteomics, peptides from proteins of interest (e.g., RDases) can be confirmed and quantified through the use of commercially available isotopically labeled peptides of the same sequence using multiple reaction monitoring (MRM) mass spectrometry.

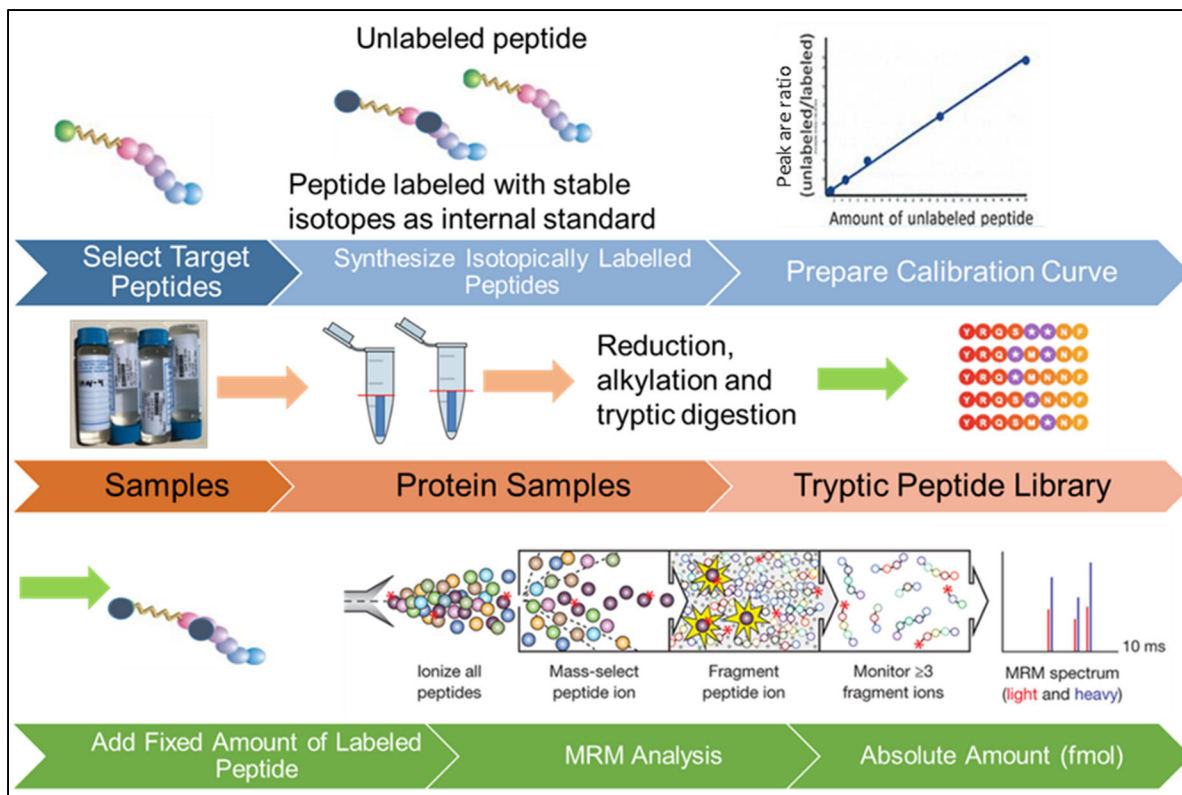


Figure 2-1. Schematic of proteomics workflow

The MRM proteomic analysis allows multiplexing of protein assay and generation of highly accurate results with near absolute specificity. This proteomic strategy is a proven and widely accepted technique for quantification of proteins [18] and has been used for decades in various matrices such as serum [19] and recently in environmental samples of groundwater and sediments [20-23]. Quantitative MRM proteomic techniques rely on targeting specific precursor peptide ions and the resulting fragment ions produced from these precursors during the analysis. Moreover, the LC-MS/MS settings can be optimized to maximize the number of precursor ions that are fragmented and scanned. Thus, in the MRM assay, specific transitions (precursor → fragment ions) for individual peptides are targeted and monitored as a function of LC retention time, which provides a highly selective, sensitive and reliable approach for quantitative analysis through integration of reproducible chromatographic peaks (Figure 2-1). With MRM, a suitable instrument such as triple-quadrupole mass spectrometer can be a priori configured to scan for a defined set of target peptides, and a selected subset of fragment ions.

Specific to this demonstration, MRM proteomic techniques have been recently used in microbial cultures to identify and quantify RDases from dechlorinators [24]. Thus, proteomics shows high potential for absolute quantification of RDases within a sample that contains mixed microbial communities. However, proteomics has not yet been exploited for the purpose of correlating cVOC

degradation rate to RDase absolute abundance. To this end, optimized protocols exist to extract proteins from biomass associated with aquifer solids or groundwater and detect and quantify key cVOC RDases with LC-MS/MS approaches [21-23].

MBTs in Assessment of cVOC Degradation Rates. Conventional nucleic-acid based MBTs can provide evidence for biodegradation, but do not aid site remediation project managers (RPMs) in prediction of contaminant longevity due to the lack of linkage to actual degradation rates. While models that include a microbial biomass, based on qPCR or total protein measurements exist [2, 17, 25, 26], their predictive power is limited. For example, batch culture/microcosm studies used biomass measurements to model cVOC degradation rates, but such models have a number of limitations and their application in support of *in situ* remediation decision making remains challenging. This limitation is due to the fact that the specific components of the microbial biomass responsible for the cVOC degradation (i.e., the RDase proteins) are not currently measured. Thus, while nucleic acid based MBTs or total biomass measurements are widely used and represent mature technologies, they may have limited value for inferring degradation rates unless combined with a more direct measure of activity (i.e., that provided by qProt). More specifically, nucleic acid-based MTBs provide a sensitive and routine means to detect and quantify DNA and transcripts of RDases, but without proteomic-based measurements, a defined correlation to cVOC degradation rate is difficult to achieve with environmental samples.

2.2 TECHNOLOGY DEVELOPMENT

At sites contaminated with chlorinated ethenes, biostimulation of indigenous dechlorinating bacteria or bioaugmentation with dechlorinating microbial consortia can achieve detoxification and environmental restoration. Contemporary bioremediation performance monitoring tools rely on nucleic acid biomarkers targeting key organohalide-respiring bacteria such as *Dhc*. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) allows the selective quantification of *Dhc* reductive dehalogenase (RDase) proteins that catalyze reductive dechlorination of chlorinated ethenes. This work applied LC-MS/MS to detect and quantify RDase peptides in the commercial bioaugmentation consortium SDC-9 comprising *Dhc* strains capable of reductive dechlorination of chlorinated ethenes and vinyl chloride to non-toxic ethene. Metagenome sequencing of the SDC-9 consortium provided a reference database for the accurate identification of target RDase peptide sequences. Shotgun proteomics workflow identified 143 RDase peptides and proteome characterization resulted in 36 distinct peptides corresponding to PceA, TceA and VcrA proteins that covered 99-100% of the annotated protein-coding sequences. From the 14 annotated RDase genes, two distinct *pceA* genes, one *vcrA* and one *tceA* gene were identified. Twelve of the 14 RDase genes were associated with RDase B. Quantification using parallel reaction monitoring (PRM) assays with ¹³C-labeled peptides determined 1.8×10^3 for TceA, and 1.2×10^2 VcrA molecules per *Dhc* cell. This approach allowed for sensitive detection and accurate quantification of relevant *Dhc* RDases and has potential utility in bioremediation monitoring regimes.

qProt has now reached a maturity level that justifies its inclusion in environmental monitoring regimes. The combined gene-, transcript-, and protein-centric approach could reveal gene presence (functional potential), transcript abundance (gene activity), and protein abundance (actual catalytic activity). The integrated analysis of these biomarkers, together with geochemical parameters, can

be used to estimate degradation rates of specific contaminants of interest (e.g., cVOCs). In an effort to move this approach into field practice, the project team completed the following tasks to generate initial qProt data and to illustrate the reductive dechlorination rate-predictive potential in the ER-201726 project proposal.

1. Metagenomic sequencing and bioinformatics analysis enabled identification of a total of 14 RDase gene sequences in the SDC-9 consortium (Table 2-1).
2. The proteomic analysis revealed more than 14 unique RDase peptides as well as peptides from accessory proteins potentially involved in transferring electrons during the reductive dechlorination process (Table 2-1).
3. RDase peptides were identified and quantified in microcosm experiments using qProt procedures.
4. Utility of the MRM proteomics approach for quantifying RDase proteins was demonstrated in microcosm studies with the commercially available cVOC biodegradation consortium SDC-9 [27].

Results of the SDC-9 metagenomic sequencing suggested that the RDase peptides were derived from three RDase proteins (highlighted in grey, Table 2-1). Results also demonstrated that only three of the 14 RDases were identified to be expressed and presumably active, even though all 14 corresponding genes would probably be detected and enumerated with qPCR.

Table 2-1. RDase genes identified in SDC-9 metagenome

RDaseA gene locus ^a	RDaseB gene locus	Number of transmembrane helices in RDaseB	Putative taxonomy	TAT signal ^b	Percent amino acid identity	Accession number of best NCBI alignment	Predicted gene
scaffold-6337_195	scaffold-6337_193	3	<i>Dehalococcoides</i>	Yes	99%	WP_081042195.1	ND
scaffold-6337_194	scaffold-6337_193	3	<i>Dehalococcoides</i>	Yes	100%	WP_081042194.1	ND
scaffold-352_158	ND	3	<i>Dehalococcoides</i>	Yes	100%	BAZ97963.1	ND
scaffold-6337_252	scaffold-6337_251	3	<i>Dehalococcoides</i>	Yes	100%	WP_010935983.1	ND
scaffold-352_212	scaffold-352_213	3	<i>Dehalococcoides</i>	Yes	99%	AEI59454.1	<i>vcrA</i>
scaffold-178_59	scaffold-178_58	3	<i>Dehalococcoides</i>	Yes	99%	WP_062900263.1	<i>tceA</i>
scaffold-3176_24	scaffold-3176_25	3	<i>Dehalobacter</i>	Yes	94%	CAD28790.2	<i>pceA</i>
scaffold-6337_160	ND	3	<i>Dehalococcoides</i>	Yes	100%	BAZ97963.1	ND
scaffold-133_66	scaffold-133_67	3	<i>Dehalobacter</i>	Yes	40%	WP_015043198.1	ND
scaffold-2271_52	scaffold-2271_51	3	<i>Dehalococcoides</i>	Yes	100%	WP_010935983.1	ND
scaffold-352_192	scaffold-352_191	3	<i>Dehalococcoides</i>	Yes	100%	WP_081042194.1	ND
scaffold-3175_18	scaffold-3175_19	3	<i>Desulfitobacterium</i>	Yes	100%	CDX01551.1	ND
scaffold-3176_29	scaffold-3176_30	3	<i>Dehalobacter/Desulfitobacterium</i>	Yes	82%	WP_025206074.1/CDX02974.1	<i>pceA</i>
scaffold-352_193	scaffold-352_191	3	<i>Dehalococcoides</i>	Yes	99%	WP_081042195.1	ND

^aThe amino acid sequence encoded by scaffold-133_66 possessed a query coverage of 99% against reference sequence WP_015043198.1. All other amino acid sequences from RDase loci reported had query coverages of 100%.

^bNo SEC signal peptides were detected in any RDase amino acid sequences examined.

ND – not determined

To date, several RDase peptides have shown good calibration linearity ($R^2 = 0.9$) and a broad dynamic range, allowing quantification of RDases from sample extracts and a comparison of their absolute abundances to dechlorination activity. In fact, quantitative analysis of two RDase peptides (TceA and PceA) in the initial proof-of-concept microcosm experiments performed using SDC-9 cell suspensions yielded good correlations between dechlorination rate and RDase concentrations (Figure 2-2). In addition to these initial proof-of-concept microcosm studies, other similar experiments have shown that RDase peptides can be identified from environmental samples [21-23]. Specifically, BvcA, VcrA and TceA peptides were identified in samples from a cVOC-contaminated site.

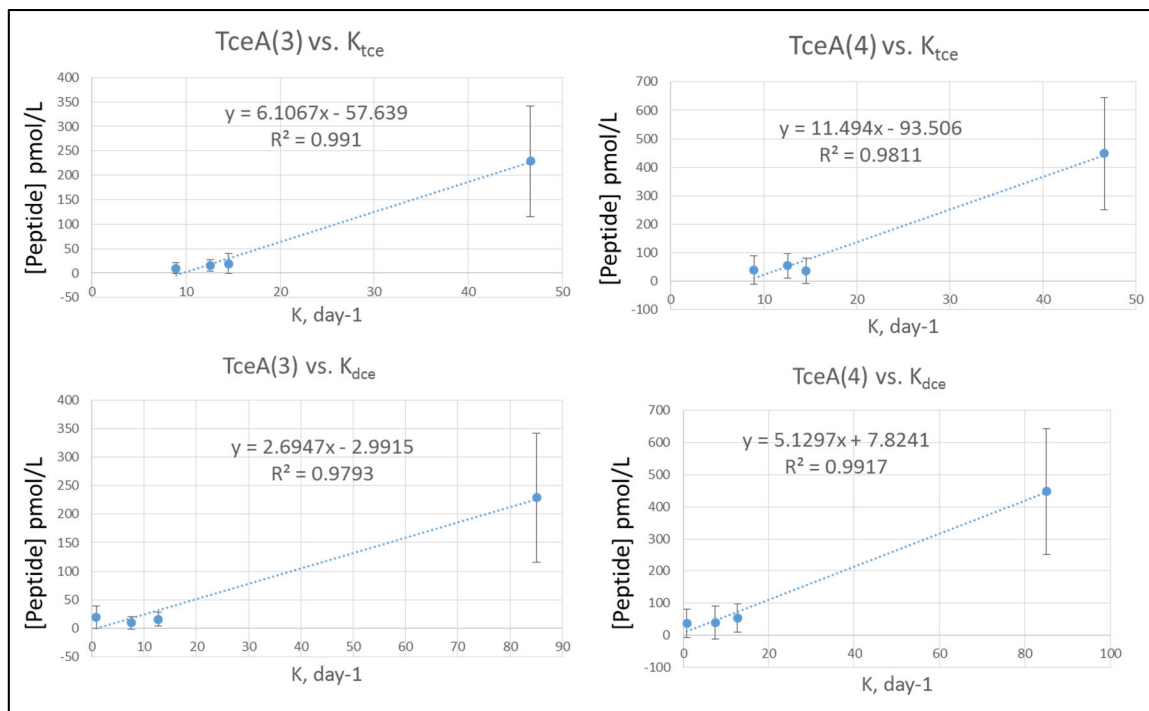


Figure 2-2. RDase peptide concentrations versus degradation rate constants
RDase peptide abundance vs. first order for TCE and cisDCE in SDC-9 microcosms.

The utility of qProt as an advanced MBT for environmental monitoring has been demonstrated. However, a quantitative link between RDase peptide abundance in environmental samples and reductive dechlorination rates has yet to be established in microcosm experiments. This demonstration (1) validated the qProt method for measuring RDase peptide abundances in environmental samples, and (2) established a quantitative link between biomarker abundance (RDase peptides and genes) and rates of *cis*-DCE and VC reductive dechlorination. This was accomplished through a series of microcosm studies performed using aquifer material from a cVOC-contaminated DoD site. A detailed description of the microcosm study design is provided in Section 5.0. Briefly, *Dhc* cell abundances were varied in each set of incubation vessels over six orders of magnitude ($10^3 - 10^9$ cells/mL) and the rate of *cis*-DCE and VC reductive dechlorination was measured in each microcosm replicate. Live and killed controls were included and all biomarker and cVOC measurements were made in triplicate. This well-controlled and replicated microcosm study used real-world aquifer materials and provided data required to establish the

quantitative link between abundances of peptides and nucleic acid biomarkers with reductive dechlorination rates. The results presented herein can now be applied in the field to validate the link between biomarker abundances and *in situ* reductive dechlorination rates at one or more cVOC-contaminated DoD sites.

2.3 ADVANTAGES AND LIMITATIONS OF THE TECHNOLOGY

The main advantage of proteomic techniques for the determination of *in situ* degradation rates is that the absolute amount of the reaction catalysts (e.g., RDase enzymes) are measured. Proteomics-based techniques are limited by the amount of biomass (more specifically, the amount of proteins of interest) collected in the sample. This limitation is due to the fact that in contrast to nucleic acids, proteins cannot be amplified, so their quantification is inherently limited by the instrument detection limit (IDL) and overall method detection limit (MDL). The IDL is a function of the LC-MS/MS instrumentation and the concentration of specific peptides to be quantified. These detection limits can approach the low attomol (10^{-18}) range for quadrupole time-of-flight tandem mass spectrometers (QTOF-MS) [28]. The MDL considers the sensitivity of the overall method, including any loss associated with extraction and purification from interfering substances (e.g., detergents that can suppress MS signals), which can reduce sensitivity to high fmol to attomol (10^{-15} - 10^{-18}) levels for environmental samples. Prior work at Battelle [21-23] and under SERDP ER-2312 [13] demonstrated that the overall MDL for the quantification of *Dhc* RDases approaches 3,000 fmol (3 pmol) RDase per liter of groundwater, which corresponds to an approximate *Dhc* biomass of 10^6 total cells. Although *Dhc* abundances are generally low at MNA sites, the qProt assay may still be reliably used at these locations by collecting suitably large sample volumes, which will ensure the sample contains sufficient RDase mass to be above the quantitation limit. Because quantitation of RDases is paramount to the proteomics method being demonstrated, additional IDL and MDL studies were completed as part of this demonstration as described in Section 5.0.

The targeted nature of qProt is both an advantage and a limitation. The main limitation is that RDases (and other proteins of interest) from indigenous (native) dechlorinating organisms may respond to biostimulation and contribute to the observed degradation activity. These native RDases may have slightly different RDase sequences and may thus not be detected in the MRM proteomic assay (i.e., false-negative results). While this is a legitimate concern, the issue can be addressed by metagenome sequencing of DNA extracted from biomass collected from site groundwater. Metagenome sequencing has become a routine procedure and can be accomplished at reasonable cost (e.g., <\$1,000). Bioinformatics pipelines to extract RDase gene fragments from metagenome datasets are available and this information can then be used to determine the exact sequences of native RDase genes of interest. With this information, the peptides of native RDases can be predicted and therefore detected and quantified with the qProt approach.

Environmental distribution of the trichloroethene reductive dehalogenase gene (*tceA*) suggests lateral gene transfer among *Dehalococcoides* [29]. Therefore, a suite of qProt assays can be developed that will be applicable to the majority of sites. Prior studies have also demonstrated allelic sequence variations of RDases genes such as *tceA*; however, we expect that the sequence variability of *tceA* gene sequences will be limited at contaminated sites and we will not find new *tceA* sequences at every site investigated. Thus, as our study demonstrates that the development of

site-specific qProt assay will be cost feasible at most sites, our knowledge of RDase gene sequence variations suggests it may be possible to design a multiplexed RDase qProt assay which will encompass several target peptides that will be useful at the majority of sites.

3.0 PERFORMANCE OBJECTIVES

This project demonstrated the utility of advanced MBTs for prediction of cVOC degradation rates in laboratory microcosms. Demonstration results highlighted the utility of this approach for estimating *in situ* reductive dechlorination rates at field sites. The following section describes quantitative and qualitative performance objectives (Table 3.1) specific to the laboratory microcosm phase of the demonstration.

Table 3-1. Demonstration performance objectives for the microcosm study

Performance Objective	Data Requirements	Success Criteria
Quantitative Performance Objectives		
Quantify rate coefficients for <i>cis</i> -DCE and VC degradation in aquifer microcosms bioaugmented with reductive dechlorinating consortium SDC-9 at varied initial cell densities. Rates of production of VC from <i>cis</i> -DCE and ethene and ethane from VC will also be quantified.	Concentrations of <i>cis</i> -DCE, VC, ethene and ethane over a minimum of six time periods in triplicate microcosms. Data will be used to estimate rate coefficients and corresponding uncertainties for each test.	Initial cell densities in the bioaugmented microcosms will be varied by 4 orders of magnitude (10^5 , 10^6 , 10^7 and 10^8 cells/mL) to ensure we obtain a range of rate coefficients and reductive dechlorination activity levels. Rate coefficients estimated using the microcosm data will be of sufficient quality if the global R^2 of the kinetic model is ≥ 0.75 , and if the average ratio of the 95% confidence interval to the rate coefficient value for both k_{cisDCE} and k_{VC} (i.e. the average of ratios in brackets $\left[\frac{95\% CI k_{cisDCE}}{k_{cisDCE}}, \frac{95\% CI k_{VC}}{k_{VC}} \right]$) is $\leq 125\%$
RDase biomarkers (RDase peptides, genes, and RNA transcripts) are quantifiable at microcosm-required and environmentally-relevant concentration levels.	Quantify initial and final RDase biomarker abundance and corresponding uncertainty for each treatment within each microcosm test.	RDase biomarker abundance measurements meet the Data Quality Objectives for this analysis. Ability to measure one or more of these RDase biomarkers at environmentally relevant <i>Dhc</i> concentrations (i.e., 10^5 to 10^6 cells/mL).
One or more of the RDase biomarkers exhibits a quantifiable, predictive association with <i>cis</i> -DCE (and/or VC) degradation rates in the microcosms.	Rate constants for each microcosm that meet objectives described above. RDase biomarker abundance (peptides, genes, and RNA transcripts) measurements that meet objectives described above.	The association between RDase biomarker abundance (RDase peptides, genes, and RNA transcripts) and the rate constants is positive and significantly different from zero at the 95% confidence level.
Qualitative Performance Objectives		
RDase biomarker abundance input to multivariate regression (or other suitable) model predicts reductive dechlorination rates with equal or better confidence than using conventional DNA-based MBTs alone.	Rate constants for each microcosm that meet objectives described above. RDase biomarker abundance (peptides, genes, and RNA transcripts) measurements that meet objectives described above.	Utility of RDase biomarkers (RDase peptides, genes, and RNA transcripts) – alone and in combination – will be quantified and documented.
Effectively communicate benefits of advance MBTs to end users – particularly managers of cVOC-contaminated DoD groundwater sites – through multiple technology transfer platforms.	Rate constants for each microcosm that meet objectives described above. RDase biomarker abundance (peptides, genes, and RNA transcripts) measurements that meet objectives described above.	At the conclusion of the 2-year microcosm test, at least one manuscript will have been submitted to a top-quality, peer-reviewed journal.

3.1 Quantify rate constants for *cis*-DCE and VC degradation in aquifer microcosms.

Rate constants were calculated for *cis*-DCE and VC degradation in each of the different microcosm treatments (Section 5.5.2). The procedure for calculating rate constants is provided in Section 5.7.

Data Required: The data required included concentrations of *cis*-DCE and VC as a function of incubation time in the microcosms. Concentrations of *cis*-DCE and VC were measured at a minimum of 8 time points in each microcosm treatment, which were prepared in triplicate. Analysis was conducted by EPA Method 8260 (Gas-Chromatography – Mass Spectrometry; GC-MS) using liquid 2-mL samples as described in Section 5.6.1.

Success Criteria: The first order rate constants were fit to the microcosm data and were considered to be of acceptable quality if the global R^2 of the kinetic model was ≥ 0.75 , and if the average ratio of the 95% confidence interval on the rate constant to the rate constant value itself for both k_{cisDCE} and k_{VC} (i.e. the average of ratios in brackets $\left[\frac{95\% CI k_{cisDCE}}{k_{cisDCE}}, \frac{95\% CI k_{VC}}{k_{VC}} \right]$) was $\leq 125\%$.

3.2 Measure target RDase biomarkers (RDase genes and proteins) at environmentally relevant abundance levels.

Abundance of RDase proteins was linked to abundance of the reductive dechlorinating microbes expressing them. The microcosm test was designed to quantify RDase biomarkers associated with *Dhc* cell densities in the $< 10^6$ cells/mL range, which is relevant to MNA sites, and up to $> 10^8$ cells/mL range, which is relevant to biostimulated and bioaugmented sites.

Data Required: Required data include abundances of RDase biomarkers (genes and proteins) in each microcosm at the time corresponding to the beginning, middle and end of the incubation used to determine the rate constants for *cis*-DCE and VC degradation. The RDase biomarkers were quantified using methods described in Section 5.6.2 and 5.6.3.

Success Criteria: Abundance of RDase biomarkers met the method detection limits and other data quality objectives summarized in Section 5. Microcosm study results were utilized to establish a lower limit of detection for quantifying peptides of interest. Results showed that single RDase protein biomarkers in the 2×10^6 *Dhc* cells/mL or more range corresponded to k_{cis} and k_{VC} rates in the range of 0.0001 day^{-1} (0.04 year^{-1}), which is relevant to sites pursuing or managing MNA remedies.

3.3 Quantify relationship between target RDase biomarker abundances and reductive dechlorination (RD) activity in aquifer materials

Abundance of RDase biomarkers (genes and proteins) were compared with the rate constants for biodegradation of *cis*-DCE and VC collected from the microcosms.

Data Required: Rate constants for *cis*-DCE and VC degradation (Objective 3.1) and concentrations of RDase peptides and genes in each microcosm at the time corresponding the beginning of the incubation used to extract the rate constants (Objective 3.2).

Success Criteria: The association between abundance of individual RDase peptides and genes, and the rate constants was tested by first performing correlation analysis. RDase biomarkers and rate constants with correlation factors that were positive and significantly different from zero at the 95% confidence interval were considered acceptable, then were carried forward into a power law least squares regression analysis where the predictive relationship was established.

3.4 Develop a multivariate regression (or other suitable) model, which predicts the *cis*-DCE and VC rate constants using RDase biomarkers as input

A simple power regression model was developed to allow an end user to predict the *cis*-DCE and VC degradation rate constant using qPCR and qProt data as model input parameters.

Data Required: Rate constants from microcosms and corresponding abundance of RDase proteins and functional genes so that the quantitative relationship between these measures can be modeled and the predictive tool can be developed.

Success Criteria: Quantify the rate-predictive power of the regression model using RDase protein abundance only, RDase functional gene abundance only, and a combination of the two together to establish the relative contribution of each measure to the predictive power of the model. This performance objective was established as a *qualitative* objective for this laboratory microcosm phase of the demonstration.

3.5 Effectively transfer the new technology to end users

Results of the microcosm study are the first to demonstrate use of qProt for predictions of reductive dechlorination rates under environmentally relevant conditions.

Data Required: Rate constants from microcosms and corresponding abundance of RDase biomarkers and functional genes so that the quantitative relationship between these measures can be modeled and the predictive tool can be developed.

Success Criteria: Distribute microcosm study findings using effective technology transfer platforms. Submit at least one manuscript describing the results and benefits of the approach to a top-quality, peer-reviewed journal. Present results at multiple national remediation conferences.

4.0 SITE DESCRIPTION

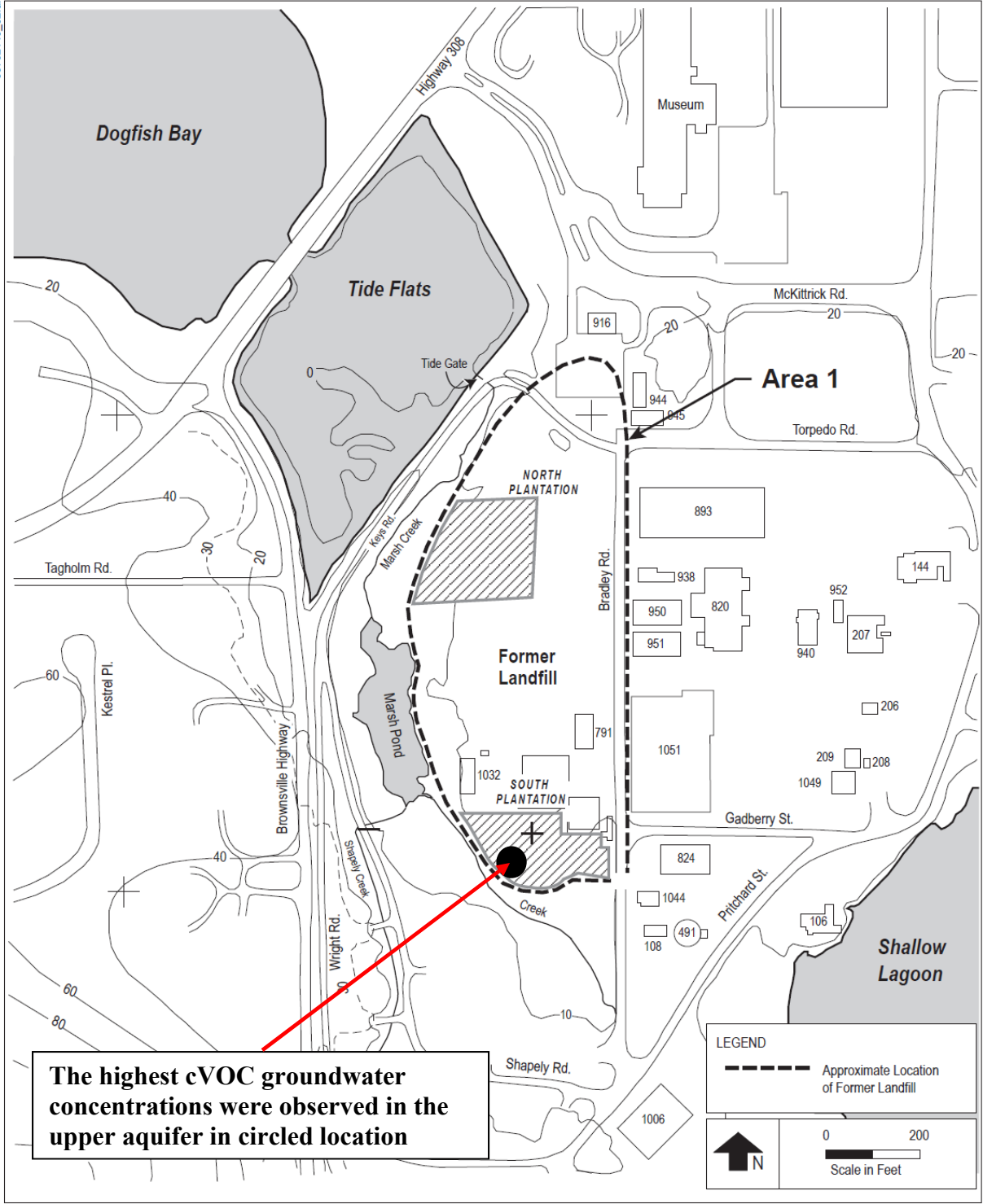
Three cVOC-contaminated DoD sites were selected for potential inclusion in this project: Naval Base Kitsap (NBK) Keyport Area 1, Joint Base Lewis-McChord (JBLM) Landfill 2, and Vandenberg Air Force Base (VAFB) Site SA288. These sites were selected because (1) collection of aquifer material and groundwater was possible with minimal cost to the project through leveraging pre-planned site characterization activities, and (2) each site is potentially suitable for a future field demonstration. The following subsections provide an overview of each DoD field site – and basis for inclusion or exclusion from this laboratory project.

4.1 NBK KEYPORT AREA 1, BREMERTON, WASHINGTON

Site Location and History. Keyport Area 1 is a former solid waste landfill at NBK Keyport, located 45 miles north of Tacoma, Washington on the Kitsap Peninsula. It comprises approximately 9 acres in the western portion of the base, next to a wetlands area and the tidal flats that flow into Dogfish Bay (Figure 4-1). The Area 1 landfill was the primary disposal area for domestic and industrial wastes generated by the base from the 1930s until 1973, when the landfill was closed. NBK Keyport became a Superfund site in 1989. The remedial investigation and feasibility study [30] identified cVOCs as contaminants of concern in site soil, sediment, tissue, groundwater, and surface water. The Record of Decision [31] for the Area 1 landfill specified cVOC hotspot treatment using phytoremediation by poplar trees in concert with natural attenuation, as well as landfill liner upgrades, monitoring and other best management practices. The landfill liner upgrade was completed in 2003 and phytoremediation was implemented in 1999 by planting two poplar plantations (Figure 4-1).

Site Geology/Hydrogeology. There are two aquifers at the site. The sandy unconfined upper aquifer is present throughout the landfill area and is 4 to 15 feet thick, with depth to water between 4 to 10 feet below ground surface (bgs) [30]. Approximately 5 feet of landfill material lies above the groundwater surface in the unsaturated zone; up to 10 feet of landfill material lies within the saturated upper aquifer. Upper aquifer groundwater generally flows west and discharges into the marsh pond (Figure 4-1). The upper aquifer is underlain by an aquitard consisting of sandy silt to clean silt, which is 4 to 15 feet thick where present. The underlying intermediate aquifer is 5 to 25 feet thick, with groundwater flow direction generally toward the tide flats (Figure 4-1). The intermediate aquifer is underlain at 25 to 40 feet bgs by a thick nonglacial silt and clay aquitard known as the Clover Park Silt, which is approximately 100 feet thick and separates the contaminated aquifers from the deeper regional water-bearing units [30].

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The highest cVOC groundwater concentrations were observed in the upper aquifer in circled location

<p>U.S. NAVY</p>	<p>OU 1 Site Map</p>	<p>NBK Keyport FOURTH FIVE-YEAR REVIEW</p>
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Figure 4-1. NBK Keyport Area 1, site map

Contaminant Distribution. In spite of a high degree of biodegradation and reductions in cVOC mass over time, groundwater concentrations of cVOCs beneath the south poplar plantation in the upper aquifer remain high and cVOC concentrations in surface water adjacent to the south plantation consistently exceed the surface water remediation goals. The maximum concentrations of cVOCs measured in upper aquifer monitoring wells in the south plantation were: TCE > 33,000 µg/L, *cis*-DCE > 55,000 µg/L, and VC > 6,000 µg/L (Figure 4-1). Aquifer material and groundwater for this project will be collected from the upper aquifer, south plantation.

Project Inclusion Decision. Aquifer solids and groundwater were collected by NBK Keyport Area 1 contractors (Battelle Memorial Institute) in July and September 2017 and shipped on ice overnight to the Aptim laboratory in Lawrenceville, NJ. The quantity of aquifer material collected in July 2017 via direct-push drilling was limited but screening results were favorable so a larger quantity of aquifer material was collected in September 2017 via hollow stem auger. Unfortunately, the larger material sample exhibited a strong odor (suspected naphthalene, not confirmed). High/potentially inhibitory levels of contamination encountered in the material, coupled with suspected presence of inhibitory co-contaminants, resulted in a decision to exclude the NBK Keyport Area 1 material during the laboratory project phase. However, results of the recent expanded site characterization make NBK Keyport Area 1 a good candidate for a future field demonstration.

4.2 VAFB SITE SA 288, VANDENBERG, CALIFORNIA

Site Location and History. The site is located 4.1 miles east of the Pacific Ocean and 2.8 miles north of the Santa Ynez River within the Cantonment Area on the Burton Mesa portion of VAFB. The site consists of three buildings that were used for various industrial processes since the 1960s. Investigation activities initiated in 2008 involved installation of soil borings and temporary wells, which identified the former chemical storage shed and the former freon processing shed as potential cVOC source areas to groundwater (Figure 4-2). Additional soil borings and monitoring wells were installed and sampled in 2016, the results of which are summarized in the contaminant distribution section below. Additional site characterization is planned to support a “remedy in place” scheduled goal of second quarter 2018.

Site Geology/Hydrogeology. The site is underlain primarily by Quaternary Orcutt Sand [32] with bedrock occurring at approximately 45 ft below ground surface. Surface water that does not infiltrate into the subsurface at the unpaved areas of the site enters a storm drain system and ultimately discharges into the Santa Ynez River to the south. Groundwater can be detected near ground surface following significant rainfall events but is typically observed within a saturated sandy silt layer, which is 2 to 5 ft thick across the site. Depth to this saturated sandy silt layer varies across the site but is typically encountered around 10 ft below ground surface. Groundwater flows in a southeast direction from the presumed source area toward New Mexico Avenue (Figure 4-2).

Contaminant Distribution. Maximum cVOC concentrations in groundwater were encountered during the 2016 monitoring event in well SA288-MW-01, which is located approximately 150 ft downgradient of the former freon processing shed [33]. Groundwater concentrations of TCE and *cis*-DCE were 2,200 µg/L and VC was 76 µg/L (Figure 4-2). Aquifer material and groundwater use during this project will be collected from the saturated sandy silt perched aquifer.

Project Inclusion Decision. Aquifer solids and groundwater were collected by VAFB SA288 contractors (Geosyntec Consultants) and shipped on ice overnight to the Aptim laboratory in Lawrenceville, NJ. Unfortunately, the groundwater and aquifer solids collected from SA288 were naturally acidic (pH ~ 3.5), which is inhibitory for *Dhc* cells, and was therefore deemed not acceptable for inclusion in the laboratory portion of the project. However, other portions of the SA288 site or even other cVOC-contaminated aquifers present at VAFB could be candidates for a future field demonstration.

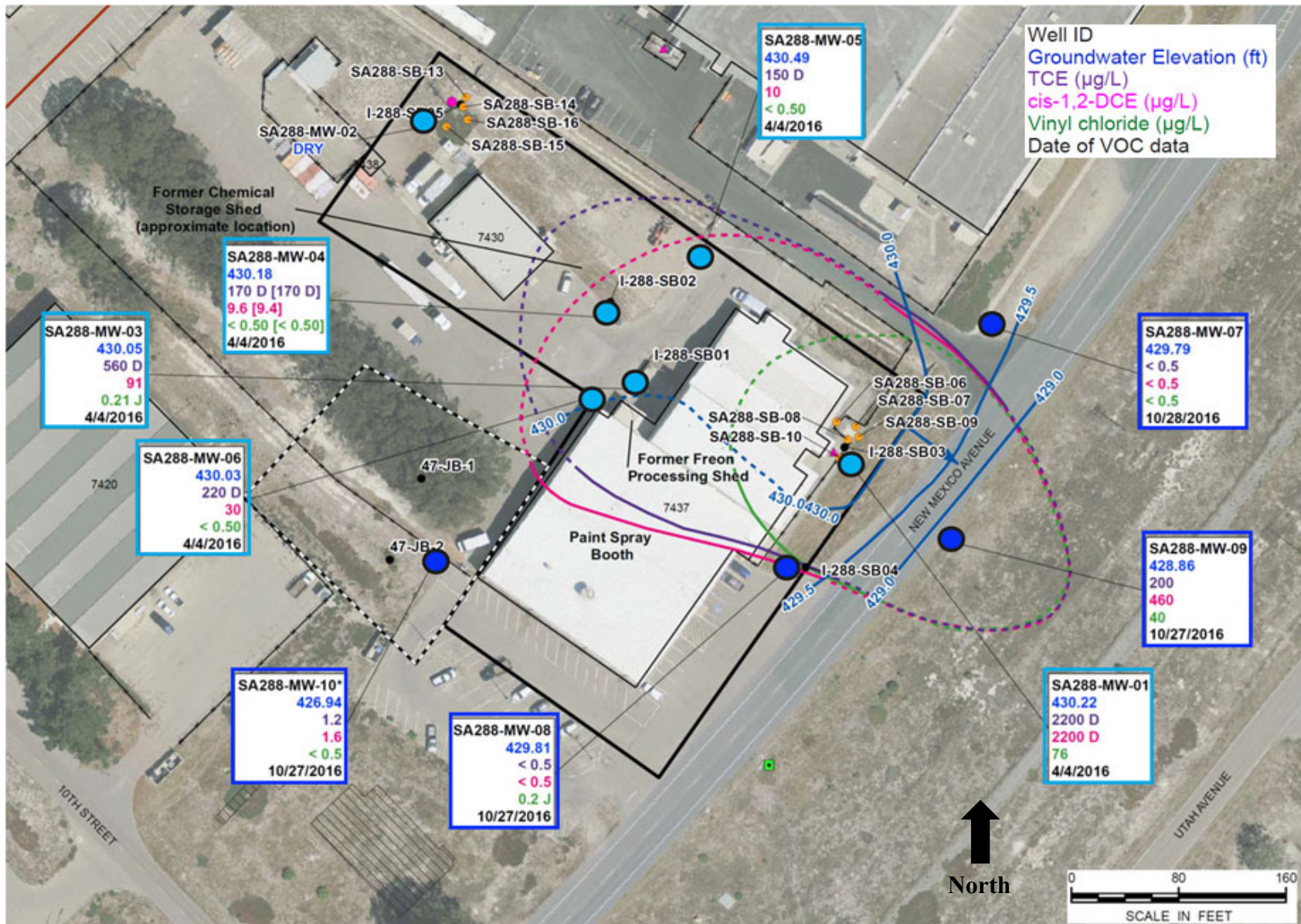


Figure 4-2. VAFB SA288 site map showing groundwater cVOC concentrations [33]

4.3 JBLM LANDFILL 2, TACOMA, WASHINGTON

Site Location and History. JBLM is a major military installation located approximately 15 miles southwest of Tacoma, Washington. Landfill 2 (LF2) was used to dispose of petroleum products and solvents generated by the Logistics Center between the 1940s to 1970s (Figure 4-3). Soils under the LF2 area are highly transmissive and the groundwater table is shallow; consequently, LF2 contributed to a very large TCE groundwater plume. The Logistics Center, which includes LF2, was listed as a Superfund site in 1989. The Record of Decision (ROD) [34] specified a pump and treat groundwater remedy for LF2. The ROD was subsequently modified to include multiple source area removal actions and enhancements to the pump and treat remedy.

Site Geology/Hydrogeology. LF2 is located within the unconfined Vashon Aquifer, which is comprised of interlayered outwash and glacial till to an approximate depth of 100 ft below ground surface. In the vicinity of the LF2 source area, the Vashon Aquifer is divided into the Upper Vashon and the Lower Vashon, which are separated by a discontinuous low permeability till layer. The Vashon Aquifer is separated from the underlying confined Sea Level Aquifer by a 10 to 20 feet thick non-glacial aquitard unit. A “window” in the aquitard unit downgradient of the LF2 source area resulted in formation of a large cVOC plume in the underlying Sea Level Aquifer.

Contaminant Distribution. Multiple source area removal actions (excavation, thermal treatment) and pump and treat remedy implementation since the 1990s have significantly reduced cVOC concentrations present in the LF2 source area wells (Figure 4-3, inset). However, only select groundwater wells are sampled during compliance monitoring events and those low concentrations (see contours, Figure 4-3) do not explain the ~ 200 µg/L sustained TCE concentrations routinely encountered in extraction well PW-1. Groundwater TCE concentrations in the ~ 1,000 µg/L range are expected in the source area vicinity. New investigation wells are being installed and a comprehensive groundwater monitoring event is being conducted to refine the LF2 conceptual site model and confirm remaining TCE concentrations in source area groundwater. These investigation activities in the LF2 source area will be leveraged to provide aquifer material and groundwater for use during this ESTCP project.

Project Inclusion Decision. Aquifer solids and groundwater were collected by U.S. Army Engineer Research Development Center (ERDC) and Seattle District U.S. Army Corps of Engineers (USACE) and shipped on ice overnight to the Aptim laboratory in Lawrenceville, NJ in May 2017. Aquifer solids and groundwater samples were stored at 4°C until use. Initial screening of the groundwater samples showed cVOC concentrations and pH were within acceptable ranges. Next, the LF2 groundwater was screened for potential reductive dechlorination inhibitory substances by conducting a simple microcosm study. SDC-9 cells (10^8 cells/mL), *cis*-DCE (10 mg/L) and lactate (500 mg/L) were added to LF2 groundwater followed by measurements of *cis*-DCE, VC and ethane/ethene concentration and pH over time. Rapid reductive dechlorination was observed in the screening microcosm, which supported a “go” decision to include LF2 materials in the laboratory project. pH reduction observed during the screening microcosm prompted inclusion of calcium carbonate buffer during subsequent microcosm experiments.

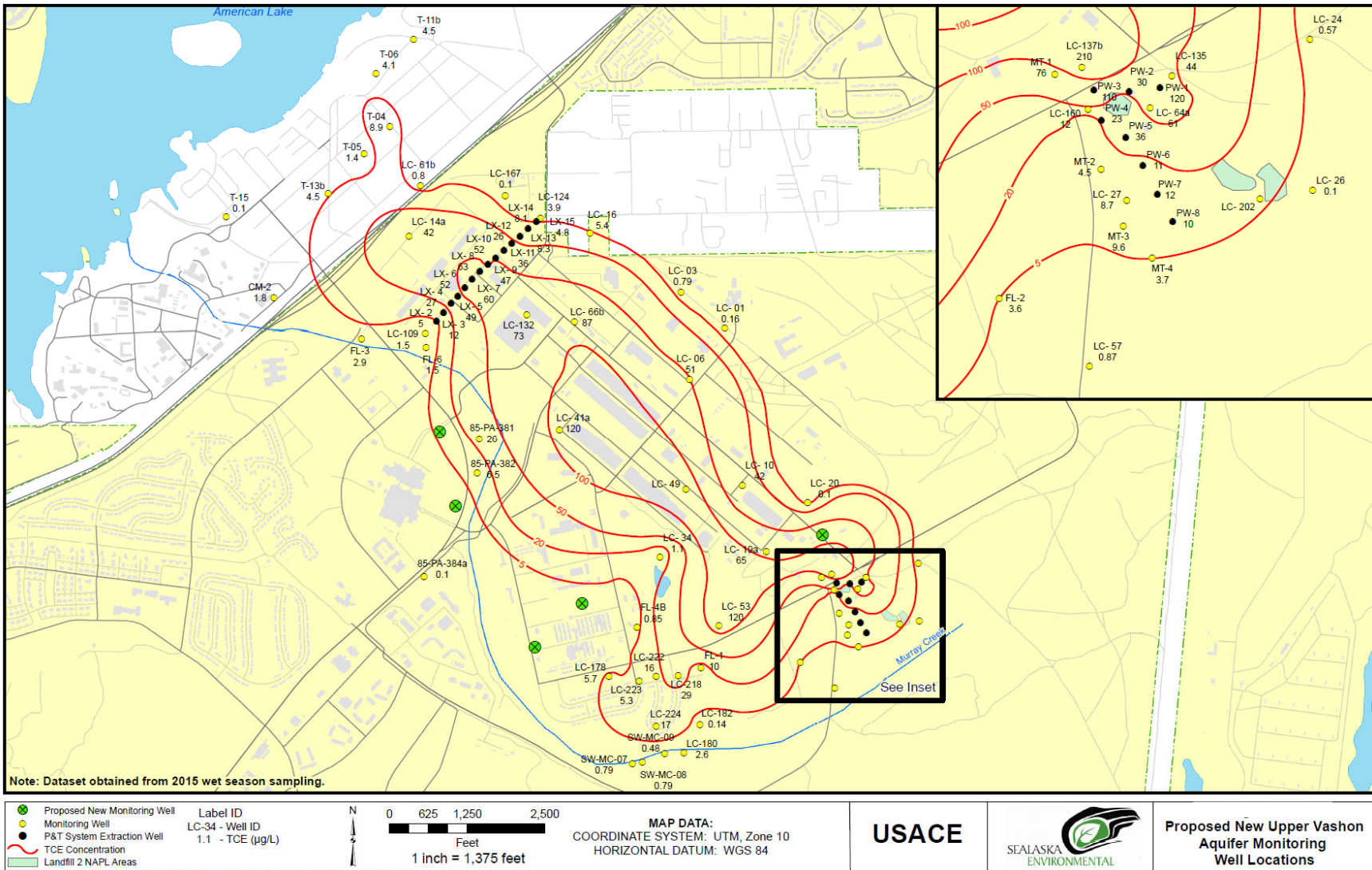


Figure 4-3. JBLM Landfill 2 TCE concentrations in Upper Vashon Aquifer source area wells (see inset)

5.0 TEST DESIGN

This section provides an overview of the experimental approach and field material collection (Sections 5.1 – 5.2), refinement and validation of the qProt assay specific to this project (Sections 5.3 – 5.5), as well as detailed experimental procedures, analytical methods and data analysis requirements for the project (Sections 5.6 – 5.8).

5.1 CONCEPTUAL EXPERIMENTAL DESIGN

The goal of this project was to demonstrate the utility of measuring RDase biomarkers (RDase genes, peptides) via qPCR and qProt to estimate *in situ* cVOC degradation rates. First, the RDase protein targets were identified to finalize the qProt assay for use during this project (Section 5.3) then MDL and IDL studies were performed to establish the quantitative framework for the qProt assay (Section 5.4). Next, we performed a study using diluted SDC-9 culture to validate quantitation limits of the qProt assay (Section 5.5). Finally, a series of microcosm studies were performed using DoD site aquifer materials where *cis*-DCE and VC degradation rates were quantified as a function of RDase biomarker gene and protein abundances (Section 5.6). Microcosms were prepared by amending JBLM LF2 aquifer material with *cis*-DCE, lactate as a growth substrate, and calcium carbonate buffer. Varied quantities of the dehalogenating consortium SDC-9, which contains *Dhc* strains carrying RDase genes including *vcrA* and *tceA*, were added to the microcosms as illustrated in Figure 5-1. Samples were collected from the microcosms over time and analyzed for cVOCs so that degradation rates of *cis*-DCE and VC could be calculated. Samples also were collected from the microcosms at multiple points for analysis of selected RDase biomarkers. As described in Section 6.0 below, results of this laboratory project illustrated that RDase biomarkers can be reliably quantified over ranges of *Dhc* cell abundances relevant to cVOC site management – from low abundance/low activity relevant to MNA to high abundance/high activity relevant to enhanced bioremediation. Furthermore, the positive and significant correlations established between the biomarker abundances and reductive dechlorination rate coefficients in this laboratory study lay the foundation for a follow-on field study where the quantitative link between RDase biomarkers and *in situ* rates can be validated.

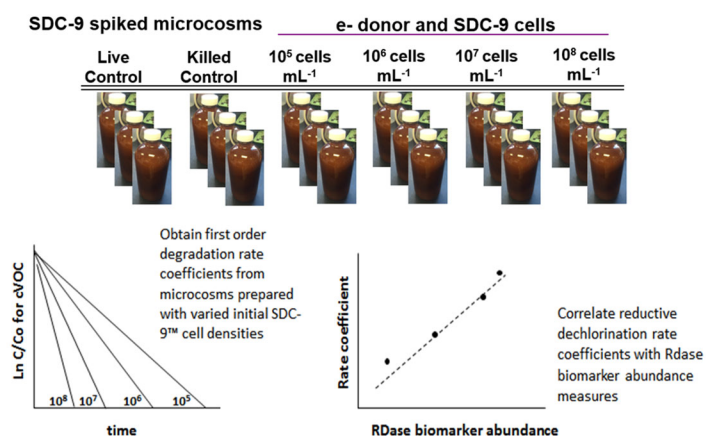


Figure 5-1. Microcosm study conceptual design

5.2 BASELINE CHARACTERIZATION ACTIVITIES

Aquifer material was collected from the saturated zone of the JBLM LF2 cVOC-contaminated aquifer the week of May 29, 2017. The aquifer materials were sieved in the field to remove gravel, collected in large Zip-Loc-type freezer bags, labeled, and then stored on ice pending overnight shipment to the Aptim laboratory (Aptim). Wet solids were shipped on ice in Zip-Loc type bags labeled “JBLM-L236 8-50’ BGS”. The solids (wet sand and some small pebbles) were homogenized under a nitrogen atmosphere at Aptim and placed in sterile 4-L glass jars. The jars were stored at 15 °C. The wet soil was allowed to settle for a week, and the water was decanted to lower the moisture content of the solids. This was repeated several times over 10 weeks until the soil moisture content was 15% (wt/wt). Groundwater was collected from the extraction well PW-1 sampling port the week of May 29, 2017, and then again the week of March 5, 2018, into 18-L stainless steel kegs that had been bleached (2,500 mg/L chlorine), rinsed with Nano-Pure water, and autoclaved (15 psi, 121 °C, 45 minutes). Groundwater samples were placed on ice and shipped overnight to Aptim. Site groundwater was analyzed for cVOCs using methods described below prior to use in microcosms. The cVOCs present in the groundwater were removed by purging with N₂ prior to groundwater use in microcosm preparation to prevent any potential impacts of the native cVOCs on the growth and activity of dechlorinators in the SDC-9 consortium. In order to ensure reductive dechlorination was not affected by inhibitors associated with the aquifer materials, a single microcosm was prepared and sampled as described in Section 5.3 below for screening purposes. This microcosm was bioaugmented to achieve a *Dhc* cell density of 10⁸ cells/mL with lactate as the electron donor, then screened for *cis*-DCE and VC degradation rates and pH changes only. If reductive dechlorination activity fell within the expected range (based upon historical data with the SDC-9 consortium), the aquifer material was considered acceptable and carried forward in the microcosm study. As described in Section 4.0, only aquifer material from JBLM LF2 was deemed acceptable to carry forward.

5.3 DEVELOPMENT OF RDASE PEPTIDE TARGETS

SDC-9 biomass was harvested during growth in a 4,000-L bioreactor maintained at Aptim at three time points and subjected to proteomic analysis. Targeted proteomic analysis was conducted using the same instrumentation and chromatographic method as used for shotgun proteomics except that the mass spectrometer was operated in parallel reaction monitoring (PRM) mode. A full scan spectrum was acquired (100 ms accumulation time) followed by product ion spectra of each target peptide (75 ms accumulation time), for a total cycle time of 1.9 seconds. The product ion scans were not time scheduled. Proteins were identified from MS/MS fragmentation data by searching the MS/MS data of the top n peaks against a custom FASTA library protein sequences acquired from the metagenome of consortium SDC-9. Searches were performed with the Paragon algorithm in Applied Biosystems ProteinPilot 4.5, with the following parameters: ID with 95% confidence, fixed modifications (carbamidomethyl), variable modifications (methionine oxidation).

ProtScore values for an identified protein were calculated by summing the ProtScore of each identified peptide after log transformation:

$$\text{ProtScore} = -\log(1 - C_n) \quad \text{equation 1}$$

where n peptides with a confidence of C_n each contributes to the ProtScore of the identified protein. For example, a protein that has four peptides with 99% confidence match has a 99.99999999% chance ($1 - 0.014$) of being a true identification. In this case, each peptide contributes 2 units to the ProtScore for every peptide identified with a 99% confidence ID. High-confidence, non-tryptic peptides were subjected to analysis using a suite of open-source software to provide explanation for the observed cleavage site as follows:

- PRED-TAT (<http://www.compgen.org/tools/PRED-TAT4>), which is used to predict signal peptide domains; and
- PROSPER (<https://prosperec.monash.edu.au/5>), PeptideCutter (http://web.expasy.org/peptide_cutter/5), and the MEROPS peptidase database (<https://www.ebi.ac.uk/merops/search.shtml6>), all of which are used to predict protease specificity for a given protein sequence.

5.4 ESTABLISHING MDL AND IDL FOR TARGET PEPTIDES

The SDC-9 culture-specific RDase peptides were identified for quantification. These specific RDases were then used in a MRM targeted proteomic assay to establish quantitative biomarker rate correlations, which are needed to generate degradation rate estimates for chlorinated ethenes. MDL/IDL study methods are summarized below; details are included in Appendix A.

For determination of the MDL, the 12.5 pmol/ μ L stock solution of isotopically labeled (IS) peptides was diluted in 50 mM ammonium bicarbonate to prepare the following concentrations (final in 25 μ L): 250, 83, 27, 9, 3, 1, 0.34, and 0.11 fmol/ μ L. Each sample was digested with trypsin overnight and desalted using C18 spin columns. To confirm instrument functionality and detectability of each IS peptide, infusion and injection steps were performed. Each IS peptide was prepared as 12.5 pmol/ μ L in dimethyl sulfoxide (DMSO)/Milli-Q water (50/50), aliquoted, and frozen at -80°C until use. Concentrated solutions for each peptide were provided to the analyst for subsequent dilution and infusion directly into the mass spectrometer (Waters Xevo TQ-XS) for confirmation of precursor (parent) ion, charge state, product ions (daughters), and optimization of collision energies (CE) (Table 2, Appendix A). This optimization step is performed to confirm that a peptide of a given sequence is detectable in the mass spectrometer and to optimize signal intensity for product ions. Each peptide was diluted to 0.5 pmol/ μ L or 1.25 pmol/ μ L in HPLC-grade water + 0.1% formic acid and was directly infused into the mass spectrometer at a flow rate of 10 μ L/min. For each peptide, a mass spectrum of the precursor ion was obtained. For each precursor ion, a mass spectrum was obtained for the product ions after fragmentation with CE of ≥ 20 V. Using Waters Intellistart software, the CE for each peptide was optimized to maximize a signal from product ions. This was performed by infusing a single peptide into the mass spectrometer while Intellistart software varied cone voltage and CE to maximize a signal for each product ion. Skyline software was also used to output optimal CE for each peptide using equation 2 with parameters (slope, intercept) that are specific to Waters Xevo mass spectrometers (Table 5-1).

$$\text{CE} = \text{slope} * (\text{precursor charge state}) + \text{intercept} \quad \text{equation 2}$$

Table 5-1. Parameters of Waters Xevo CE Equation

Precursor Charge State	Slope	Intercept
+2	0.037	-1.066
+3	0.036	-1.328

After optimization of CE per each IS peptide further development of multiple reaction monitoring assay was performed, including optimization of dwell time, CE, and solvent program. During this phase, peptides with relatively poor response were dropped from the MRM method file. The Skyline-optimized CEs were used in initial MRM method development. Comparison to Intellistart-optimized CEs was performed later in MRM development, however improvements in signal intensity were insignificant.

For MRM method development, peptides were prepared as a mixture at 1.25 pmol/ μ L in HPLC-grade water + 0.1% formic acid from a 12.5 pmol/ μ L mixture in DMSO/Milli-Q water. The solvent program and modified versions thereof were used (see Appendix A, Table 3). The chromatographic system used was the Waters M-Class equipped with a trap column (Acquity UPLC M-Class Trap Symmetry® C18; 5 μ m particle size, 100Å pore size; 0.3 mm x 50 mm) and an analytical column (Acquity UPLC M-Class HSS T3 C18; 1.8 μ m particle size, 0.3 mm x 50 mm). Based on the observed maximum peak heights of each peptide at 1.25 pmol/ μ L prepared in HPLC-grade water + 0.1% formic acid (MS Parameters from September 5, 2017: 123 transitions; 30 ms dwell time; 3.7 s cycle time), some peptides were removed from the transition list based on poor response (peak height or peak area) relative to other peptides. Only those peptides with the largest responses were retained on the transition list.

Using the modified transition list and a 1.25 pmol/ μ L standard prepared in HPLC-grade water + 0.1% formic acid, three dwell times (20 ms, 50 ms, and 70 ms) were examined to assess the sensitivity of the signal to variation in dwell time. Based on the quality of the output data (peak height, peak shape, and points across a peak), the 50 ms dwell time was pursued for MDL experiments. The dwell time parameter was adjusted to 30 ms after further method development was prompted by failure of the first MDL set.

To establish IDL, IS peptides were prepared as a mixture at 12.5 pmol/ μ L in DMSO/Milli-Q water (50/50), aliquoted, and frozen at -80°C until use. A mixed, concentrated solution (12.5 pmol/ μ L) was provided fresh to the analyst during each day of analysis. The analyst diluted the sample to 250 fmol/ μ L in HPLC-grade water + 0.1% formic acid and serially diluted this solution three-fold to prepare the following concentrations: 83, 27, 9, 3, 1, 0.34, and 0.11 fmol/ μ L. The lowest measurable concentration for each peptide, defined as $S/N \geq 3$ (as measured by MassLynx) for the primary and secondary ion, represents the IDL for each peptide.

To determine the MDL of peptide targets, IS peptides were prepared as a mixture at 12.5 pmol/ μ L in DMSO/Milli-Q water (50/50), aliquoted, and frozen at -80°C until use. A mixed, concentrated solution (12.5 pmol/ μ L) was provided fresh to the analyst during each day of analysis. The analyst diluted the sample to 1.25 fmol/ μ L in HPLC-grade water + 0.1% formic acid to use as a control during the analysis sequence. The 12.5 pmol/ μ L stock solution was diluted in ammonium bicarbonate to prepare the following concentrations (final in 25 μ L): 250, 83, 27, 9, 3, 1, 0.3, and

0.1 fmol/ μ L. Each sample was digested with trypsin overnight and desalted using C18 spin columns. The lowest measurable concentration for each peptide, defined as $S/N \geq 3$ (as measured by MassLynx) for the primary and secondary ion, represents the MDL for each peptide.

A detailed report characterizes each step of system and IS peptide optimization (Appendix A). Data pertaining to system resolution check, calibration, and chromatograms of peptide detections are grouped per sample set.

5.5 VALIDATION OF QPROT ASSAY QUANTITATION LIMITS

After the development of the MRM assay and after the IDL and MDL values had been established for each RDase peptide, a study was performed with the SDC-9 consortium to identify the lowest *Dhc* cell titer that generated detectable and quantifiable concentrations of the RDase peptides selected for quantification. Validation study methods are summarized below; details are included in Appendix B.

To correlate the number of RDase proteins to *Dhc* cell abundances, qPCR was performed with the same samples. Briefly, to determine initial *Dhc* cell density, culture suspension (1 mL) of freshly grown SDC-9 consortium was filtered through 0.22 μ m Durapore membrane filters (25 mm, Millipore, Billerica, MA) in triplicate to collect biomass, and then DNA was extracted by using a DNeasy PowerLyzer PowerSoil Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions except for application of bead-beating method for enhanced cell lysis (OMNI Bead Rupter Homogenizer, OMNI International, GA) at 5 m/s for 3 min. Total DNA concentrations were determined using the Qubit dsDNA BR Assay (Invitrogen, Carlsbad, CA). TaqMan qPCR analysis of DNA was performed to determine the *Dhc* cell abundances using a *Dhc* 16S rRNA gene-targeted primer-probe set and qPCR conditions given in Section 5.6.3.

Based on initial *Dhc* cell density/mL determined by TaqMan qPCR assay, the SDC-9 culture was diluted to *Dhc* cell densities of 10^5 , 10^6 and 10^7 cells/mL using reduced mineral salt medium [35] inside an anoxic chamber. Sample preparations were performed in triplicate in sterile 50-mL Falcon plastic tubes. Samples (15 mL) were taken from each dilution and filtered through a 0.2 μ m filter (0.22 μ m, Millipore, Billerica, MA) at low flow speed for qPCR and qProt analyses. Each filter was placed in a sterile 50-mL Falcon tube and stored at -80°C immediately.

For qProt analysis, the filters were sent to Battelle on dry ice with an overnight carrier. Proteins were extracted with the Protein Extraction Kit (MoBio) and protein concentration was calculated using published methods [36]. An aliquot corresponding to 100 μ g of protein was mixed with 100 mM ammonium bicarbonate, 10 μ g bovine serum albumin (BSA) and isotopic peptide mix, reduced with dithiothreitol (10 mM), and incubated for 30 minutes at 57°C . Proteins were then alkylated with iodoacetamide (50 mM) for 30 min at room temperature in the dark. Excess iodoacetamide was quenched with dithiothreitol (16 mM final concentration). Peptides were digested with trypsin added in a 1:50 trypsin/protein ratio for 10 hours at 37°C . Samples were then acidified with an equal volume of 3% trifluoroacetic acid (TFA), dried via SpeedVac, then suspended in 270 μ L of 0.1% TFA. Samples were loaded on a C18 XTerra column (1 \times 100 mm, 5 μ m pore size, 100 \AA ; Waters Corporation, Milford, MA, USA), desalted using 0.1% TFA, and peptides were eluted with 70% acetonitrile. Samples were dried via SpeedVac, then suspended in

a 2% indexed Retention Time (iRT) solution (Waters) prior to injection onto a Xevo TQ-XS Triple Quadrupole Mass Spectrometer.

5.6 LABORATORY MICROCOSM TESTING

The microcosms utilized for this project consisted of 500 mL (groundwater only) or 1000 mL (groundwater + soil) amber, narrow mouth glass bottles. Each bottle was fitted with a Teflon[®]-lined screw-cap. Microcosm tests were performed using materials from the JBLM LF2 field site (Section 4). One of the microcosm tests was performed using groundwater only with no aquifer solids. Two of the microcosm tests were performed using a mixture of groundwater and aquifer solids. In order to most effectively simulate a groundwater aquifer, a high ratio of aquifer solids/groundwater was used in the solids-containing microcosms. All microcosms were placed on a bottle roller to ensure adequate mixing during incubation. Subsamples were collected as described in Section 5.6.3 and were subject to chemical, geochemical and molecular parameter analysis described in Section 5.7.

5.6.1 Growth of the SDC-9 Inoculum

The SDC-9 culture was inoculated in microcosms at varying densities to quantify and correlate rates of cVOC degradation with quantities of key RDase biomarkers. The SDC-9 inoculum was grown in reduced basal salts medium [37] in a 4,000-L fermenter using lactate as a source of carbon and electrons, PCE as a sole electron acceptor, and yeast extract as a source of nutrients. Further details concerning the fermentation and growth of SDC-9 are provided elsewhere [38]. For the current study, a volume of the culture was removed from the fermenter (or from a keg of culture previously grown and stored at 4°C for < 1 month), centrifuged, and suspended in medium to a *Dhc* density of $\sim 1 \times 10^{10}$ cells/mL based upon optical density (OD), $\alpha = 600$ nm [38]. The culture was then diluted for addition to microcosms as described below. Initial studies were conducted to estimate biodegradation rates of *cis*-DCE by SDC-9 prior to microcosm preparation.

5.6.2 Microcosm Preparation and Treatments

Microcosm #1, Groundwater Only Treatments. Six treatments (1-6) were tested in triplicate (a,b,c) microcosms. Microcosm construction and sampling was performed in a Coy anoxic chamber with a pure N₂ headspace. No H₂ gas was used in the chamber, which was thoroughly purged with N₂ prior to use to minimize any residual O₂. Microcosms were constructed in 500 mL amber Boston Round analytical bottles with Teflon[®]-lined septa caps. Microcosm bottles were bleached, rinsed with deionized water (DI) and autoclaved prior to use. Due to the relatively low alkalinity and pH of the site water, 1.0 g calcium carbonate (CaCO₃) was added to each microcosm as a slow release buffer to maintain a neutral pH during incubation. Site water (475 mL) was added to each of the bottles. Bottles then received the following amendments to bring the final aqueous concentration in each to ~ 490 mL:

1. Lactic acid sodium salt (LASS) to 500 mg/L final concentration of lactate (4.1 mL of 6% LASS).
2. NaBr to a final Br⁻ concentration of 10 mg/L: (0.49 mL of 10,000 mg/L Br⁻ stock).
3. To individual bottles, washed SDC-9 culture was added in medium to achieve nominal *Dhc* titers listed below:

- | | |
|--------|--|
| 1a,b,c | Add 4.9 mL of 10^{10} <i>Dhc</i> /mL (final = 10^8 /cells <i>Dhc</i> per mL) |
| 2a,b,c | Add 4.9 mL of 10^9 <i>Dhc</i> /mL (final = 10^7 /cells <i>Dhc</i> per mL) |
| 3a,b,c | Add 4.9 mL of 10^8 <i>Dhc</i> /mL (final = 10^6 /cells <i>Dhc</i> per mL) |
| 4a,b,c | Add 4.9 mL of 10^7 <i>Dhc</i> /mL (final = 10^5 /cells <i>Dhc</i> per mL) |
| 5a,b,c | Add 4.9 mL of site water |
| 6a,b,c | Add 4.9 mL of site water and 0.333 g HgCl ₂ |
4. A 5 mg/L final concentration of *cis*-DCE was added to each bottle (2.45 mL of 1,000 mg/L *cis*-DCE in DI water).
 5. Small amounts of additional site water were added to achieve a final volume of 490 mL in each incubation vessel.

After all amendments were added, the microcosms were “topped off” with groundwater so that < 1 mL headspace was present in each bottle. The bottles were then tightly sealed with Teflon[®]-lined caps and removed from the anoxic chamber. Each bottle was then placed at 15 °C on a bottle roller operating at ~0.5 rotations per minute for incubation for an appropriate period of time depending on the treatment prior to sampling again for analysis of *cis*-DCE. The sampling procedure and analytes measured are provided in Section 5.6.3 and 5.7, respectively.

Microcosm #2, Groundwater Only and Groundwater + Solids Treatments. Ten treatments were established in the JBLM 2 microcosms, which were prepared in 1000 mL clean, sterile Boston Round Bottles. All microcosms were amended with 500 mg/L LASS, 10 mg/L *cis*-DCE, and 10 mg/L Br⁻ (NaBr) prepared as previously described. Microcosms received SDC-9 at four expected *Dhc* cell titers; 10^7 *Dhc*/mL (1a, b, c and 1d, e, f), 10^6 *Dhc*/mL (2a, b, c and 2d, e, f), 10^5 *Dhc*/mL (3a, b, c) and 10^4 *Dhc*/mL (4a, b, c) as previously described except that dilutions were prepared in site groundwater rather than medium. Live (5a, b; 5 c, d) and killed (6 a, b; 6c, d) controls were also prepared. For replicates a,b,c in treatments 1-4 and a,b in treatments 5-6, only groundwater was added to the bottles. The remaining bottles (replicates d,e,f in treatments 1 and 2 and replicates c, d in treatments 5 and 6) received 353 g of aquifer solids at 15% moisture content (300 g dry weight). After all amendments had been added, the microcosms were completely filled with site groundwater, sealed and placed on a bottle roller (1 rpm) at 15 °C. After 10 days of incubation, 2 g of CaCO₃ (solid) was added to all microcosms due to an observed decline in pH in some bottles with SDC-9 added. Treatment 1, with the highest *Dhc* concentration, was set up a second time with an initial 2 g of CaCO₃ added to ensure that the declining pH did not affect degradation kinetics. A photograph of the JBLM 2 microcosm bottles with and without sediments is provided in Figure 5-2.

5.6.3 Microcosm Sampling Procedure

Water samples were collected from microcosms in order to measure contaminant degradation and RDase biomarkers as described below.

Microcosm #1 Sampling. All sampling was performed in a Coy anoxic chamber with a N₂ headspace. Liquid samples for chemical analysis were removed from the microcosms with gas-tight syringes to appropriate sample containers. Samples volumes consisted of the following: 2 mL for cVOCs (EPA Method 8260); 4 mL for methane, ethane and ethene (EPA 3810/RSK-175); 1 mL for anions (EPA Method 300.0) and volatile fatty acids (EPA 300m); and 5 mL for pH determination. The methods of analysis are provided in Section 5.7 and Appendix C.

Duplicate 15-mL aqueous samples, one for qPCR and the other for proteomic analysis, were removed using a glass pipette and transferred to sterile screw-cap 50-mL conical tubes. Cells were collected by centrifugation for 40 min at 11,000 rpm using a refrigerated Sorvall Lynx 6000 Centrifuge and a F21-8x50y rotor (Thermo Scientific). Immediately after centrifugation, the supernatant was aspirated from the cell pellets and the samples frozen at -80 °C. Microcosms were refilled with site water removed during sampling, punctured septa were replaced, and bottles were returned to rollers operating at 1 rpm and 15 °C.

Samples from the microcosm treatments were collected at different intervals based upon the initial concentration of *Dhc* added. See summary of cVOC sampling times bulleted below. Samples for qPCR and proteomic analysis were not collected and/or analyzed at each of these time points, but generally at the beginning, middle, and end of the incubating periods. Specific times for sample collection from each microcosm bottle are provided in Appendix E.

- Set 1: 0, 1, 2, 4, 6, 8, and 24 hours
- Set 2: 0, 8, 24, 48, 72, 96, 120 hours
- Set 3: 0, 6, 9, 13, 16, 20, 23, 27, 36, 55, and 83 days
- Set 4: 0, 6, 13, 20, 27, 36, 55, and 83 days
- Set 5 and Set 6: 0, 6, 9, 13, 16, 20, 23, 27, 55, and 83 days



Figure 5-2. Photograph of microcosms with and without sediments on the bottle roller (left) and settling for sample collection (right).

Microcosm #2 Sampling. Microcosm sampling was conducted as described for Microcosm #1, except that bottles with solids were removed from the rollers for 30 minutes to allow solids to settle prior to liquid sampling. See summary of cVOC sampling times bulleted below. Samples for qPCR and proteomic analysis were not collected and/or analyzed at each of these time points, but generally at the beginning, middle, and end of the incubating periods.

- Set 1: 0, 3, 7, 10, 14, 17, 21, 24, and 28 days
- Set 1 (duplicate): 0, 0.3, 1, 1.3, 2, 2.3, 3, 3.3, and 7 days
- Set 2: 0, 3, 7, 10, 14, 17, 21, 24, 28, 31, 35, 38, and 42 days
- Set 3 and Set 4: 0, 7, 14, 21, 28, 35, and 42 days
- Set 5 and Set 6: 0, 3, 7, 9, 14, 17, 21, 24, 28, 31, 35, 38, and 42 days

5.7 SAMPLING & ANALYSIS METHODS

This section provides a summary of all samples collected during the laboratory project (Table 5-2), as well as a summary of the analysis methods used (Table 5-3). Method SOPs and detailed QC procedures are included in Appendices C and D, respectively.

Table 5-2. Number of microcosm test samples by analysis

Component	Matrix	Number of Samples	Analyte	Location ^a
Screen aquifer material from 3 candidate cVOC-contaminated aquifer sites	Groundwater	3	cVOCs, pH	JBLM LF2, VAFB SA288, NBK Keyport Area 1
	Groundwater/Aquifer Material Slurries	3	cVOCs, pH	JBLM LF2, VAFB SA288, NBK Keyport Area 1
	<i>Dhc</i> activity inhibitor screening microcosm	3	cVOCs, pH	JBLM LF2 only
JBLM Microcosm#1, Groundwater only	Groundwater	24	peptides	JBLM LF2
	“	24	genes	“
	“	159	anions	“
	“	159	cVOCs	“
	“	159	VFAs	“
JBLM Microcosm#2, Groundwater only treatments	Groundwater	18	peptides	JBLM LF2
	“	18	genes	“
	“	182	anions	“
	“	182	cVOCs	“
	“	182	VFAs	“
JBLM Microcosm#2, Groundwater+ aquifer solids treatments	Groundwater ^b	18	peptides	JBLM LF2
	“	18	genes	“
	“	145	anions	“
	“	145	cVOCs	“
	“	145	VFAs	“
JBLM Microcosm#2, Groundwater+ aquifer solids treatments	Groundwater ^b	18	peptides	JBLM LF2
	“	18	genes	“
	“	145	anions	“
	“	145	cVOCs	“
	“	145	VFAs	“
JBLM Microcosm#2, Groundwater+ aquifer solids treatments	Groundwater ^b	18	peptides	JBLM LF2
	“	18	genes	“
	“	145	anions	“
	“	145	cVOCs	“
	“	145	VFAs	“

a. Field materials were collected from candidate sites described in Section 4.0.

b. Groundwater collected after allowing solids to settle per Section 5.6.3.

Table 5-3. Summary of standard analytical and RDase biomarker methods

Sample Type	Analyte	Method	Container	Preservative	Hold Time
Microcosm Samples	peptides	Proteomics (Section 5.4.2, Appendix B)	Sterile 15 mL plastic Falcon tube	-80°C	N/A
	genes	qPCR (Section 5.4.3, Appendix B)	Sterile 15 mL plastic Falcon tube	-80°C	N/A
	Anions	EPA 300.0	1 mL	4°C	7 days
	cVOCs	EPA Method 8260B	2 mL	HCl	28 days
	VFAs	EPA 300 m	< 100 µL (aliquot from anion sample)	4°C	7 days
	Dissolved gasses	EPA Method 3810, RSK-175	5 mL w/1 mL headspace	HCl	28 days

N/A reflects direct injection analysis for cVOCs and dissolved gasses upon sample collection

5.7.1 Analytical Methods: Standard Geochemical cVOC Analyses

Microcosm samples (groundwater/sediment mixture) were analyzed using the following standard EPA procedures or modifications of these procedures for the analytes of interest at Aptim. Detailed

method procedures are included in Appendix C. Inorganic anions were determined according to EPA Method 300.0, which uses ion chromatography. cVOCs were determined by EPA Method 8260B using gas chromatography and mass spectrometry. cVOCs were introduced into the gas chromatograph by the purge-and-trap method 5030B. Dissolved gases including methane, ethane and ethene were analyzed according to EPA Method 3810, RSK-175 [39]. For this method, a 4-mL volume of water from the microcosm was added to a 5 mL serum vial, and the vial was then sealed and shaken to equilibrate the headspace with the aqueous phase. The headspace was then analyzed for dissolved gases by GC using direct injection. The Henry's law coefficient for each gas was then used to calculate the aqueous concentration. Volatile fatty acids (VFAs) including acetate, lactate, formate, and propionate were measured using ion chromatography via modification of EPA method 300 (EPA 300m). pH was measured using a pH meter and microprobe.

5.7.2 Analytical Methods: Proteomics

Samples collected during the microcosm study were frozen at -80 °C then shipped on dry ice overnight to Battelle Memorial Institute for proteomic analyses. Proteins were extracted from lyophilized groundwater/sediment slurry samples, reduced, alkylated, trypsin digested, and subjected to LC-MS/MS using a Nano 415 LC system in line with an ABI Sciex Triple TOF 5600 high resolution MS instrument (Sciex, Concord, Canada) (Figure 5-3). During processing, the entire sample was subjected to protein extraction since protein and peptide concentration determination is a prerequisite for optimal protein digestion and optimal sample loading amount in bottom-up proteomics. The protein and peptide concentrations were calculated with a tryptophan assay [36]. For qProt, samples were spiked with selected isotopically labeled peptides at the digestion step for quantification of native peptide equivalents.

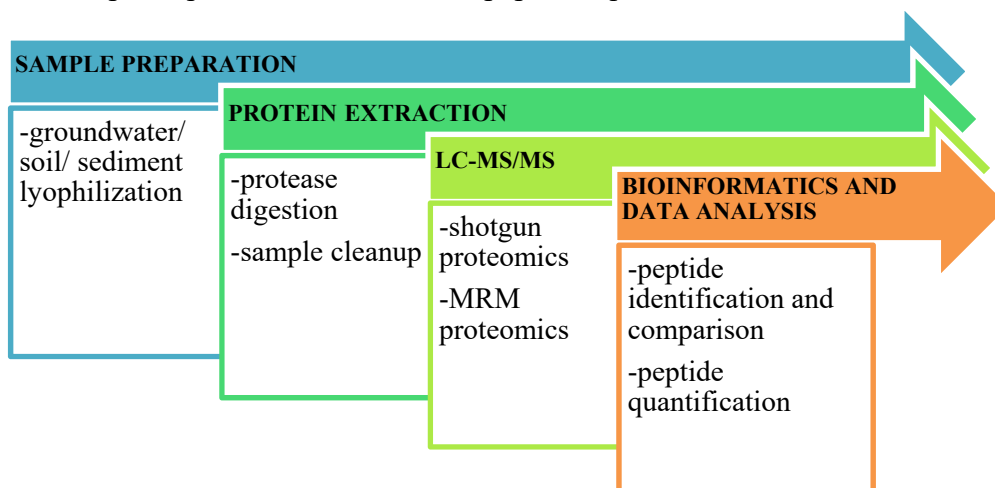


Figure 5-3. Steps involved in proteomic analysis of microcosm test samples

Sample MS and MS/MS data were acquired using an Eksigent Nano 415 liquid chromatograph system (Sciex, Concord, Canada) directly connected to a quadrupole time-of-flight (QqTOF) TripleTOF 5600 mass spectrometer (Sciex, Concord, Canada). The instrumentation is controlled using Analyst TF 1.6 and Eksigent software. A total of 25 µL of sample was injected onto a 0.3 mm x 150 mm Eksigent C18-CL-120 analytical column (3 µm particle size, 120 Å pore size,) using a trap-and-elute method. Peptide separation was achieved using a linear gradient of acetonitrile containing 0.1% (v/v) formic acid of different lengths depending on the acquisition

mode. Solvents used included 0.1% formic acid (v/v) (solvent A) and 0.1% formic acid (v/v) in acetonitrile (solvent B). Peptides were trapped on the loading column using 100% solvent A at a flow rate 5 $\mu\text{L}/\text{min}$ for 5 min. Trapped peptides were then separated at a predetermined flow rate using the following conditions: (1) 5% solvent B in A (from 0-5 min), (2) 5-35% solvent B in A (from 5-65 min), (3) 35-90% solvent B in A (from 65-66 minutes), and (4) 90% solvent B in A (from 66-70 minutes), with a total runtime of 90 min, including mobile phase equilibration.

Continuing mass calibration of the TOF MS and TOF MS/MS was performed throughout the analysis sequence by analyzing a digested β -galactosidase standard (Sciex, Concord, Canada). Mass spectrometric analysis was performed using data dependent acquisition (referred to as information dependent acquisitions, or IDA). Full scan spectra were acquired for specific m/z with a 250-millisecond acquisition time. For collision induced dissociation tandem mass spectrometry (CID MS/MS) in IDA mode, the mass window for precursor ion selection of the quadrupole mass analyzer was set to unit resolution ($\pm 0.5 m/z$). For MS/MS analysis, precursor ions were fragmented in a collision cell using nitrogen as the collision gas. For IDA analysis, the instrument was set to trigger product ion scans (from 100 to 1500 m/z) only after specific criteria were met by the precursor ions. These criteria were defined during the IDL and MDL analyses. The Rolling CE algorithm was used to determine the appropriate collision energy for each precursor mass.

For quantification, labeled RDase conserved peptides selected based on multiple sequence alignment of known RDase protein sequences and an internal bovine serum albumin control were spiked into sample extracts immediately prior to the protease digestion step. Native peptide concentrations were determined by comparing peak ratios of native and isotopically labeled peptides. Proteins were identified from LC-MS/MS spectra by searching against a database of protein sequences constructed from the metagenome sequences of the SDC-9 microbial community. In addition, sequences of protein contaminants typical for proteomic experiments (e.g., keratin and trypsin) were added to the database. The proteomic LC-MS/MS data were queried against this database and searched against the library of known selected enzymes involved in cVOC degradation. Only peptides with the “Protscore” for a particular protein higher than 1.3 were considered true positives. Statistical analyses of proteomic data were performed using Protein Pilot (confidence score and false discovery rate).

5.7.3 Analytical Methods: Quantitative PCR

Reductive dechlorination biomarker genes were enumerated in aqueous samples with qPCR following established procedures [7, 14]. Frozen cell pellets collected from the microcosms (details in Section 5.6.3) incubated in the Aptim laboratory were shipped overnight in a cooler with dry ice to the University of Tennessee. The samples were stored frozen at -80°C until analysis.

DNA extraction. The cell suspensions were thawed, and DNA was isolated from cell pellets with DNeasy PowerLyzer PowerSoil Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions except for using a bead-beating method (OMNI Bead Rupter Homogenizer, 5 m/s for 3 min) (OMNI International, GA) for enhanced cell lysis. DNA was eluted into nuclease-free water and DNA concentration and quality were determined with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and a Qubit fluorometer (Invitrogen) using

double stranded DNA (dsDNA) Broad-Range assay kit according to the manufacturer’s manual. DNA was stored at -80°C until analysis.

qPCR. Primers and probes to enumerate total bacterial 16S rRNA, *Dhc* 16S rRNA, *tceA* and *vcrA* genes have been reported [3, 7, 10, 40]. In addition, new primer and probe combinations have been designed for SDC9_24_ *pceA* and *fdhA* (Table 5-4).

Table 5-4. Summary of specific qPCR assays run for microcosm samples

Assay ID	Organism(s)	Target Gene
Bac_16S	Total Bacteria	16S rRNA
Dhc_16S	<i>Dhc</i> -specific	16S rRNA
SDC9_24_ <i>pceA</i>	<i>Dehalobacter restrictus</i> / <i>Desulfitobacterium hafniense</i>	PCE reductive dehalogenase
SDC9_59_ <i>tceA</i>	<i>Dhc</i>	TCE reductive dehalogenase
SDC9_212_ <i>vcrA</i>	<i>Dhc</i>	VC reductive dehalogenase
<i>fdhA</i> (<i>omeA</i>)	<i>Dhc</i> -specific	Molybdoenzyme involved in electron transfer to the RDase

Specific primers and TaqMan probe sequences targeting SDC9_24 *pceA* and *fdhA* were designed using Geneious R11.0.2 (<http://www.geneious.com>, [41]) and primers were synthesized by IDT (Integrated DNA Technologies). In order to ensure specific hybridization at a uniform temperature, probes with Minor Groove Binder (MGB) modification were synthesized by Thermo Fisher Scientific. Design parameters for the target assays included primer and probe annealing temperature close to 60°C, primer and probe lengths ranged between 14-30 and 16-25 base pairs (bp), respectively and parameters were set to ensure that it was thermodynamically unlikely to form hairpin structures, self-dimers and heterodimers for primers and TaqMan probes. The specificity of the primers and probes was also verified using primer-BLAST analysis [42].

(i) For regular qPCR, every 20-μL reaction had 10 μL of 2×TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA), 2 μL of diluted (1:10 and 1:100) DNA template, and forward and reverse primers and probe at final concentrations of 300 nM each. Reactions were initially held for 2 min at 50°C and 10 min at 95°C following 40 cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 min. The qPCR assay results were analyzed using the ViiA7 Software (Applied Biosystems, Carlsbad, CA). All qPCR assays were performed in triplicate.

(ii) For high-throughput qPCR in 384-well microtiter plates, all qPCR reactions were performed with the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems, Carlsbad, CA), a flexible platform enabling the instrument to accommodate one 384-well plate. Every 10-μl reaction contained 5 μL of 2×TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA), 2 μL of diluted (1:10 and 1:100) DNA template, and forward and reverse primers and probe at final concentrations of 300 nM each. Reactions were initially held for 2 min at 50°C and 10 min at 95°C following 40 cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 min. The qPCR assay results were analyzed using the QuantStudio 12K Flex Real-Time PCR System Software (Applied Biosystems™, Carlsbad, CA).

Plasmid DNA containing each of the cloned target gene was used as templates for standard curves. Standard curves were included with every qPCR plate using 10-fold serial dilutions of plasmid DNA over a 7 orders of magnitude range beginning at a $1 \text{ ng } \mu\text{L}^{-1}$ concentration ($\sim 8 \text{ log gene copies}$) and decreasing to $10^{-7} \text{ ng } \mu\text{L}^{-1}$. All standard curves had a total of eight calibration points and were run in triplicate. To calculate the number of gene copies in a known amount of DNA and gene copies per sample, previously published equations were applied [7].

From this original dilution series, Level of Detection (LOD) and Level of Quantitation (LOQ) were determined experimentally as 1-10 copies/ μL and 10-50 copies/ μL , respectively based on targeted assay. Examples of qPCR standard curves are given in Appendix C.

To further understand RDase expression and regulation as well as potentially correlate degradation rate to gene expression, transcript (mRNA) measurements were planned. Using transcript measurements, some correlation between dechlorination activity and RDase gene expression has been demonstrated, but the correlation is highly inconsistent and currently unpredictable [5]. This inconsistency is due to the fact that RDase gene expression is regulated by various environmental factors (e.g., growth phase, contaminant concentrations, etc.) through poorly understood accessory proteins. Further, reproducible and quantitative RNA extraction remains challenging due to the inherent susceptibility of RNA to degradation (i.e., unintended loss of biomarker) [43]. Thus, while informative, assays that use ribonucleic acid provide no reliable correlation to degradation rate and no direct information about the catalysts (i.e., the enzymes) that actually perform the biodegradation reaction, and measurements of transcripts to determine gene activity were not applied to samples in this study.

5.8 DATA ANALYSIS

Estimating Reductive Dechlorination Rates. First-order rate coefficients for biological reductive dechlorination of *cis*-DCE and VC (k_{DCE} and k_{VC}) in the microcosms were estimated by fitting a numerical approximation of the first order reaction equations for sequential degradation of *cis*-DCE to VC and then to ethene to the change in molar concentrations of *cis*-DCE, VC, and ethene (target compounds) over time in the microcosms. Microsoft Excel Solver was used to minimize the sum of squares error between the measured and model-estimated values to obtain the best fit. Measured values were corrected for dilution by applying a dilution factor equal to the total moles of target compounds present in the microcosm when the sample was collected divided by the total moles of target compounds present at time zero. Dilution correction using bromide concentrations yielded similar results. The number of moles in individual target compounds in microcosms that contained aquifer solids were corrected for sorption to the solids by assuming linear partitioning and using equation 3 below,

$$M_{tot, sorption\ corrected} = C_w V_w + C_w M_s (0.63 K_{ow} f_{oc}) \quad \text{equation 3}$$

where C_w is the dilution-corrected molar concentration in water, V_w is the volume of water present in the microcosm, M_s is the mass of solids in the microcosm, K_{ow} is the VOC-specific octanol-water partitioning coefficient, and f_{oc} is the fraction of organic carbon present in the solids, which was assumed to be 0.001. The constant 0.63 converts units of the product of K_{ow} and f_{oc} into L/kg. K_{ow} values used for *cis*-DCE, VC, and ethane were 72.4, 28.8, and 13.5, respectively [44]. Rate coefficients for each test replicate microcosm were determined separately using this approach; rate

coefficients for live and killed controls were not determined. The 95% confidence interval associated with each fitted rate constant was determined using the approach described in Smith et al. 1998 [45].

Correlating Reductive Dechlorination Rates and Biomarker Abundances. Log-transformed rate coefficients and biomarker abundances were subject to the Shapiro-Wilk normality test. The Spearman Rank Order Correlation analysis followed by power law least squares regression analysis was used to quantify the relationship between biomarker abundances and rate coefficients. Rate coefficients represent an integrated measure of activity in each microcosm over time, whereas biomarker abundances provide a single measure at various time points during the incubation. Correlation analysis was performed twice: once using the global data, which included biomarker abundances collected at early, mid and late time points from each microcosm with the corresponding integrated rate coefficients measured for that microcosm, and again using only mid-point biomarker abundances and the corresponding rate coefficients.

Assessing Predictive Power of Biomarker Abundances. The microcosm experiments were divided into a “training set” and an “evaluation set.” Rate coefficients and biomarker abundances from the “training set” microcosms were subject to power law least squares regression analysis of rate constants on abundance. Only data from microcosms where non-zero rate coefficients were obtained were included in the analysis. Measured biomarker abundances in the “evaluation set” of microcosms were entered as “x” variable in the regression equations to obtain a predicted rate coefficient. The predicted rate coefficient was then compared to the measured rate coefficient for that “evaluation set” microcosm. Two types of training sets were established. The first training set featured randomly selected microcosms that contained either 10^6 , 10^7 or 10^8 *Dhc* cells/mL. The second training set featured the global average of biomarker abundances and rate coefficients for all microcosms that contained either 10^6 , 10^7 or 10^8 *Dhc* cells/mL. The predictive power of the biomarkers were evaluated by comparing (1) the measured vs. predicted rate constants for each individual biomarker, (2) the measured vs. the average of the predicted rate constants for the various gene biomarkers, (3) the measured vs. the average of the predicted rate constants for the various peptide biomarkers, and (4) the measured vs. the average of the predicted rate constants for all of the biomarkers.

6.0 PERFORMANCE ASSESSMENT

6.1 DETERMINATION OF RDASE TARGETS WITH SHOTGUN PROTEOMICS

Shotgun proteomic analyses of SDC-9 extracts identified 35 RDase peptides. The tryptic peptides included the most abundant peptides of TceA, PceA and VcrA, which were detected with 99-100% peptide coverage. Five non-tryptic peptides (i.e., truncated from one end [either N-terminal or C-terminal] of the tryptic peptide), were observed with confidence levels exceeding 90% (three corresponding to PceA and two corresponding to TceA). A single peptide corresponding to VcrA was observed with low confidence (<50%). Sixteen peptides corresponding to PceA and fourteen peptides corresponding to TceA were identified with maximum confidence (100% sequence identity). The final list of SDC-9 unique RDase peptide targets and their transitions downselected for the MRM work are listed in Table 6-1.

Table 6-1. PRM transitions of selected RDase SDC-9 endogenous peptides

Protein	Peptide Sequence	Precursor <i>m/z</i>	RT ^d	Product <i>m/z</i> ^c	Ion	CV ^a	R ²	IDL ^b	Accession Number
PceA	IATQIPLLQDAAR	705.4 [M+2H] ²⁺	45.3	1225.7	y11	4.3	0.98	5	CAD28790.2 WP_025206074.1
				996.6	y9	3.5			
				883.5	y8	5.1			
	LESGYVQNMVK	634.3 [M+2H] ²⁺	33.2	1025.5	y9	2.2	0.98	5	CAD28790.2 CDX02974.1 WP_025206074.1
				938.5	y8	0.8			
				718.4	y6	3.2			
VYTDLELAPDKPR	506.3 [M+3H] ³⁺	33.2	925.5	y8	7.3	0.97	1	CAD28790.2 CDX02974.1 CDX02974.1	
			683.4	y6	5.1				
			612.3	y5	5.9				
TceA	VNNEPWWVTTR	701.3 [M+2H] ²⁺	43.6	1188.6	y11	4.9	0.97	5	WP_062900263.1
				945.5	y7	1.7			
	YFGASSVGAIK	550.3 [M+2H] ²⁺	34.5	848.4	y6	4.1	0.98	0.5	WP_062900263.1
				936.5	y10	2.9			
				789.4	y9	3.4			
	YSGWNNQGAYFL PEDYLSPTYTGR	933.8 [M+3H] ³⁺	54.5	661.4	y7	1.3	NA	100	WP_062900263.1
978.4				b9	4.5				
1172.6	y10	1.6							
			1057.5	y9	3.4				
VcrA	VVTDLPIAPTPPID AGMFEFCK	806.7 [M+3H] ³⁺	56.7	1104.4	y9	2.6	NA	100	See peptide BLAST results in SI; no match with sequences from metagenomic hits
				989.4	y8	3.2			
				918.4	y7	3.2			
	SLNNFPWYVK	634.3 [M+2H] ²⁺	48.1	1067.5	y8	3.1	0.98	50	See peptide BLAST results in SI; no match with sequences from metagenomic hits
				839.4	y6	6.8			
				692.4	y5	6.6			
GLGLAGAGIGAVA	1086.9 [M+3H] ³⁺	ND	1211.1	y23		ND		AEI59454.1	
ASAP- VFHDIDEFVSSEA			1161.5	y22					
NSTK			1126.0	y21					

a. CV calculated from n=3 replicates from a 25, 50, 250 or 500 fmol/μL isotopically-labeled standard; b. units are in fmol/μL and values represent IDL for the isotopically-labeled standards; c. bolded product *m/z* represent those used for quantitation; d. retention time, minutes

6.2 METHOD DETECTION AND INSTRUMENT DETECTION LIMIT RESULTS

RDase peptide hits identified from shotgun mass spectrometry experiments (Table 6-1) and additional FdhA peptides were selected for targeted quantification based on the following selection criteria: confidence score >90%, no missed cleavages, non-tryptic, no methionine oxidation, and no carbamidomethylation. Peptides were then searched against the National Center for Biotechnology Information (NCBI) protein database to assess specificity to RDase targets. Three peptides per protein were monitored, and the three most sensitive transitions per peptide were reported. Isotopically labeled peptide standards were used to verify transitions, quantify selected peptides and determine retention time on the liquid chromatography system for all peptides. Instrument detection limits were reported as the lowest isotopically labeled standard that satisfied a signal-to-noise ratio ≥ 3 across three separate analyses. The most sensitive transition per peptide was used for quantification, and the remaining transitions were used as qualifiers. More specifically, several transitions for a given peptide ion were measured to validate the identification of a peptide, otherwise known as qualifiers. Only subsets of the transitions, typically the transition(s) with the highest intensities, were used for quantification of the peptide, also known as the quantifier. For example, the y9 ion of YFGASSVGAIK was the most sensitive ion for that peptide and was used for quantification, while detection of y7 and y10 ions were requisite to increase confidence in protein quantifications.

To determine the limit of detection, triplicate measurements of standard peptides at eight different concentrations in 0.1% formic acid were performed. The signal to noise ratio per each peptide was measured, and the standard deviations of the response at each concentration were calculated. CEs were optimized for initial 28 isotopically-labeled peptides to maximize the resulting signal from product ions. Following the optimization step, dwell time and solvent program were optimized and peptides that demonstrated poor signal response were discarded from the list. In total, 10 peptides were discarded from the list after optimization steps were completed and IDLs and MDLs were developed for the remaining peptides. Most peptides were observed in experimental samples during the IDL and MDL analysis, however VcrA peptides were observed exclusively in MDL experiments and not in IDL experiments (not observed is denoted as <250 fmol/ μ L), suggesting that sample digest and cleanup enhance the peptide signals for VcrA peptides. Two VcrA peptides (DQPWYVK and VPDHAVPINFK) were detectable in all MDL experiments while they were not detected in IDL experiments. Performance variation between IDL and MDL experiments are likely due to matrix effects. Some peptides performed similarly between MDL replicates (e.g., DQPWYVK) while others did not. Within the MDL set, inconsistencies were observed for sensitive peptides (e.g., TSPSLISSATVGK, VSSIIEPR, YFGASSVGAIK). This variation represents the variation present in the preparatory methods and instrumental analysis; it is unlikely that instrumental variation resulted in decreased sensitivity as control samples did not reveal loss of chromatographic quality or loss in mass spectrometer signal during the MDL runs.

Overall, the experiments performed allowed for identification of the most sensitive RDase and FdhA peptides for targeted quantification. MDL experiments resulted in detection of up to three of the most sensitive peptides per protein with up to three of the most intensive transition ions per peptide (Table 6-2).

Table 6-2. MDL for peptides analyzed for SDC-9 culture

Protein	ID	Peptide ¹	MDL 1	MDL 2	MDL 3	Established MDL
FdhA	FdhA2	SGSEIAFTGGLIK ¹	3	3	3	3
	FdhA5	ALGIVYLD SQAR	3	3	1	3
	FdhA8	NQAVSAPGEAK	3	3	3	3
PceA	PceA4	IATQIPLLQDAAR	9	9	9	9
	PceA5	LESGYVQNMVK	3	3	3	3
	PceA7	DFWNNPEPIK	1	1	1	1
	PceA8	TSPSLISSATVGGK	0.3	0.3	1	1
TceA	TceA2	DVDDLLSAGK	0.3	3	3	3
	TceA3	VSSIIIEPR	0.3	0.3	1	1
	TceA4	VNNEPWWVTTR	9	9	9	9
	TceA5	YFGASSVGAIK	0.3	0.3	1	1
VcrA	VcrA1	WGLYGPPHDSAPPDGSVPK	9	9	3	9
	VcrA2	YFGAGDVGALNLADPK	27	27	27	27
	VcrA3	VPDHAVPINFK	0.3	0.3	1	1
	VcrA4	GVYEGPPDAPFTSWG NR	83	27	27	83
	VcrA6	DQPWYVK	1	1	1	1

Units are fmol-peptide. A 1.0 mL sample was extracted to determine the MDLs.
¹ **Bolded letter denote heavy ¹³C and ¹⁵N labeled amino acid**; the maximum of three MDL test replicates was established as the MDL

The data generated during the optimization and calibration experiments were built into the MRM method (i.e. the “qProt assay”) used for quantification of RDase peptides in the microcosm experiment samples obtained during this laboratory study.

6.2 VALIDATION OF MRM ASSAY QUANTITATION LIMITS RESULTS

Quantitation limits of the qProt MRM assay for FdhA, PceA, TceA and VcrA proteins were validated by analyzing triplicate samples of SDC-9 culture diluted to 10⁵, 10⁶, or 10⁷ *Dhc* cells/mL.

Only two out of three FdhA peptides, namely FdhA2 and FdhA5, were observed in the analyzed samples. The FdhA8 peptide was not detected. FdhA2 and FdhA5 peptides were detected in all SDC-9 cell dilutions (10⁵ to 10⁷ *Dhc* cells). However, only 10⁷ *Dhc* cells concentrations rendered quantifiable concentrations above the lower limit of MDL for these two peptides. FdhA2 peptide was selected as a quantifier based on the detection of the fragment ions showing the lowest LOQ. Table 6-3 shows FdhA peptide concentrations per total *Dhc* cell number. Peptides PceA4, PceA7 and PceA8 showed lower sensitivity than the PceA5 peptide, which was detected in samples with 10⁵ *Dhc* cells/mL. The lowest abundance of *Dhc* cells that generated detectable and quantifiable concentrations of PceA4, PceA7 and PceA8 was 10⁶, while 10⁵ *Dhc* cells was needed to quantify the PceA5 peptide. The, PceA5 and PceA4 peptides were selected as quantifiers for the proceeding studies. The TceA2, TceA3 and TceA5 peptides had highest sensitivity and were detected and quantified in samples containing 10⁵ *Dhc* cells (Table 6-3). However, the TceA5 peptide had a relatively inconsistent retention time variation and the peptides TceA2 and TceA3 were selected as quantifiers for the proceeding studies. The TceA4 peptide was detected in samples with 10⁷ *Dhc* cells/mL. The VcrA peptides were least sensitive, with VcrA1, VcrA2, VcrA3 and VcrA6 peptides detected in the 10⁶ and 10⁷ *Dhc* cells and with small number of transition ions passing the accuracy

criteria. The VcrA4 peptide was detected but below the quantification limit in this study and most likely requires higher number of *Dhc* cells for quantification. Of all VcrA peptides, the VcrA3 was selected as a quantifier due to its highest sensitivity, detection of the highest number of transitions and its low MDL of 1 fmol/mL.

The analysis of the compiled data shows that the most sensitive peptides for quantification were FdhA2, PceA4 and PceA5, TceA2 and TceA3 and VcrA3 and required abundances of 2.2×10^6 *Dhc* cells/mL or more in the 15 mL sample that was extracted (corresponding to 10^7 *Dhc* cells or more extracted) to be detected (Table 6-3). These peptides served as quantifiers in the next set of experiments. The remainder of qualifier peptides was used to confirm accuracy of the detection method and were analyzed in all subsequent experiments. Additionally, four to six transition ions were analyzed per peptide ion to confirm accuracy and sensitivity of the method and to confirm peptide sequence. In this study, up to six transitions for a given peptide ion were measured to validate the identification of a peptide.

The required lowest concentration of *Dhc* cells for detection of the remainder of peptides varied per protein, for example, to detect other TceA peptides a minimum of 10^5 *Dhc* cells are required, but to detect VcrA specific peptides the cell concentrations need to be an order of magnitude higher. Thus, the total recommended *Dhc* cells in a sample for targeted proteomics is 10^7 cells, regardless of sample volume.

Table 6-3. Results of qProt assay quantitation limit validation study

Protein	Peptide ID	<i>Dhc</i> abundance in sample, <i>Dhc</i> cells/mL ^b						Previously reported [46, 47] protein concentrations in culture, fmol/mL (protein/cell)
		1.3x10 ⁵	2.2x10 ⁶	3.1x10 ⁷	1.3x10 ⁵	2.2x10 ⁶	3.1x10 ⁷	
		Peptide concentration in culture, fmol/mL			Protein concentration in culture, fmol/mL (protein/cell) ^c			
FdhA	FdhA2 ^a	<3.0x10 ⁰	<3.0x10 ⁰	8.5x10 ⁰	<3.0 x10 ⁰	<3.0 x10 ⁰	8.5x10 ⁰ (3.8x10 ³)	[46]KB1, D2 culture (TCE): 9.0x10 ¹ – 1.0x10 ² (2.3x10 ³ – 3.5x10 ³)
	FdhA5	<3.0x10 ⁰	<3.0x10 ⁰	1.1x10 ¹				
PceA	PceA4 ^a	<9.0x10 ⁰	<9.0x10 ⁰	2.1x10 ¹	6.3x10 ²	1.9x10 ²	4.4x10 ¹	
	PceA5 ^a	6.3x10 ²	1.9x10 ²	6.8x10 ¹				
	PceA7	<1.0x10 ⁰	2.2x10 ¹	1.9x10 ¹				
	PceA8	<1.0x10 ⁰	1.7x10 ¹	1.2x10 ¹				
TceA	TceA2 ^a	<3.0x10 ⁰	1.9x10 ¹	1.3x10 ¹	<1.0x10 ⁰	2.5x10 ¹ (1.1x10 ⁴)	1.7x10 ¹ (7.7x10 ³)	(47)2.3x10 ³
	TceA3 ^a	<1.0x10 ⁰	3.1x10 ¹	2.1x10 ¹				
	TceA4	<9.0x10 ⁰	<9.0x10 ⁰	2.3x10 ¹				
	TceA5	<1.0x10 ⁰	2.4x10 ¹	1.7x10 ¹				
VcrA	VcrA1	<9.0x10 ⁰	1.7x10 ²	1.8x10 ¹	<1.0x10 ⁰	5.7x10 ¹ (2.6x10 ⁴)	9.3x10 ⁰ (4.2x10 ³)	Difficult to quantify due to low peptide sensitivity
	VcrA2	<2.7x10 ¹	7.6x10 ¹	4.9x10 ¹				
	VcrA3 ^a	<1.0x10 ⁰	5.7x10 ¹	9.3x10 ¹				
	VcrA4	<8.3x10 ¹	<8.3x10 ¹	<8.3x10 ¹				
	VcrA6	<1.0x10 ⁰	5.8x10 ¹	1.1x10 ¹				

^aQuantifier peptides used to estimate protein abundance, in cases where multiple quantifier proteins exist those abundances are averaged to obtain protein abundance, a 1:1 peptide to protein ratio is assumed; ^b15mL of each cell density were extracted, corresponding to 2×10^6 to 5×10^8 *Dhc* cells extracted; ^cDetected proteins were expressed in both fmol/mL and protein/cell concentration units

6.3 MICROCOSM STUDY RESULTS

Reductive dechlorination of *cis*-DCE and VC and subsequent production of ethene were observed in the microcosm experiments as a general function of the SDC-9 inoculum concentration; Figure 6-1 provides an example of relevant microcosm data. Losses in uninoculated-live and killed-control microcosms were comparatively small and similar in magnitude, presumably due to volatilization during sample collection (see panel C in Figure 6-1 for data from an uninoculated-live microcosm). The data indicate that indigenous dechlorinating organisms did not contribute significantly to the observed rates of *cis*-DCE or VC degradation. In microcosms inoculated with SDC-9, lactate was generally fermented to acetate and propionate (Figure 6-1, panel B), which then declined slowly over time. Bromide was used as a conservative tracer to document losses of VOCs and fatty acids due to dilution as all water in the bottles was replaced with VOC- and VFA-free site water after sample collection (Figure 6-1, panel D).

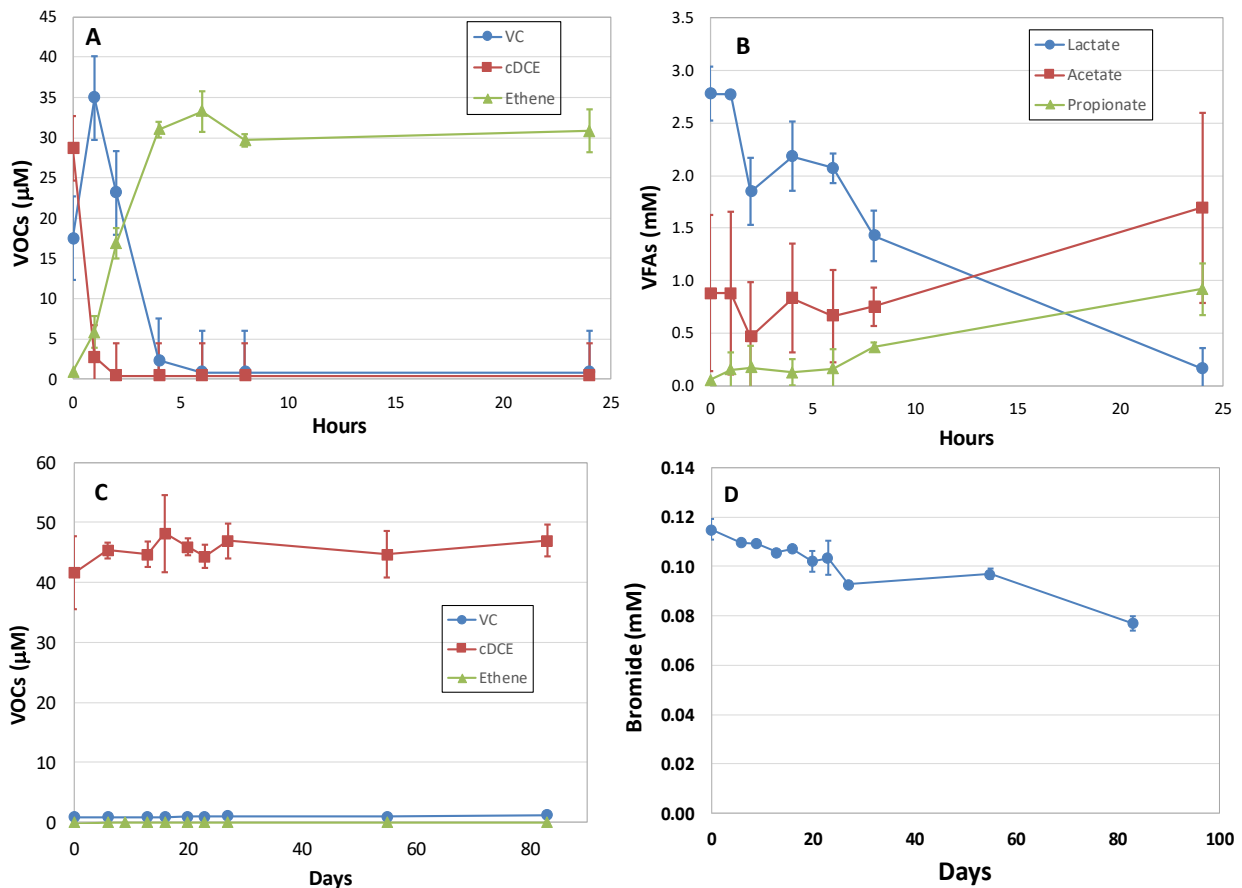


Figure 6-1. Concentrations of VOCs, VFAs and bromide in select JBLM #1 microcosms. Panel A and panel B show VOC and VFA concentrations, respectively, in microcosms receiving the highest SDC-9 inoculum ($\sim 10^8$ cells/ml). Panel C shows VOC concentrations in the live microcosms that were not inoculated with SDC-9, and Panel D shows bromide concentrations in these same microcosms (uninoculated) over time as a measure of dilution during sampling.

Example measured and model-fitted time-series *cis*-DCE, VC and ethene concentrations from selected microcosm sets are shown in Figure 6-2. Initial acceptance criteria for fitted rate constants required that the 95% confidence interval on the fitted rate constant be \leq the rate coefficient value itself. However, because best fit k_{cisDCE} , k_{vc} and global model R^2 are not independent, (i.e. reflect tradeoff between goodness of fit to parent and daughter product time-series concentrations subject to mass balance constraints), a more appropriate acceptance criterion was established to require (1) a global R^2 value of ≥ 0.75 , and (2) an average ratio of the 95% confidence interval on the k_{cisDCE} and k_{vc} rate constants of $\geq 125\%$. Of the 40 microcosm tests performed (excluding live and killed controls), 26 and 15 tests respectively yielded acceptable quality k_{cisDCE} and k_{vc} rate coefficient data for further evaluation during this study (Table 6-4). A summary of all microcosm analytical data, as well as gene and protein abundance average and standard deviation values (triplicate analyses of single samples) obtained for each microcosm set at each time point sampled, is included in Appendix E.

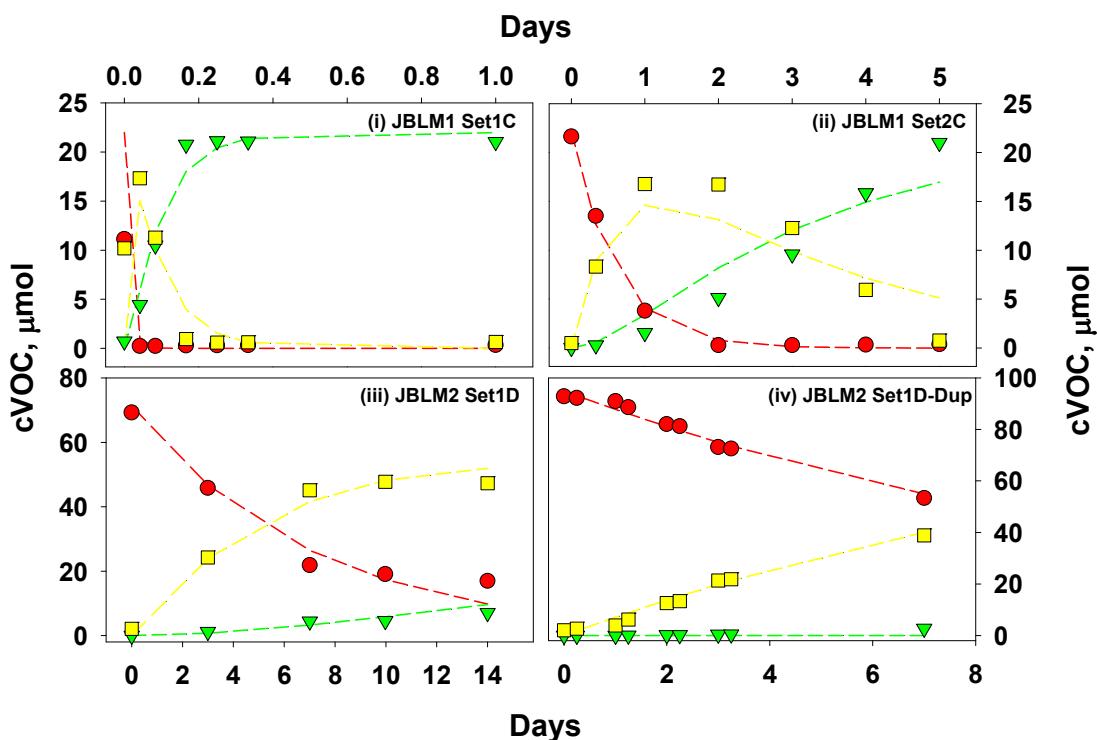


Figure 6-2. Measured and model-fitted cVOC concentrations in microcosms

Measured (symbols) and fitted (dashed line) time series cVOC and ethene mass values measured in selected microcosms inoculated with 10^9 (i), 10^7 (ii), 10^6 (iii), 10^7 (iv) *Dhc* cells/mL. Symbols for chlorinated ethenes are red circles (*cis*-DCE), yellow squares (VC), and ethene (green triangles).

Table 6-4. Summary of fitted k_{cisDCE} and k_{vc} by microcosm test

Microcosm Test Replicate	^a k_{cisDCE} , day ⁻¹	^a k_{vc} , day ⁻¹	R ² model	Average Ratio of 95% Confidence Interval to Rate Constant for k_{cisDCE} and k_{vc}
JBLM1				
JBLM1_Set1A	62.6 ± 73.1	10.2 ± 3.9	0.85	77%
JBLM1_Set1B	57.0 ± 53.7	10.2 ± 3.6	0.86	65%
^a JBLM1_Set1C	73.6 ± 146.0	11.3 ± 5.9	0.77	125%
JBLM1_Set2A	1.48 ± 0.50	0.31 ± 0.073	0.88	29%
JBLM1_Set2B	1.49 ± 0.48	0.34 ± 0.078	0.89	28%
JBLM1_Set2C	1.68 ± 0.53	0.34 ± 0.070	0.91	26%
JBLM1_Set3A	0.024 ± 0.0026	0.0015 ± 0.0018	0.90	65%
JBLM1_Set3B	0.016 ± 1.20x10 ⁻⁸	0.00058 ± 6.6x10 ⁻⁸	0.88	0.0%
JBLM1_Set3C	0.026 ± 2.20x10 ⁻⁷	0.0037 ± 6.6x10 ⁻⁷	0.89	0.0%
JBLM1_Set4A	--	--	--	--
JBLM1_Set4B	0.0013 ± 0.00013	-- ^b	0.91	--
JBLM1_Set4C	0.0014 ± 0.0014	0.000001 ± 0.026	0.91	--
JBLM2				
JBLM2_Set1A	1.05 ± 0.94	0.11 ± 0.018	0.94	53%
JBLM2_Set1B	1.05 ± 1.14	0.13 ± 0.028	0.92	65%
JBLM2_Set1C	1.13 ± 1.28	0.17 ± 0.041	0.83	68%
JBLM2_Set1A_Dup	0.28 ± 0.038	0.013 ± 0.031	0.99	128%
JBLM2_Set1B_Dup	0.30 ± 0.031	0.000027 ± 0.023	0.99	43591%
JBLM2_Set1C_Dup	0.22 ± 0.017	0.0067 ± 0.021	1.00	161%
JBLM2_Set2A	0.0028 ± 0.00040	0.0031 ± 0.0086	0.49	145%
JBLM2_Set2B	0.0018 ± 0.00024	0.0026 ± 0.0083	0.53	168%
JBLM2_Set2C	0.00244 ± 0.00039	0.0042 ± 0.0099	0.34	125%
JBLM2_Set3A	0.0010 ± 0.00026	0.0028 ± 0.013	0.05	255%
JBLM2_Set3B	0.00088 ± 0.00022	0.0033 ± 0.013	0.13	210%
JBLM2_Set3C	0.00096 ± 0.00025	0.0017 ± 0.013	0.06	401%
JBLM2_Set4A	0.0011 ± 0.00030	0.0037 ± 0.014	0.03	197%
JBLM2_Set4B	0.00082 ± 0.00021	0.0037 ± 0.013	0.12	189%
JBLM2_Set4C	--	--	--	--
JBLM2_Set5A	Live controls			
JBLM2_Set5B	Live controls			
JBLM2_Set6A	Killed controls			
JBLM2_Set6B	Killed controls			
^c *JBLM2_Set1D	0.14 ± 0.016	0.019 ± 0.0081	0.97	27%
*JBLM2_Set1E	0.088 ± 0.013	0.018 ± 0.015	0.92	51%
*JBLM2_Set1F	0.096 ± 0.015	0.021 ± 0.015	0.92	44%
^d *JBLM2_Set1D_Dup	0.078 ± 0.0046	0.00001 ± 0.016	0.97	81907%
*JBLM2_Set1E_Dup	0.059 ± 0.0038	0.018 ± 0.020	0.96	57%
*JBLM2_Set1F_Dup	0.057 ± 0.0035	0.0027 ± 0.018	0.96	343%
*JBLM2_Set2D	0.001 ± 0.00026	0.00009 ± 0.015	-0.26	8447%
*JBLM2_Set2E	0.001 ± 0.00029	0.00001 ± 0.017	-0.15	83025%
*JBLM2_Set2F	0.00001 ± 0.00048	0.0001 ± 2.88	-0.22	1439925%
*JBLM2_Set5C	Live controls			
*JBLM2_Set5D	Live controls			
*JBLM2_Set6C	Killed controls			
*JBLM2_Set6D	Killed controls			

-- indicates no rate was computed; ^aBest fit rate constants ± the 95% confidence interval on the rate constant; grey-highlighted values did not meet acceptance criteria and were excluded from further analysis; ^c "*" indicates microcosm included groundwater and aquifer solids

6.4 BIOMARKERS AS PREDICTORS OF REDUCTIVE DECHLORINATION RATES

Gene and protein abundances collected from early, middle and late time points from each microcosm were positively and significantly correlated with the k_{cisDCE} and k_{vc} rate coefficients extracted from those microcosms (Table 6-5). The correlation coefficients between biomarker abundances and rate coefficients were generally highest when only the middle time point biomarker abundances were included in the correlation analysis. For example, the correlation coefficient for VcrA protein and the k_{cisDCE} rate coefficient increased from 0.374 to 0.725 when only the midpoint protein abundances were considered (Table 6-5). This may reflect the general metabolic status of the dechlorinating organisms in the batch system (i.e., organisms had time to assimilate to the groundwater environment after inoculation and were actively biodegrading *cis*-DCE). This status may most effectively simulate that found in a flow-through aquifer system, where electron acceptor (e.g., *cis*-DCE) and nutrients are resupplied by groundwater flow. Accordingly, the rate coefficient vs. biomarker abundances regression analyses were performed using the midpoint biomarker abundances only. Note that while the *pceA* gene is carried by reductive dechlorinators present in the SDC-9 consortium, and both *pceA* gene and PceA protein abundances were found to be positively correlated with reductive dechlorination rates, these RDase biomarkers are not present in *Dhc* cells and are not reflective of complete dechlorination to ethene. Therefore, *pceA* gene and PceA protein abundances were not carried forward during rate and biomarker abundance regression analysis.

Table 6-5. Rate coefficients and biomarker correlations

Biomarker abundance correlations with rate coefficients (biomarker abundance, all microcosm time points)									
	FdhA	PceA	TceA	VcrA	DHC 16S gene	<i>tceA</i>	<i>vcrA</i>	<i>fdhA</i>	<i>pceA</i>
Log k_{cis}	0.737	0.571	0.575	0.374	0.844	0.859	0.856	0.801	0.804
p value	2×10^{-7}	6×10^{-6}	1×10^{-6}	6×10^{-3}	2×10^{-7}	2×10^{-7}	2×10^{-7}	2×10^{-7}	2×10^{-7}
n	57	55	62	54	64	64	64	64	62
Log k_{vc}	0.774	0.797	0.652	0.678	0.932	0.934	0.93	0.905	0.91
p value	2×10^{-7}	2×10^{-7}	3×10^{-5}	3×10^{-5}	2×10^{-7}	2×10^{-7}	2×10^{-7}	2×10^{-7}	2×10^{-7}
n	35	33	34	30	36	36	36	36	36
Biomarker abundance correlations with rate coefficients (biomarker abundance, microcosm mid-points only)									
	FdhA	PceA	TceA	VcrA	DHC 16S gene	<i>tceA</i>	<i>vcrA</i>	<i>fdhA</i>	<i>pceA</i>
Log k_{cis}	0.852	0.793	0.755	0.725	0.863	0.905	0.918	0.881	0.854
p value	2×10^{-7}	2×10^{-7}	2×10^{-7}	7×10^{-4}	2×10^{-7}	2×10^{-7}	2×10^{-7}	2×10^{-7}	2×10^{-7}
n	21	21	23	17	23	23	23	23	23
Log k_{vc}	0.925	0.836	0.765	0.916	0.934	0.953	0.962	0.966	0.943
p value	2×10^{-7}	2×10^{-7}	4×10^{-4}	2×10^{-7}	2×10^{-7}	2×10^{-7}	2×10^{-7}	2×10^{-7}	2×10^{-7}
n	15	15	15	11	15	15	15	15	15

Results of the power law least squares regression analysis of *cis*-DCE and VC rate coefficients versus gene and protein abundances are presented in Figure 6-3. Following the regression trends of target proteins down to the highest of the method detection limit for targeted proteins in this study of 3 fmol (e.g. TceA2, Table 6-1), which is equivalent to a typical 1-L groundwater sample containing 2×10^6 proteins/mL, would translate to k_{cisDCE} and k_{vc} rate constants both in the range of 0.0001 day^{-1} ($\sim 0.04 \text{ yr}^{-1}$), which is suitably low to be relevant to MNA sites¹. Thus, the proteomics

¹ For example, an apparent first order degradation rate coefficient of 0.04 yr^{-1} means 500 $\mu\text{g/L}$ VC would decrease to the 2 $\mu\text{g/L}$ VC maximum contaminant level in 138 years.

assay is sensitive enough to quantify proteins over a wide range of *Dhc* abundances and activities relevant to both biostimulated or bioaugmented sites where biomarker abundances and rates of reductive dechlorination are high, as well as to MNA sites where biomarker abundances and reductive dechlorination rates are quite low.

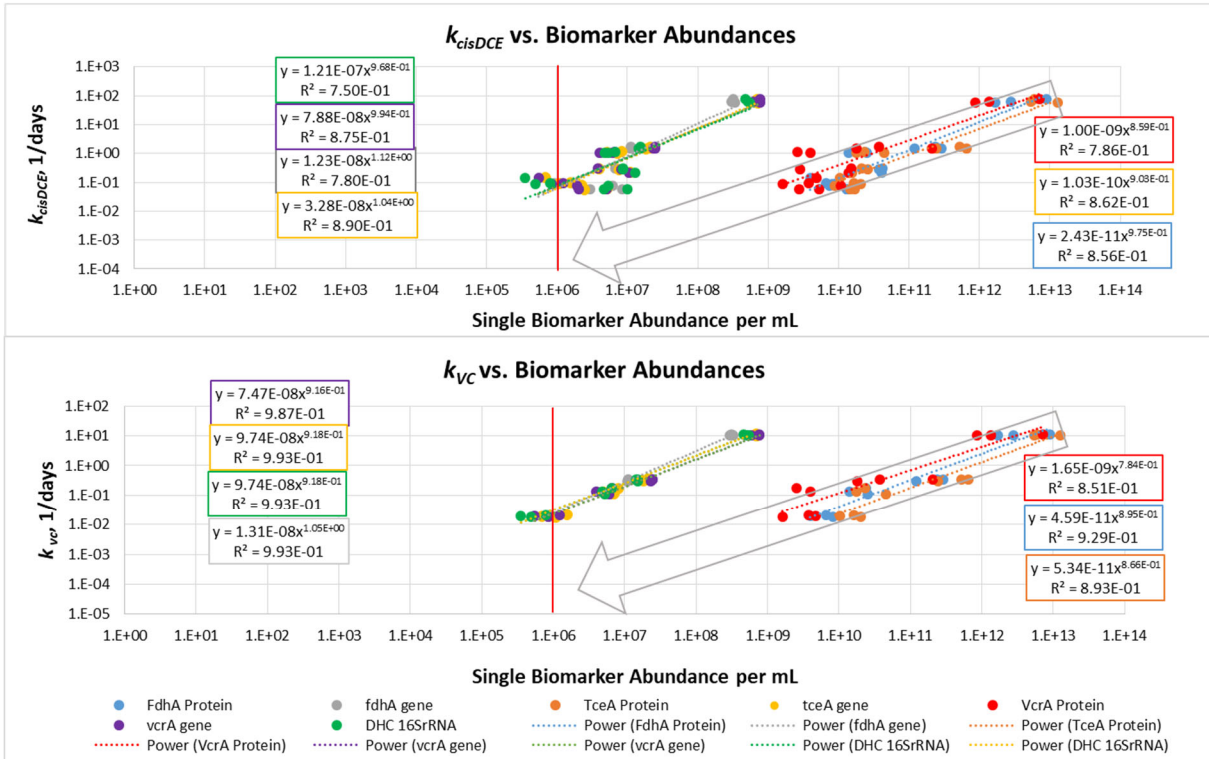


Figure 6-3. Rate coefficients vs. biomarker regression results

Microcosms that yielded acceptable rate coefficients (Table 6-4) and their corresponding mid-point RDase gene and biomarker abundances were used to complete regression results shown above.

The predictive power of gene and protein biomarkers was tested using regression equations featured in Figure 6-3 and biomarker abundances from microcosm tests that met the data quality acceptance screening criteria (Table 6-4) but that were not included in the regression analysis. The test was performed in two ways (1) using randomly selected biomarker abundances corresponding to a range of *Dhc* cell abundances, and (2) using global averages of all biomarker abundances that corresponded to *Dhc* cell abundances at 10^6 , 10^7 , 10^8 cells/mL. The randomly-selected biomarker abundances corresponded to the time zero sampling from microcosm sets JBLM1_Set1B, JBLM1_Set2C, and JBLM2_Set1F. As shown in Figure 6-4, protein-based rate predictions (white bars with black dots) were within an order of magnitude of measured rate coefficients (green boxes) for all tests. Rates predicted using a combination of genes and proteins (green bars, Figure 6-4) were generally better than those predicted using proteins alone.

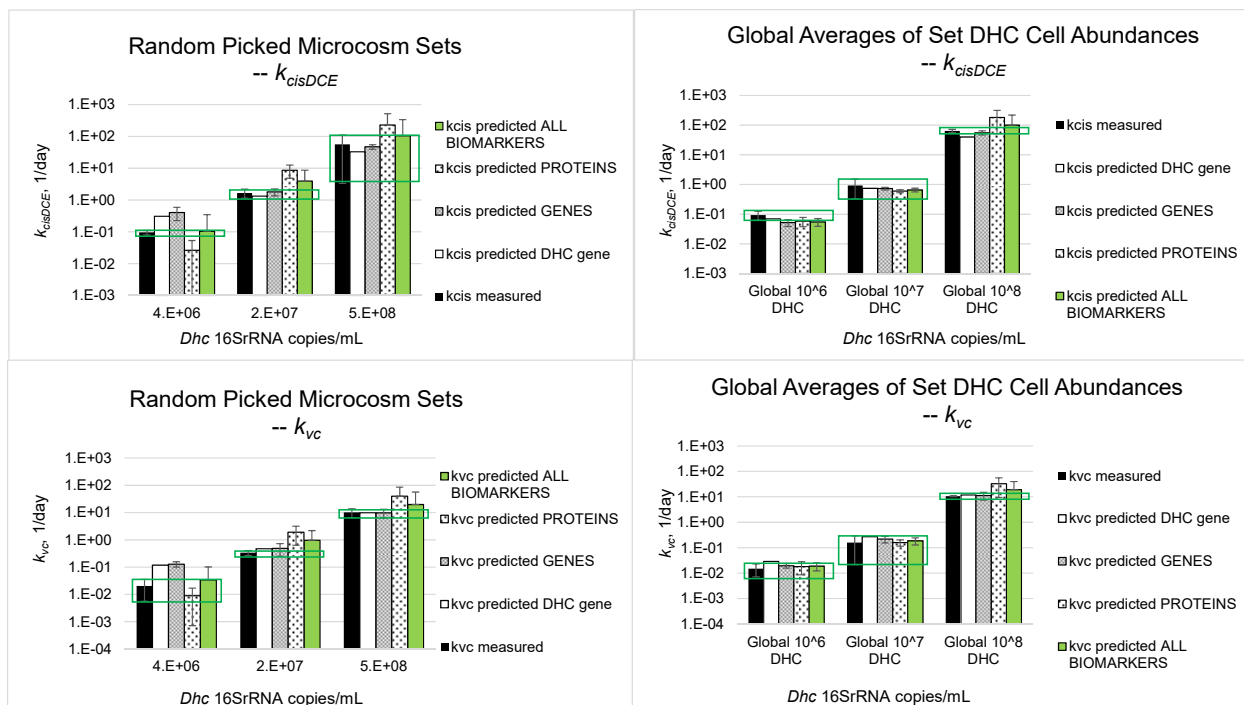


Figure 6-4. Biomarker-based rate predictions vs. measured rate coefficients

Green boxes reflect the error range associated with the measured rate coefficients during the study. Rates predicted using a combination of RDase genes and proteins (green bars) generally yielded an improved rate prediction compared to RDase proteins alone.

6.5 CONSIDERING APPLICABILITY OF THE LABORATORY STUDY RESULTS TO FIELD SITES

The simple laboratory microcosm systems were appropriate for confirming a quantitative relationship between biomarker abundances and reductive dechlorination rates mediated by *Dhc* cells from the SDC-9 consortia. However, conditions in the laboratory microcosms do not perfectly emulate conditions in aquifers at cVOC-contaminated field sites, which is where the qProt tool must be useful to provide maximal benefit. Here we consider how the quantitative link established between the biomarker abundances in the laboratory might be different than the link established in the field.

First, the majority of the bioaugmented microcosm test completed under this project featured only groundwater; relatively few microcosms featured groundwater with aquifers solids. Results of the rate coefficient vs. protein biomarker abundance regressions are reproduced in Figure 6-5 below, here with the groundwater-only (blue symbols) and groundwater with soil (orange symbols) differentiated for each biomarker. Microcosms that featured groundwater with soil generally clustered at the low end of the biomarker abundances and activity rates. Although the plus soil treatments tended to have lower biomarker abundances and activities, the results were generally consistent with the entirety of the data set, suggesting no significant difference in the presence of aquifer solids. It should be noted that all microcosm tests with soil were performed relatively quickly, under continuously mixed conditions, and were not designed to assess or account for *Dhc* cell attachment that may occur overtime; attachment was assumed to be negligible during these

tests. The contribution of attached vs. planktonic *Dhc* cells was beyond the scope of this laboratory study but will be addressed in the pending follow-on field demonstration.

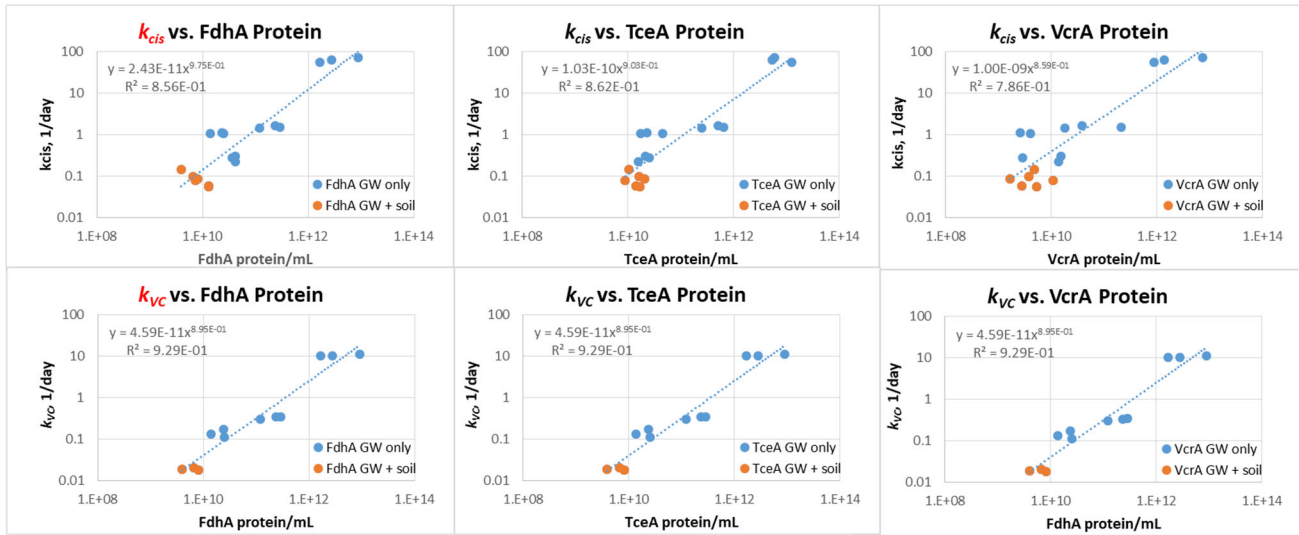


Figure 6-5. Rate coefficients vs. biomarker abundances with treatments distinguished.

Blue and orange symbols represent biomarker and rate coefficient results from groundwater-only microcosms and groundwater with aquifer solids microcosms, respectively.

Second, in a natural aquifer system, the abundance of bacteria will adjust to a level where growth sustained by the supply of the limiting electron acceptor (e.g., *cis*-DCE) is balanced by maintenance and cell death (e.g., predation). Indeed, viable microbes present in a natural system may consume dead cells as a nutrient source [48] and thereby “turn over” the dead cells. This cell “turn over” in natural systems will, in theory, minimize the chance of detecting “carcass proteins” (i.e., in this case RDases associated with dead cells). While the presence of such RDases in nonviable cells would be difficult to quantify, we did evaluate whether the protein levels detected were reasonable based upon the density of *Dhc* cells measured. If protein to *Dhc* cell abundances were greater than physiologically expected, or if ratios were much larger than have been previously published for RDase proteins in *Dhc*, the presence of RDases not associated with viable cells could be a confounding factor in RDase biomarker vs. rate coefficient regressions established in this study. Observed ratios were generally between 10^3 and 10^5 proteins/cell (Figure 6-6), which is in the range of previously published values of 7.6×10^3 and 2.60×10^4 for TceA reported for KB-1 and D2 cultures, respectively [46]. Further, the theoretical maximum number of proteins that could “fit” in the periplasmic space of a *Dhc* cell was estimated using the computational approach of Milo 2013 [52] to be 10^5 proteins. If we assume 10% of the proteins in a *Dhc* cell are RDases, the maximum RDases per *Dhc* cell would be 10^4 . Therefore, the observed range of RDase proteins per *Dhc* cell during this study do not exceed the realm of physiological possibility.

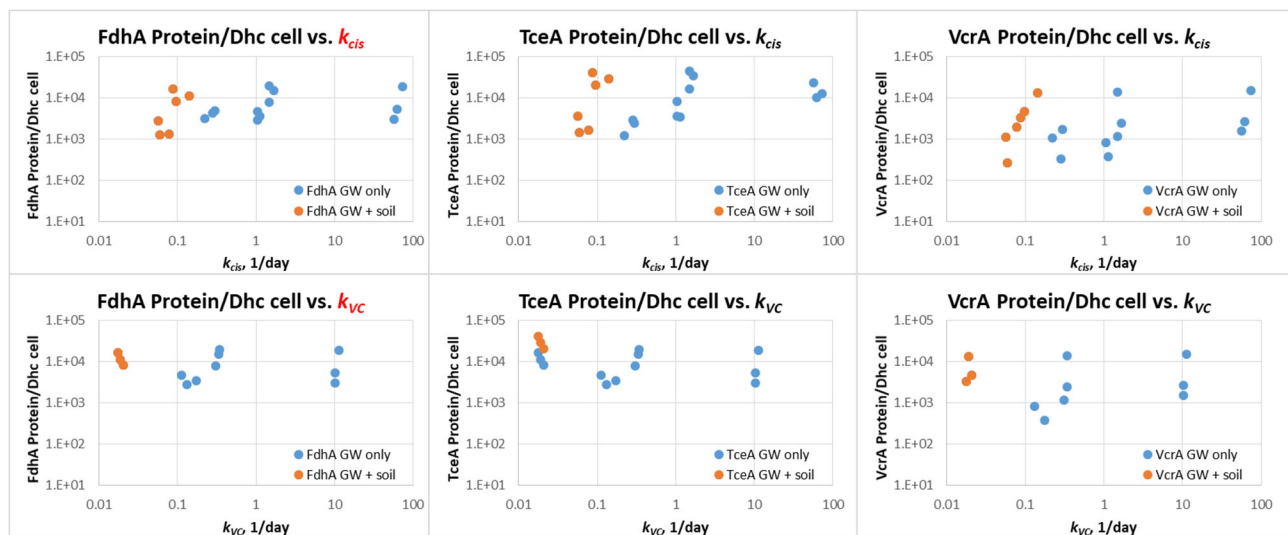


Figure 6-6. Protein/*Dhc* cell ratios vs. rate coefficients for microcosm tests

Blue and orange symbols represent biomarker and rate coefficient results from groundwater-only microcosms and groundwater with aquifer solids microcosms, respectively.

Finally, because first order-based kinetics often describe reductive dechlorination at field scales reasonably well, our first order-based regressions (Figures 6-3 and 6-5) will be directly applicable for supporting qProt assay interpretation at many field sites. In a natural system – in this case a cVOC-contaminated aquifer undergoing monitored natural attenuation – *Dhc* abundance will adjust to a level where growth sustained by the supply of chlorinated ethenes is balanced by self-consumption to sustain metabolism, and predation. In this case the abundance of *Dhc* and concentration of chlorinated ethenes remain relatively constant at any one position along the flow path. In such cases cVOC degradation kinetics can be described by a pseudo first-order kinetic model; i.e. at any one location along the flow path, the overall rate of degradation of the substrate in the groundwater ($\mu\text{g/L per day}$) divided by the concentration of the substrate ($\mu\text{g/L}$) is a fixed ratio. If an end user of the qProt assay wishes to use an alternative kinetic model to interpret test- or field site-specific results, published kinetic constants in the literature could be used to do so.

7.0 COST ASSESSMENT

7.1 COST MODEL

Standard analytical costs to obtain RDase activity rate estimates during the microcosm studies, as well as costs to obtain RDase biomarker abundance, were tracked as part of this laboratory project (Table 7-1). Costs to perform the microcosm study, analyze the data, and prepare a memorandum were tracked and recorded as well. Capital and operation and maintenance (O&M) costs were *not* tracked or reported. A field demonstration is necessary to a complete cost assessment with sufficient detail that a future “end user” of proteomics technology could compare costs between proteomics and existing MBTs and develop a reasonable cost estimate for conducting proteomic analysis at a cVOC-contaminated site. It should be noted that the microcosm testing conducted during this phase of the ESTCP Project would not be required during a field application of proteomic technology, so the associated costs are not relevant.

Table 7-1. Cost model for proteomics

Cost Element	Details	Tracked Demonstration Data	Discounted Costs	
Capital Costs				
System Design	Labor	These data were not tracked as part of the laboratory project but would be tracked during subsequent field demonstration		
Well Installation and Development	Labor			
	Materials			
	Subcontracts			
System Installation	Labor			
	Equipment & Materials			
	Subcontracts			
Travel				
Subtotal				
Operation and Maintenance (O&M) Costs				
Groundwater Sampling	Labor	These data were not tracked as part of the laboratory project but would be tracked during subsequent field demonstration		
	Materials			
- Analytical	In-house Labor			
	Laboratory			
System O&M	Labor			
	Materials			
Reporting & Data Management	Labor			
Travel				
Subtotal				
Other Technology-Specific Costs				
Site Selection	Labor & Travel	The cost to set up and run all the microcosm tests, including all the cVOC analysis and preparing the concentration data for analysis, was \$215K. Cost per sample for RDase biomarker analysis is described separately in Section 7.3 below.		
Site Characterization	Labor			
	Materials			
	Subcontractor			
Treatability Testing	Labor			
	Materials			
	Subcontractor			
Meetings & Reporting	Labor & Travel			
Technology Transfer	Labor & Travel			
Demonstration Plan/Work Plan	Labor			
Final Report	Labor			
Cost and Performance Report	Labor			
Subtotal				
TOTAL COSTS				
ESTIMATED TREATMENT VOLUME (CY)				
ESTIMATED TREATMENT VOLUME (GAL)				
APPROXIMATE TREATMENT COST (PER CY)				
APPROXIMATE TREATMENT COST (PER GAL)				

7.2 COST DRIVERS

Implementation of advanced MBTs during the assessment phase, remedy implementation and monitoring of the project are impacted by the factors as described above. Although there are currently no regulatory requirements that specifically mandate advanced MBTs be used to assess a site, the data provided by the MBTs are meant to supplement and possibly replace other forms of data that provide lines of evidence that MNA is occurring and to estimate a degradation rate. Hence, the total sampling and analytical cost is driven by number of sample locations at a site and total number of samples collected (i.e., a greater number of samples equates to a higher cost). It should be noted however that the individual cost per sample for analyses with advanced MBTs may decrease based on a greater number of total samples requiring analyses since the lab work is highly specialized and cost efficiencies generally can be realized for a larger quantity of analyses.

7.3 COST ANALYSIS

The microcosm studies conducted for this project to correlate rates of cVOC biodegradation with RDase abundance were extensive and would not be required for field implementation. The cost for the microcosm work including analytical (cVOCs, VFAs, dissolved gases, anions, pH) for all studies, collection and shipping of samples for qPCR and proteomic analysis, as well as preparation of the treatability study workplan and keeping a project database was \$215K. This included \$209K in labor and \$6K in materials, supplies, equipment, and shipping costs. No subcontracts were issued as all work including analytical was conducted at Aptim's laboratory in Lawrenceville, NJ. Collection of site materials for the microcosm studies is not included in these costs.

With the exception of metagenomics and metaproteomics, the techniques used to assess the contaminant degradation and continued potential for natural attenuation are common and costs to apply these techniques are well documented in the literature [49-51]. As discussed in Section 7.2, costs are highly dependent on the number of samples collected, frequency of sampling, and number/types of analytes, which are primarily dictated by the nature/diversity of the contaminants of concern (COCs), size of the site, proximity of receptors, and regulatory requirements. Hence, it is not the intent of this demonstration report to generate a life-cycle cost estimate for a hypothetical site at which these techniques are applied to evaluate remedial performance and subsequent natural attenuation of the remaining COCs to achieve site remedial action objectives (RAOs).

Table 7-2 provides a general cost comparison of conventional MBTs (e.g., qPCR) to the advanced MBTs, including proteomics. As indicated in the last column of the table, many of these techniques have only limited commercial availability and/or are available through a university or other research laboratory. As such, application costs remain relatively high. It is expected as these techniques mature, they will become more widely available and the analytical cost per sample will decrease substantially. For comparison purpose, the cost of shotgun and quantitative metaproteomic analyses based on cost data collected during this demonstration were \$1,200 and \$800 per sample, respectively, assuming analysis of a batch of 12 samples. The cost of the metaproteomic analyses included use of an existing metaproteomic platform but assumed development of a workflow specific for cVOCs.

Table 7-2. Cost comparison of conventional MBTs (e.g., qPCR) to the advanced MBTs.

Molecular Tool	Identity/ Potential Activity/ Expressed Activity^a	Quantitative, Qualitative (QA/QL)	Cost Range (\$) ^b	Availability^c
Conventional MBTs				
Compound specific isotope analysis	E	QA	100 to 2,500	C/R
Quantitative polymerase chain reaction	I/P/E	QA	275 to 425	WC
Microarrays	I/P/E	QL	1,250 to 5,000	C/R
Stable isotope probing	I/P/E	QA/QL	1,500 and up	C/R
Enzyme activity probes	E	QA	250 to 2,500	C/R
Advanced (omic) MBTs^d				
Metagenomics (16S Sequencing)	I	QL	150 to 500	WC/R
Shotgun Metaproteomics	E	QL	800 to 1,500	C/R
MRM Metaproteomics	E	QA	500 and up	C/R

Adapted from ITRC (2011). ^aI - identity of microorganisms (i.e., genus or species), P - potential activity (i.e., genetically capable of completing the activity), E - expressed activity (i.e., actually completing the activity at a given time). ^bEstimated price per sample. Low end represents compound specific restricted analysis. ^cWC - widely commercially available, C- minimally commercially available, R - available through university or other research laboratory. ^dThe cost of advanced omic MBTs represents cost from two commercial laboratories and Battelle metagenomic and proteomic lab. These costs are based on current costs from 2017 and higher number of batches (20 samples). These costs elements are reduced since the methods are maturing and proteomic analyses becomes more routinely used.

8.0 IMPLEMENTATION ISSUES

This section focuses on proteomic analysis and the potential of this technology to facilitate assessment of MNA. The primary end users of qProt are expected to be DoD site managers, consultants and their contractors. The general concerns of these end users are likely to include the following: (1) regulatory acceptance; (2) insufficient confidence in results and access to specialized laboratories; and (3) technology cost compared to other more conventional monitoring options. These implementation issues are addressed in the following sections.

8.1 REGULATORY ACCEPTANCE

Proteomics is a new tool in environmental assessment and one which requires validation. The relationship between specific proteins and protein levels and degradation rates of various cVOCs is now being established, with the work reported herein providing key data in this regard. At present, proteomics can be used to provide a direct line of evidence that biodegradation is actively occurring based on the detection of proteins that are produced during the degradation process. However, in the future, it is conceivable that proteomics could provide a direct measure of degradation rates based on the concentrations of proteins that are measured in a sample, which could eliminate or reduce the need to measure concentrations of cVOCs. It is therefore expected that regulatory acceptance of this technology will in part be based on the application and end use of the resulting data.

As with any new technology, detailed demonstration and validation are required to ensure accuracy and precision of results for both techniques before widespread regulatory acceptance can be obtained. Standardized methods and procedures for sample collection and shipping, analytical methods, QA/QC and data evaluation must be further developed and validated to help ensure regulatory acceptance. In addition, technology transfer through SERDP/ESTCP, peer reviewed journal articles, webinars, conferences, and other meetings will play an important role to facilitate understanding and acceptance of these powerful tools.

8.2 LIMITED AVAILABILITY

Proteomic analysis of enzymes involved in the reductive dehalogenation of cVOCs in field samples is a relatively new endeavor. The results provided in this report provide a strong basis for moving forward with this MBT for site assessment purposes. However, as with any new technology, availability and data quality are important concerns. Due to the young state of the practice, QA/QC guidelines for environmental applications of proteomic analysis are not yet available. In addition, only few analytical environmental laboratories offer advanced MBTs, and qProt is not yet commercially available. As with other important MBTs (e.g., qPCR) it is anticipated that these issues will be resolved over time as the method becomes more widely accepted and commercially applied.

8.3 COST COMPARED TO OTHER MONITORING TOOLS

The costs for qProt analysis are high compared to conventional technologies but are expected to decrease substantially as the technology continues to advance. Although costs per sample currently range from several hundred dollars to about \$1,000 for these types of analyses, MBTs help to answer a variety of management questions and facilitate decision making that can result in a reduction of the life-cycle cost of a remedy. For instance, MBTs such as metagenomics and metaproteomics, may be used instead of laborious microcosm studies to definitively state if microorganisms of interest are performing required activities and are actively degrading specific contaminants. These new MBTs characterize the contaminant-degrading *in situ* microbiome with unprecedented resolution. Information provided by these new MBTs, together with data from conventional MBTs, provides a comprehensive assessment and enables site management decisions to be made with greater confidence. Not only will this likely result in a direct cost savings to the project since microcosm studies can be more costly than the MBT analyses, but it also reduces the time required for assessment because microcosm studies generally take 60 to 90 days to perform.

During remediation efforts, MBT data help to design the remedy, to optimize remedial strategies, and to troubleshoot unsuccessful treatment approaches. Results can be used to determine when to reapply amendments to optimize growth and distribution of the target organisms, which can help to minimize the time required for the active portion of the remedy. Conceivably, in the near future, proteomics may provide the necessary means to directly calculate degradation rates, which then can be augmented during the active portion of the remedy to facilitate removal of cVOCs, thereby reducing application time and life-cycle cost.

Proteomics can facilitate long-term monitoring efforts by confirming that active degradation is occurring across the site, and eventually may aid to estimate the rate of degradation to decide if site-specific cleanup goals can be achieved within a desired timeframe. This could result in less frequent monitoring events and or a reduced number of analytes, which may reduce the life-cycle long-term monitoring cost and may support more rapid site closure. As metagenomics and proteomics are increasingly used in environmental applications, and as more laboratories begin to offer these analyses, competition increases, and techniques are refined, which will bring down the costs.

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APPENDIX A
QUANTITATIVE PROTEOMICS METHOD DETECTION LIMIT
AND INSTRUMENTATION DETECTION LIMIT STUDY RESULTS

Study No. W912DW-17-P-0036

Assessment of Instrument Detection Limit and Method Detection Limit for Reductive Dehalogenase Isotopic Peptides

REPORT

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Table of Contents

List of Acronyms	ii
1. <u>Introduction</u>	3
2. <u>RDase Peptide Selection, Protein Extraction and Quantification</u>	5
2.1 <u>RDase Peptide Selection</u>	5
2.2 <u>Protein Extraction and Quantification</u>	5
3. <u>Infusion and Injection of Isotopic Peptides</u>	6
4. <u>Multiple Reaction Monitoring (MRM) Method Development</u>	7
5. <u>Calibration and System Suitability</u>	8
6. <u>Instrument Detection Limit (IDL)</u>	12
7. <u>Method Detection Limit (MDL)</u>	13
8. <u>Summary</u>	17

List of Tables

Table 1. <u>List of SDC-9 Peptides Selected for Quantification</u>	6
Table 2. <u>Parameters of Waters Xevo CE Equation</u>	7
Table 3. <u>Solvent Program for M-Class Chromatographic System</u>	7
Table 4. <u>List of SDC-9 Peptides Used in Optimization Experiments</u>	9
Table 5. <u>Selected Peptides Used in IDL/MDL Experiments</u>	10
Table 6. <u>IDL Replicate Experiment Results</u>	12
Table 7. <u>MDL Replicate Experiment Results</u>	13

List of Figures

Figure 1. <u>Optimization of Instrument Parameters and Isotopic Standards Characteristics</u>	Error!
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Figure 2. <u>Representative Total Ion Chromatogram Displaying Detected RDase peptides; BSA peptides not shown as detection signals are significantly larger than for RDase peptides; the two panels to the right are more detailed chromatograms displaying the signal from each product ion for the VcrA peptide VPDHAVPINFK</u>	14
Figure 3. <u>BSA Peptides Detected in MDL Samples</u>	15
Figure 4. <u>Representative detailed chromatogram, peak area contributions, and retention time variation for 0.11-250 fmol/μL VcrA peptide VPDHAVPINFK standard from MDL experiment 1 (9/11/2017)</u>	16

List of Appendices

APPENDIX A: Protein Extraction, Quantification and Cleanup.....	A
APPENDIX B: List of all Isotopic Peptides and Their Transitions.....	B
APPENDIX C: IDL Data Set 1.....	C
APPENDIX D: IDL Data Set 2.....	D
APPENDIX E: IDL Data Set 3.....	E
APPENDIX F: MDL Data Set 1.....	F
APPENDIX G: MDL Data Set 2	G

APPENDIX H: MDL Data Set 3.....H

List of Acronyms

CEs	collision energies
DHC	<i>Dehalococcoides</i>
IDL	instrument detection limit
IS	internal standards
LOD	limit of detection
LOQ	limit of quantification
LLOQ	Lower level of quantification
MRM	multiple reaction monitoring-based
m/z	mass-to-charge ratios
PCE	tetrachloroethene
RDase	reductive dehalogenase
S/N	Signal-to-noise
TCE	trichloroethene
VC	vinyl chloride

Introduction

In preparation for microcosm experiments planned for this project, the Battelle proteomics team, in collaboration with the University of Tennessee, has identified SDC-9 culture-specific reductive dehalogenase (RDase) peptides for quantification. These specific RDases are used in a multiple reaction monitoring-based (MRM) targeted proteomic assay to establish quantitative biomarker rate correlations, which are needed to generate in situ degradation rate estimates of chlorinated ethenes.

During the development phase, the MRM assay was thoroughly evaluated for limit of detection (LOD) and limit of quantification (LOQ). To maximize precision, stable isotope labeled internal standards (IS) are frequently used to account for errors and losses that can occur during sample handling and variability in peptide ionization in the analysis of peptides. Because of the rigors of establishing these assays and successfully performing them in complex matrices, they tend to be implemented on only a selected number of analytes in parallel. With only a small number of analytes measured, it is common to expend considerable time optimizing tune parameters and collision energies (CEs) of each analyte individually to attain the highest sensitivity possible.

Prior to the use of IS peptides for MRM proteomics, general MS instrument parameters that work well with the broad diversity of peptides to be targeted needed to be determined (**Figure 1**). Peptide standards are directly infused to optimize these parameters empirically. CE is an instrument parameter that is frequently optimized to maximize fragment ion intensities. Multiple instrument manufacturers offer automated routines for CE optimization by peptide infusion as part of the instrument tuning software.

Once the system is optimized (resolution and calibration) and optimal CEs are determined, instrument detection limits and method detection limits are established for each IS peptide. Most analytical instruments produce a signal even when a blank (matrix without analyte) is analyzed. This signal is referred to as the instrument background level. Noise is a measure of the magnitude of the background signal. It is generally measured by calculating the standard deviation of a number of consecutive point measurements of the background signal or by measuring the magnitude of a defined region of background. Signal-to-noise (S/N) is obtained by calculating the ratio between the magnitude of the signal and the magnitude of the noise. Thus, the instrument detection limit (IDL), also known as LOD, is the analyte concentration required to produce a signal that is distinguishable from the noise level.

For most applications, required sample preparation methods may result in alteration of clean analyte prepared in solvent. It may be necessary to remove unwanted matrix components, digest, extract and concentrate the analyte, or even derivatize the analyte for improved chromatography or detection. The analyte may also be further diluted or concentrated prior to analysis on an instrument. Additional steps in a sample preparation method add additional opportunities for error (losses). Determination of the detection limit when sample preparation/manipulation steps are incorporated into the preparatory and analysis scheme results in an identified method detection limit (MDL), also known as LOQ. An MDL or LOQ accounts for additional losses that occur during the course of sample manipulation/preparation. Theoretically, the IDL is lower than the MDL for a given target analyte.

In this report, each step of system and IS peptide optimization is characterized in the sections below. Data pertaining to system resolution check, calibration, and chromatograms of peptide detections are grouped per sample set in **Appendices C through H**.

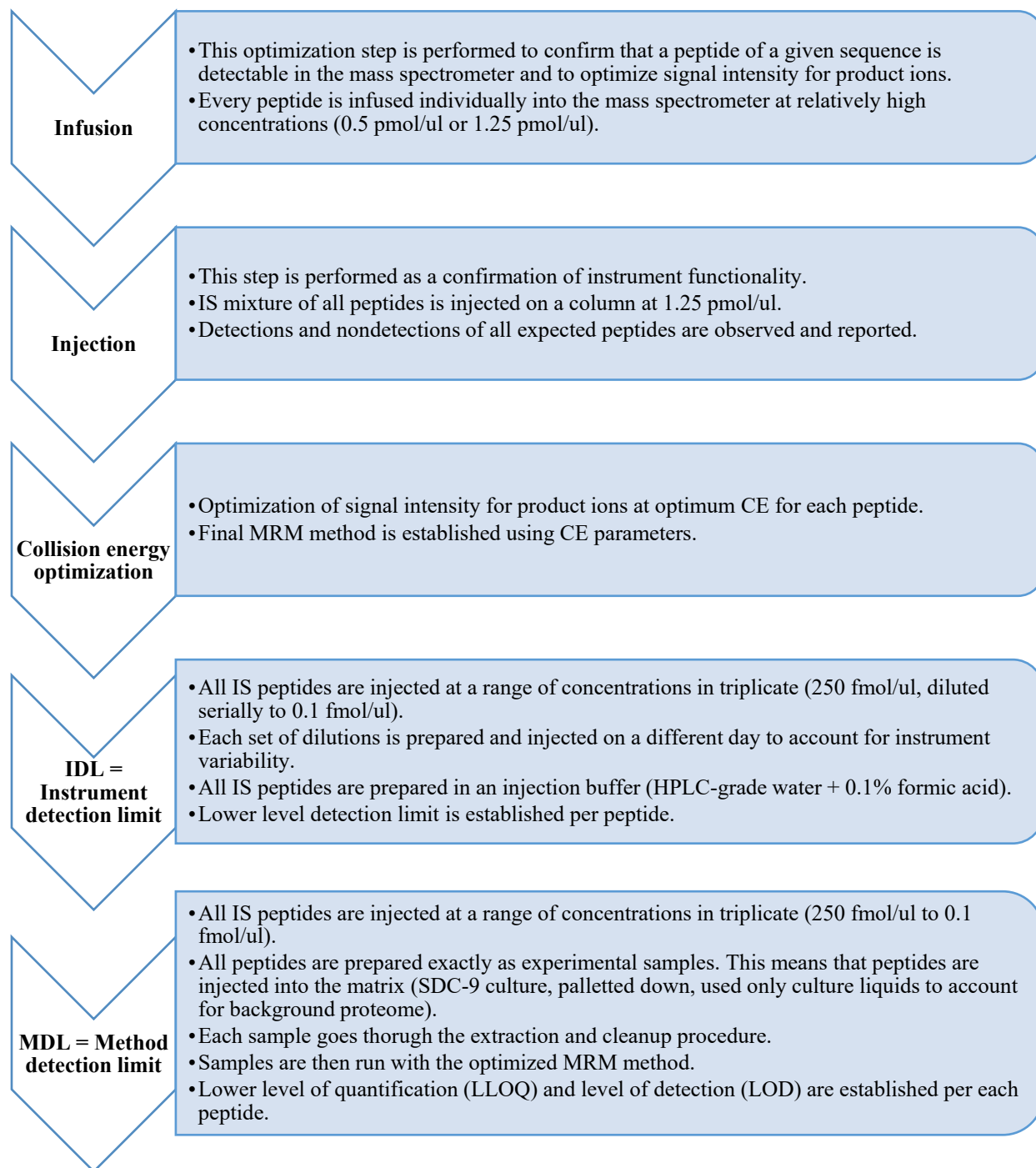


Figure 1. Optimization of Instrument Parameters and Isotopic Standards Characteristics

RDase Peptide Selection, Protein Extraction and Quantification

RDase Peptide Selection

Dehalococcoides (DHC) comprise a genus-level group of bacteria within the phylum *Chloroflexi*, notable for their ability to respire halogenated compounds including recalcitrant groundwater contaminants. Their obligate use of halogenated organic compounds as an energy source has allowed successful development of DHC-containing enrichment cultures for bioaugmentation of chlorinated ethene-contaminated sites, such as SDC-9 consortium. Each DHC strain contains a unique complement of genes that are homologs of known RDases, the genes required for respiration of halogenated organic compounds. The SDC-9 consortium is a well-defined enrichment culture with a variety of robust tetrachloroethene (PCE) – vinyl chloride (VC) dechlorinators. A metagenome sequencing project for the SDC-9 consortium was completed by Battelle Memorial Institute and the University of Tennessee to determine specific RDase genes that could serve as targets in proteomic analyses.

Overall, 14 genes encoding RDases were identified, 10 of which best matched previously identified RDases from members of the genus *Dehalococcoides*. Of these 14 sequences, one *vcrA* and one *tceA* gene were identified, as well as two separate *pceA* genes. Of these 14 RDases, 12 were associated with small, protein coding RDase B genes that are predicted to have three membrane-spanning helices. Peptide sequences of RDases and several sequences of FdhA protein that encodes for formate dehydrogenase were selected for targeted proteomic analysis. The detailed list of peptide sequences is presented in **Table 1**.

Protein Extraction and Quantification

For determination of the MDL, the 12.5 pmol/μL stock solution was diluted in ammonium bicarbonate to prepare the following concentrations (final in 25 μL): 250, 83, 27, 9, 3, 1, 0.34, and 0.11 fmol/μL. Each sample was digested with trypsin overnight and desalted using C18 spin columns. Protein extraction protocol, including protein detection with tryptophan assay and sample cleanup, has been developed for work with environmental samples and is located in **Appendix A** of this report. Copies of laboratory pages of sample extraction for MDL are also in **Appendix A**.

Table 1. List of SDC-9 Peptides Selected for Quantification

Protein	Peptide	Precursor (m/z)
FdhA	SELEVISSLLSR	671.9
	SGSEIAFTGGLIK	644.4
	SWDWALGEIANK	699.4
	AAGASDWEEK	536.2
	ALGIVYLDLSQAR	658.4
	VSSLQQLESPEELR	812.9
	LSWTYSTNPSAADVAK	859.9
	NQAVSAPGEAK	540.3
	TDTNTDYSYVNAIK	806.9
PceA	VETWNHDVAR	412.9
	FDEWFGYSGPVNPEER	969.9
	LLPWDLPK	495.3
	IATQIPLLQDAAR	710.4
	LESGYVQNMVK	638.3
	VYTDLELAPDKPR	509.6
	DFWNNPEPIK	634.3
	TSPSLISSATVGK	628.4
TceA	FLGADLVGIAPYDER	823.4
	DVDDLLSAGK	520.8
	VSSIIEPR	455.8
	VNNEPWWVTTR	706.4
	YFGASSVGAIK	554.3
VcrA	WGLYGPPHDSAPPDGSPVK	662.3
	YFGAGDVGALNLADPK	808.4
	VPDHAVPINFK	415.6
	GVYEGPPDAPFTSWG NR	930.4
	TGAAIHWK	446.2
	DQPWYVK	472.2
BSA*	LVNELTEFAK	582.3
	AEFVEVTK	461.7
	EYEATLEECCA K	751.8
	QTALVELLK	507.8

***BSA peptides were added to the method as internal standard for peptide recovery based on discovery data run previously**

Infusion and Injection of Isotopic Peptides

To confirm instrument functionality and detectability of each IS, infusion and injection steps were performed. Each isotopically labeled (IS) peptide was prepared as 12.5 pmol/ μ L in dimethyl sulfoxide (DMSO)/Milli-Q water (50/50), aliquoted, and frozen at -80°C until use. Concentrated solutions for each peptide were provided to the analyst for subsequent dilution and infusion directly into the mass spectrometer (Waters Xevo TQ-XS) for confirmation of precursor (parent) ion, charge state, product ions (daughters),

and optimization of CE (**Table 2**). This optimization step is performed to confirm that a peptide of a given sequence is detectable in the mass spectrometer and to optimize signal intensity for product ions. Each peptide was diluted to 0.5 pmol/ μ L or 1.25 pmol/ μ L in HPLC-grade water + 0.1% formic acid and was directly infused into the mass spectrometer at a flow rate of 10 μ L/min. For each peptide, a mass spectrum of the precursor ion was obtained (**Appendix B**). For each precursor ion, a mass spectrum was obtained for the product ions after fragmentation with CE of ≥ 20 V.

Using Waters Intellistart software, the CE for each peptide was optimized to maximize a signal from product ions. This was performed by infusing a single peptide into the mass spectrometer while Intellistart software varied cone voltage and CE to maximize a signal for each product ion. Skyline software was also used to output optimal CE for each peptide using the following equation with parameters (slope, intercept) that are specific to Waters Xevo mass spectrometers (**Table 2**).

$$CE = \text{slope} * (\text{precursor charge state}) + \text{intercept}$$

Table 2. Parameters of Waters Xevo CE Equation

Precursor Charge State	Slope	Intercept
+2	0.037	-1.066
+3	0.036	-1.328

Multiple Reaction Monitoring (MRM) Method Development

After optimization of CE per each IS peptide further development of multiple reaction monitoring assay was performed, including optimization of dwell time, CE, and solvent program. During this phase, peptides with relatively poor response were dropped from the MRM method file. The Skyline-optimized CEs were used in initial MRM method development. Comparison to Intellistart-optimized CEs was performed later in MRM development, however improvements in signal intensity were insignificant.

For MRM method development, peptides were prepared as a mixture at 1.25 pmol/ μ L in HPLC-grade water + 0.1% formic acid from a 12.5 pmol/ μ L mixture in DMSO/Milli-Q water. The solvent program and modified versions thereof listed in **Table 2** were used. The chromatographic system used was the Waters M-Class equipped with a trap column (Acquity UPLC M-Class Trap Symmetry® C18; 5 μ m particle size, 100Å pore size; 0.3 mm x 50 mm) and an analytical column (Acquity UPLC M-Class HSS T3 C18; 1.8 μ m particle size, 0.3 mm x 50 mm). **Table 3** displays the solvent program used where A = HPLC-grade water + 0.1% formic acid and B = HPLC-grade acetonitrile + 0.1% formic acid.

Table 3. Solvent Program for M-Class Chromatographic System

Time (min)	Flow Rate (μ L/min)	% A	% B
-	10	95	5
5	10	95	5
65	10	35	65
66	10	10	90
70	10	10	90
80	10	95	5
85	10	95	5

Based on the observed maximum peak heights of each peptide at 1.25 pmol/μL prepared in HPLC-grade water + 0.1% formic acid (MS Parameters from September 5, 2017: 123 transitions; 30 ms dwell time; 3.7 s cycle time), some peptides were removed from the transition list based on poor response (peak height or peak area) relative to other peptides. Only those peptides with the largest responses were retained on the transition list as specified in **Table 4** and **Table 5**.

Using the modified transition list and a 1.25 pmol/μL standard prepared in HPLC-grade water + 0.1% formic acid, three dwell times (20 ms, 50 ms, and 70 ms) were examined to assess the sensitivity of the signal to variation in dwell time. Based on the quality of the output data (peak height, peak shape, and points across a peak), the 50 ms dwell time was pursued for MDL experiments. The dwell time parameter was adjusted to 30 ms after further method development was prompted by failure of the first MDL set.

Calibration and System Suitability

Prior to each analytical run for IDL and MDL samples, the instrument was calibrated using a commercially available tuning solution (NAIRB) and a resolution check was performed. Instrument calibration ensures that the proper mass-to-charge ratios (m/z) have been assigned. The instrument is tuned in both MS1 and MS2 modes. Source and lens parameters are adjusted to optimize peak intensity and shape and the resolution and ion energy parameters are set for unit mass resolution on MS1 and MS2. This is performed by infusing a calibrant solution of NAIRB into the mass spectrometer and allowing the software to calibrate across the specified mass range (100-2000 m/z). Although triple quadrupole instruments are known to hold their calibrations for weeks to months, calibrations are performed or verified prior to each analysis sequence for sample sets. For infusion and optimization experiments, the instrument may or may not be calibrated prior to use each day. After instrument calibration, the mass accuracy (residuals) should be ± 0.2 Da.

System suitability was determined by injecting a commercially available retention time synthetic peptide mixture (Pierce™ Peptide Retention Time Calibration Mixture, Thermo Fisher Scientific) and a solvent spike (1.25 pmol/uL peptide mixture in HPLC-grade water + 0.1% formic acid) followed by solvent blank(s) (HPLC-grade water) before each sample set. Experimental samples were bracketed by injections of the retention time peptide mixture and the solvent spike to ensure the instrument functioned as anticipated and to track any loss of sensitivity or signal if observed during the course of the run. Checks for sensitivity, peak width, retention time, and carryover were performed qualitatively by inspection of the chromatograms of retention time peptide injections, solvent spike injections, and solvent blanks. While instrument sensitivity can vary day to day, no significant losses in instrument performance were observed during the course of IDL and MDL runs.

Table 4. List of SDC-9 Peptides Used in Optimization Experiments

Protein	Peptide	Precursor (m/z)	Observed (Y/N)	Retained (Y/N)
FdhA	SELEVISSLLSR	671.9	N	N
	SGSEIAFTGGLIK	644.4	Y	Y
	SWDWALGEIANK	699.4	N	N
	AAGASDWEEK	536.2	Y	Y*
	ALGIVYLD SQAR	658.4	Y	Y
	VSSLQQLSPEELR	812.9	Y	N
	LSWTYSTNPSAADVAK	859.9	Y	N
	NQAVSAPGEAK	540.3	Y	Y
	TDTNTDYSYVNAIK	806.9	Y	N
PceA	VETWNHDVAR	412.9	N	N
	FDEWFGYSGPVNPEER	969.9	N	N
	LLPWDLPK	495.3	Y	N
	IATQIPLLQDAAR	710.4	Y	Y
	LESGYVQNMVK	638.3	Y	Y
	VYTDLELAPDKPR	509.6	N	N
	DFWNNPEPIK	634.3	Y	Y
	TSPSLISSATVGK	628.4	Y	Y
TceA	FLGADLVGIAPYDER	823.4	Y	Y
	DVDDLLSAGK	520.8	Y	Y
	VSSIIER	455.8	Y	Y
	VNNEPWWVTTR	706.4	Y	Y
	YFGASSVGAIK	554.3	Y	Y
VcrA	WGLYGPPHDSAPPDGSVPK	662.3	N	Y
	YFGAGDVGALNLADPK	808.4	N	Y
	VPDHAVPINFK	415.6	N	Y
	GVYEGPPDAPFTSWG NR	930.4	N	Y
	TGAAIHWK	446.2	N	N
	DQPWYVK	472.2	Y	Y
BSA[‡]	LVNELTEFAK	582.3	N	Y
	AEFVEVTK	461.7	N	Y
	EYEATLEECCA K	751.8	N	Y
	QTALVELLK	507.8	N	Y

Bolded letters represent isotopically labeled amino acids
***Peptide was added to the transition list at a later date**
‡BSA peptides were added to the method based on discovery data run previously

Table 5. Selected Peptides Used in IDL/MDL Experiments

ID	Peptide	Precursor m/z	Product m/z	Ion	Charge
FdhA2	SGSEIAFTGGLIK	644.4 [M+2H] ²⁺	1056.6	y10	+1
			927.6	y9	+1
			814.5	y8	+1
			743.5	y7	+1
			1133.6	b12	+1
FdhA4	AAGASDWEEK	536.2 [M+2H] ²⁺	929.4	y8	+1
			872.4	y7	+1
			801.4	y6	+1
			714.3	y5	+1
			599.3	y4	+1
FdhA5	ALGIVYLD SQAR	658.4 [M+2H] ²⁺	1131.6	y10	+1
			961.5	y8	+1
			862.4	y7	+1
			699.4	y6	+1
			730.4	b7	+1
FdhA8	NQAVSAPGEAK	540.3 [M+2H] ²⁺	837.5	y9	+1
			766.4	y8	+1
			667.4	y7	+1
			580.3	y6	+1
			571.3	b6	+1
PceA4	IATQIPLLQDAAR	710.4 [M+2H] ²⁺	1235.7	y11	+1
			1134.7	y10	+1
			1006.6	y9	+1
			893.5	y8	+1
			796.5	y7	+1
PceA5	LESGYVQNMVK	638.3 [M+2H] ²⁺	1162.6	y10	+1
			1033.5	y9	+1
			946.5	y8	+1
			889.5	y7	+1
			726.4	y6	+1
PceA7	DFWNNPEPIK	634.3 [M+2H] ²⁺	1005.5	y8	+1
			819.4	y7	+1
			705.4	y6	+1
			591.4	y5	+1
			677.3	b5	+1
PceA8	TSPSLISSATVGK	628.3 [M+2H] ²⁺	1067.6	y11	+1
			970.6	y10	+1
			883.5	y9	+1
			770.4	y8	+1
			657.4	y7	+1
TceA1	FLGADLVGIAPYDER	823.4	1385.7	y13	+1

		$[M+2H]^{2+}$	1029.5	y9	+1
			930.5	y8	+1
			873.4	y7	+1
			886.5	b9	+1
TceA2	DVDDLLSAGK	520.8 $[M+2H]^{2+}$	826.4	y8	+1
			711.4	y7	+1
			596.4	y6	+1
			483.3	y5	+1
TceA3	VSSIIEPR	455.8 $[M+2H]^{2+}$	811.5	y7	+1
			724.4	y6	+1
			637.4	y5	+1
			524.3	y4	+1
TceA4	VNNEPWWVTTR	706.4 $[M+2H]^{2+}$	411.2	y3	+1
			1198.6	y9	+1
			1084.5	y8	+1
			955.5	y7	+1
TceA5	YFGASSVGAIK	554.3 $[M+2H]^{2+}$	858.4	y6	+1
			1025.5	b8	+1
			944.5	y10	+1
			797.5	y9	+1
VcrA1	WGLYGPPHDSAPPDGSVP K	662.3 $[M+3H]^{3+}$	740.4	y8	+1
			669.4	y7	+1
			582.4	y6	+1
			899.9	y18	+2
VcrA2	YFGAGDVGALNLADPK	808.4 $[M+2H]^{2+}$	871.4	y17	+2
			814.9	y16	+2
			733.4	y15	+2
			704.9	y14	+2
VcrA3	VPDHAVPINFK	415.6 $[M+3H]^{3+}$	1305.7	y14	+1
			1177.6	y12	+1
			1005.6	y10	+1
			906.5	y9	+1
VcrA4	GVYEGPPDAPFTSWG NR	930.4 $[M+2H]^{2+}$	1364.6	b14	+1
			725.4	6	+1
			626.4	5	+1
			573.3	10	+2
VcrA6	DQPWYVK	472.2	467.3	8	+2
			520.3	5	+1
			1411.7	y13	+1
			1354.6	y12	+1
			1254.6	y11	+1
			1045.5	y9	+1
			974.5	y8	+1
			700.4	y5	+1

		[M+2H] ²⁺	603.3	y4	+1
			417.3	y3	+1
			811.4	y6	+1

Instrument Detection Limit (IDL)

To establish IDL, IS peptides were prepared as a mixture at 12.5 pmol/μL in DMSO/Milli-Q water (50/50), aliquoted, and frozen at -80°C until use. A mixed, concentrated solution (12.5 pmol/μL) was provided fresh to the analyst during each day of analysis. The analyst diluted the sample to 250 fmol/μL in in HPLC-grade water + 0.1% formic acid and serially diluted this solution three-fold to prepare the following concentrations: 83, 27, 9, 3, 1, 0.34, and 0.11 fmol/μL. The lowest measurable concentration for each peptide, defined as S/N ≥ 3 (as measured by MassLynx) for the primary and secondary ion, represents the IDL for each peptide. Three trials were performed with the same dilution scheme; results generated are displayed in **Table 6** below.

Table 6. IDL Replicate Experiment Results

Protein	Peptide	IDL 1 (fmol/μL)	IDL 2 (fmol/μL)	IDL 3 (fmol/μL)
FdhA	SGSEIAFTGGLIK	83 ^g	83	27
	AAGASDWEK	>250	>250	>250
	ALGIVYLD SQAR	83	250	83
	NQAVSAPGEAK	27 [£]	27	27
PceA	IATQIPLLQDAAR	83 ^g	250 ^g	83 ^g
	LESGYVQNMVK	250	250	250 ^g
	DFWNNPEPIK	>250	>250	>250
	TSPSLISSATVGK	27	27 [¥]	27 [£]
TceA	FLGADLVGIAPYDER	>250	>250	250
	DVDDLLSAGK	250	250 ^A	83
	VSSIIEPR	9	3 ^³	9
	VNNEPWWVTTR	>250	>250	>250
	YFGASSVGAIK	250	>250	250
VcrA	WGLYGPPHDSAPPDGSPVK	>250	>250	>250
	YFGAGDVGALNLADPK	>250	>250	>250
	VPDHAVPINFK	>250	>250	>250
	GVYEGPPDAPFTSWG NR	>250	>250	>250
	DQPWYVK	>250	>250	>250

IDL units are fmol/μL
>250 fmol/μL denotes peptide was “not observed”
[¥] Primary and secondary ion pass at 3 fmol/μL with failure at 9 fmol/μL
^A Primary ion meets criteria at 3 fmol/μL
[£] Primary ion meets criteria at 9 fmol/μL
^g Primary ion meets criteria at 27 fmol/μL
^³ Secondary ion fails to meet criteria at 9 fmol/μL

Method Detection Limit (MDL)

To determine the MDL of peptide targets, IS peptides were prepared as a mixture at 12.5 pmol/ μ L in DMSO/Milli-Q water (50/50), aliquoted, and frozen at -80°C until use. A mixed, concentrated solution (12.5 pmol/ μ L) was provided fresh to the analyst during each day of analysis. The analyst diluted the sample to 1.25 fmol/ μ L in HPLC-grade water + 0.1% formic acid to use as a control during the analysis sequence. The 12.5 pmol/ μ L stock solution was diluted in ammonium bicarbonate to prepare the following concentrations (final in 25 μ L): 250, 83, 27, 9, 3, 1, 0.3, and 0.1 fmol/ μ L. Each sample was digested with trypsin overnight and desalted using C18 spin columns. The lowest measurable concentration for each peptide, defined as S/N \geq 3 (as measured by MassLynx) for the primary and secondary ion, represents the MDL for each peptide. Three trials were performed with the same dilution scheme; results generated are displayed in **Table 7**. An example total ion chromatogram displaying RDase peptides detected in the IS mix during the MDL study is shown in **Figure 2**.

Table 7. MDL Replicate Experiment Results

Protein	Peptide	MDL 1 (fmol/ μ L)	MDL 2 (fmol/ μ L)	MDL 3 (fmol/ μ L)
FdhA	SGSEIAFTGGLIK	27	83	83
	AAGASDWE EK	>250	>250	>250
	ALGIVYLD SQAR	3	83	83
	NQAVSAPGEAK	>250	>250	>250
PceA	IATQIPLLQDAAR	27 ^F	250 ^G	250
	LESGYVQN MVK	27 ^D	250 ^C	>250
	DFWNNPEPIK	27 ^D	250	250 ^C
	TSPSLISSATV GK	9	83	27
TceA	FLGADLVGIAPYDER	27	250 ^C	250 ^C
	DVDDLLSAGK	1	83	250 ^B
	VSSIIIEPR	3	27	83
	VNNEPWWVTTR	83	>250	>250
	YFGASSVGAIK	9	83 ^A	250 ^G
VcrA	WGLYGPPHDSAPPDG SVPK	83	>250	>250
	YFGAGDVGALNLADPK	83	>250	>250
	VPDHAVPINFK	3	9	9
	GVYEGPPDAPFTSWGNR	250	>250	>250
	DQPWYVK	27	27	27

MDL units are fmol/ μ L
>250 fmol/ μ L denotes peptide was “not observed”
^A Primary and secondary ion pass at 3 fmol/ μ L with failure at 9 and 27 fmol/ μ L
^B Primary and secondary ion pass at 9 fmol/ μ L with failure at 27 and 83 fmol/ μ L
^C Primary ion meets criteria at 83 fmol/ μ L
^D Primary ion meets criteria at 9 fmol/ μ L
^F Primary ion meets criteria at 3 fmol/ μ L
^G Primary ion meets criteria at 27 fmol/ μ L

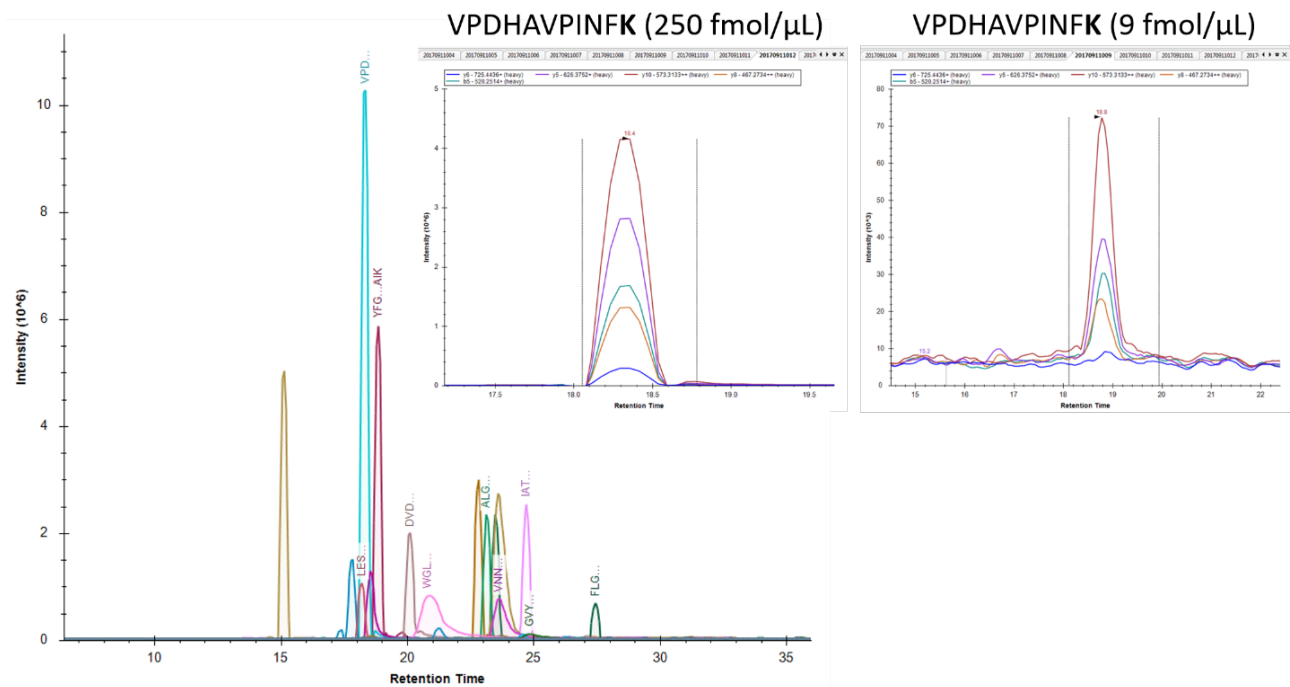


Figure 2. Representative Total Ion Chromatogram Displaying Detected RDase peptides; BSA peptides not shown as detection signals are significantly larger than for RDase peptides; the two panels to the right are more detailed chromatograms displaying the signal from each product ion for the VcrA peptide VPDHAVPINFK.

All BSA peptides were observed during MDL analysis. An example chromatogram of BSA peptides (10 μ g of BSA was added prior to each MDL sample prior to digestion) is displayed in **Figure 3** (from the MDL dataset 9/18/2017).

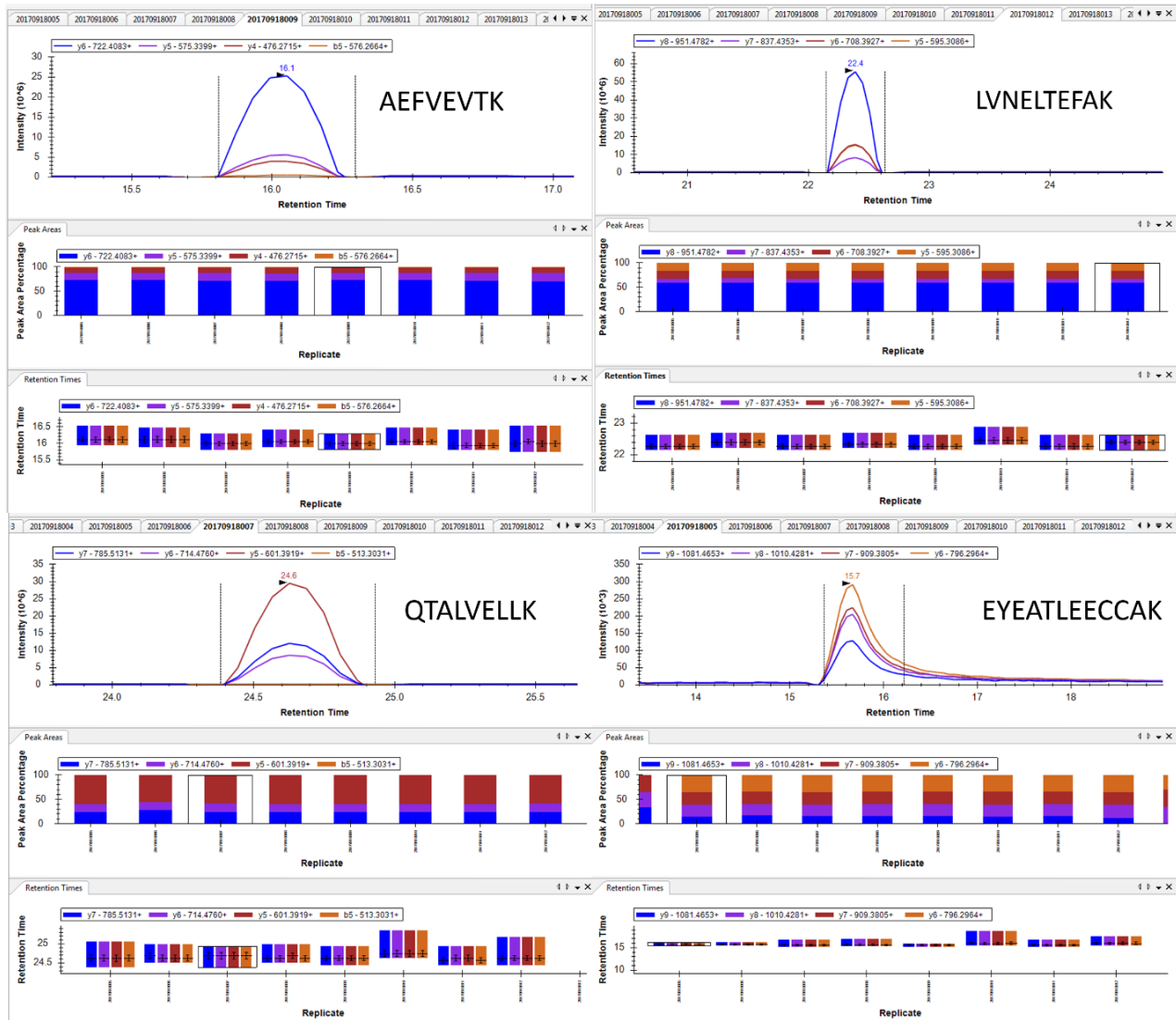


Figure 3. BSA Peptides Detected in MDL Samples

Two VcrA peptides (DQPWYVK and VPDHAVPINFK) were detectable in all MDL experiments while they were not detected in IDL experiments (**Figure 4**). The causes of performance variation between IDL and MDL experiments are likely due to matrix effects. Some peptides performed similarly between MDL replicates (e.g., DQPWYVK) while others did not. Within the MDL set, inconsistencies were observed for sensitive peptides (e.g., TSPSLISSATVGGK, VSSIIIEPR, YFGASSVGAIK). This variation represents the variation present in the preparatory methods and instrumental analysis; it is unlikely that instrumental variation resulted in decreased sensitivity as control samples of 1.25 pmol/uL mixed peptide prepared in HPLC-grade water + 0.1% formic acid and a Thermo Retention Time Peptide Mixture that bracketed samples did not reveal loss of chromatographic quality or loss in mass spectrometer signal during the course of the MDL runs.

For peptides that have poor secondary ion responses (e.g., YFGASSVGAIK), a more restrictive quality control scheme (that is, requiring two product ions to be present at $S/N \geq 3$) will result in higher detection limits. It is proposed that during sample analysis, the S/N of all analytes be calculated using MassLynx to satisfy the following acceptance criteria: those samples with $S/N \leq 3$ would be categorized as not detected, and those that meet $S/N \geq 3$ would be accepted as true detections and would be reported with quantitative values.

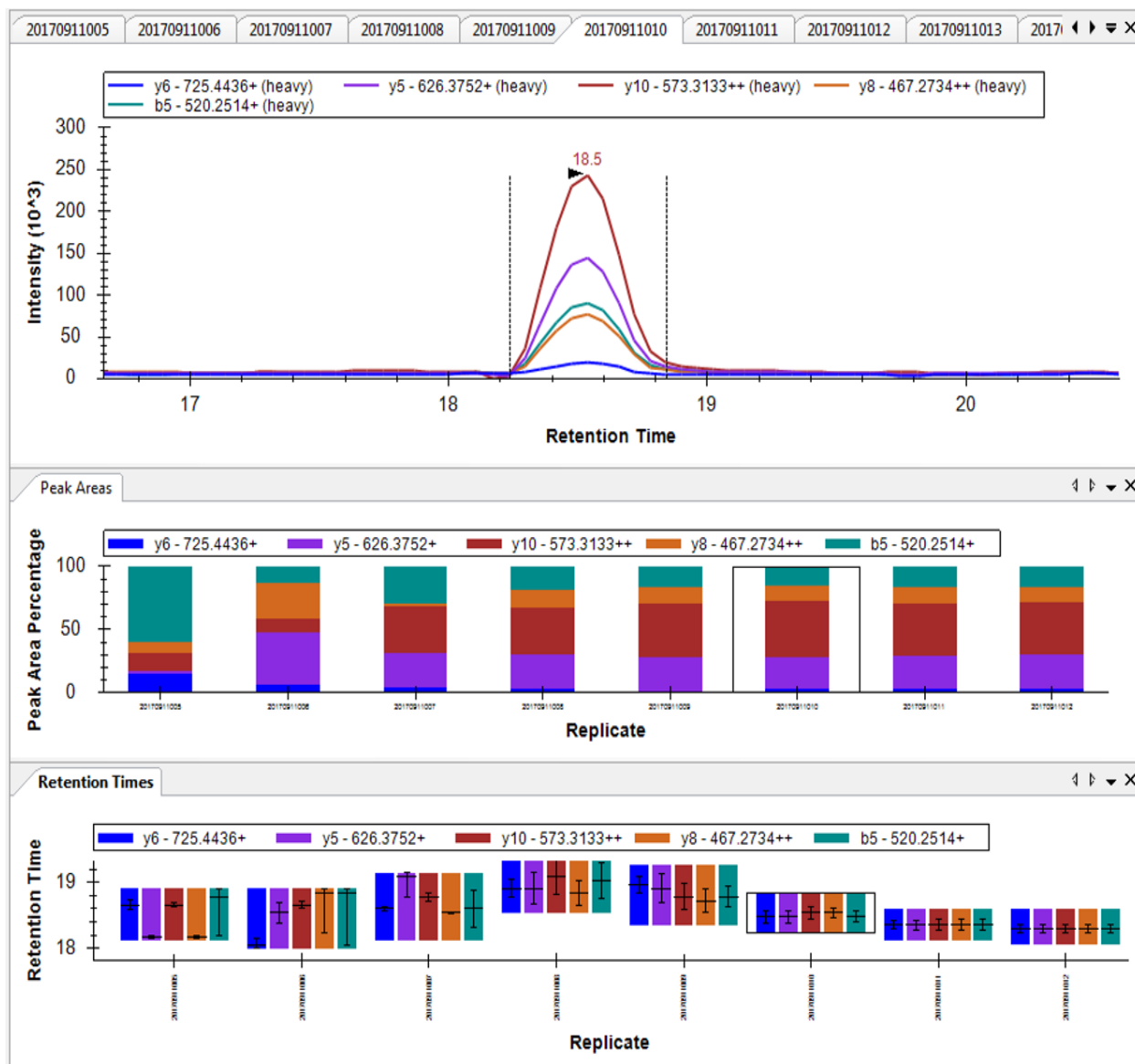


Figure 4. Representative detailed chromatogram, peak area contributions, and retention time variation for 0.11-250 fmol/ μ L VcrA peptide VPDHAVPINFK standard from MDL experiment 1 (9/11/2017).

Summary

In groundwater contaminated with chlorinated ethenes, the dominant and most productive biodegradation mechanism is typically reductive dechlorination. This is a process whereby the parent compound(s) PCE and/or TCE are sequentially dehalogenated to *cis*-1,2-dichloroethene (*cis*-DCE), vinyl chloride (VC) and finally ethene and/or ethane, which are considered environmentally benign. A number of different dehalogenating bacteria catalyze one or more steps throughout this process, with DHC being the only organismal group known to complete the entire pathway. In an effort to provide a robust and specific measurement that directly correlates to degradation rates, our team has developed a proteomics approach to quantify RDase proteins using a targeted MRM assay.

Prior to development of the MRM assay for targeted quantification, SDC-9 culture-specific RDase peptides were identified. Overall, a total of 28 peptide sequences, including those encoding for FdhA protein, were selected for quantification. During the initial steps of MRM assay development confirmation of instrument functionality and detection of peptide targets were performed. For that purpose, isotopically labeled peptide standards were injected into the mass spectrometer and their CE were optimized. Instrument and MDLs were subsequently established for each respective peptide.

In this study, CEs were optimized for initial 28 IS peptides to maximize the resulting signal from product ions. Following the optimization step, dwell time and solvent program were optimized and peptides that demonstrated poor signal response were discarded from the list. In total, 10 peptides were discarded from the list after optimization steps were completed and IDLs and MDLs were developed for the remaining peptides. Most peptides were observed in experimental samples during the IDL and MDL analysis, however VcrA peptides were observed exclusively in MDL experiments and not in IDL experiments (not observed is denoted as >250 fmol/μL), suggesting that sample digest and cleanup enhance the peptide signals for VcrA peptides. Two VcrA peptides (DQPWYVK and VPDHAVPINFK) were detectable in all MDL experiments while they were not detected in IDL experiments. Performance variation between IDL and MDL experiments are likely due to matrix effects. Some peptides performed similarly between MDL replicates (e.g., DQPWYVK) while others did not. Within the MDL set, inconsistencies were observed for sensitive peptides (e.g., TSPSLISSATVGK, VSSIIEPR, YFGASSVGAIK). This variation represents the variation present in the preparatory methods and instrumental analysis; it is unlikely that instrumental variation resulted in decreased sensitivity as control samples did not reveal loss of chromatographic quality or loss in mass spectrometer signal during the MDL runs.

Overall, the experiments performed during this study allowed for identification of the most sensitive RDase and FdhA peptides for targeted quantification. MDL experiments resulted in detection of up to three most sensitive peptides per protein with up to three most intensive transition ions per peptide. The data generated during the optimization and calibration experiments will be built into the MRM method used for quantification of native RDase peptides in microcosm experiment samples planned to be tested in the next phase of this project.

Study No. W912DW-17-P-0036

Re-Assessment of Instrument Detection Limit and Method Detection Limit for Reductive Dehalogenase Isotopic Peptides

REPORT

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Introduction

A suite of SDC-9 culture-specific reductive dehalogenase (RDase) peptides have been previously identified for quantification. These specific RDases are used in a multiple reaction monitoring-based (MRM) targeted proteomic assay to determine their quantities. During the initial development phase, the MRM assay was evaluated for empirical limits of detection (LOD) and limit of quantification (LOQ). While LOD and LOQ data were obtained during initial method development, the data was poorly reproducible among replicates (see Table 1 and 2 below) with some peptides not being observed even at the highest calibration level. Preparation and analysis of a second batch of samples was initiated to improve the reproducibility of the data. Changes to instrumental methods and updated tables are discussed below.

Table 1. Previously Determined Limits of Detection for RDase peptides

Protein	Peptide	IDL 1	IDL 2	IDL 3
FdhA	SGSEIAFTGGLIK	83 ^g	83	27
	AAGASDWEEK	>250	>250	>250
	ALGIVYLDLQAR	83	250	83
	NQAVSAPGEAK	27 [£]	27	27
PceA	IATQIPLLQDAAR	83 ^g	250 ^g	83 ^g
	LESGYVQNMVK	250	250	250 ^g
	DFWNNPEPIK	>250	>250	>250
	TSPSLISSATVGK	27	27 [¥]	27 [£]
TceA	FLGADLVGIAPYDER	>250	>250	250
	DVDDLLSAGK	250	250 ^A	83
	VSSIIIEPR	9	3 [§]	9
	VNNEPWWVTTR	>250	>250	>250
	YFGASSVGAIK	250	>250	250
VcrA	WGLYGPPHDSAPPDGSVPK	>250	>250	>250
	YFGAGDVGALNLADPK	>250	>250	>250
	VPDHAVPINFK	>250	>250	>250
	GVYEGPPDAPFTSWGNR	>250	>250	>250
	DQPWYVK	>250	>250	>250

Units are fmol/mL
 >250 fmol/ mL denotes peptide was “not observed”
 ¥ Primary and secondary ion pass at 3 fmol/µL with failure at 9 fmol/mL
 A Primary ion meets criteria at 3 fmol/mL
 £ Primary ion meets criteria at 9 fmol/mL
 g Primary ion meets criteria at 27 fmol/mL
 § Secondary ion fails to meet criteria at 9 fmol/mL

Table 2. Previously Determined Limits of Quantification for RDase peptides

Protein	ID	Peptide	MDL 1	MDL 2	MDL 3	MDL 4	MDL 5
FdhA	FdhA2	SGSEIAFTGGLIK	27	83	83	83	250 ^{A,G}
	FdhA5	ALGIVYLDLQAR	3	83	83	83	27
	FdhA8	NQAVSAPGEAK	>250	>250	>250	>2250	>2250
PceA	PceA4	IATQIPLLQDAAR	27 ^F	250 ^G	250	83 ^G	750 ^G
	PceA5	LESGYVQNMVK	27 ^D	250 ^C	>250	250 ^C	750 ^H
	PceA7	DFWNNPEPIK	27 ^D	250	250 ^C	83	250
	PceA8	TSPSLISSATVVGK	9	83	27	27	27
TceA	TceA1	FLGADLVGIAPYDER	27	250 ^C	250 ^C	750 ^H	750 ^H
	TceA2	DVDDLLSAGK	1	83	250 ^B	83 ^D	83 ^G
	TceA3	VSSIIIEPR	3	27	83	9	9
	TceA4	VNNEPWVVTTR	83	>250	>250	750	1000 ^I
	TceA5	YFGASSVGAIK	9	83 ^A	250 ^G	83 ^G	83 ^F
VcrA	VcrA1	WGLYGPPHDSAPPDGSVPK	83	>250	>250	750	750
	VcrA2	YFGAGDVGALNLADPK	83	>250	>250	750 ^H	1000
	VcrA3	VPDHAVPINFK	3	9	9	27	27
	VcrA4	GVYEGPPDAPFTSWGNR	250	>250	>250	2250 ^I	2250
	VcrA6	DQPWYVK	27	27	27	250 ^G	750 ^D

Units are fmol/mL

>250 fmol/ μ L denotes peptide was “not observed”

^A Primary and secondary ion pass at 3 fmol/ μ L with failure at 9 and 27 fmol/mL

^B Primary and secondary ion pass at 9 fmol/ μ L with failure at 27 and 83 fmol/mL

^C Primary ion meets criteria at 83 fmol/mL

^D Primary ion meets criteria at 9 fmol/mL

^F Primary ion meets criteria at 3 fmol/mL

^G Primary ion meets criteria at 27 fmol/mL

^H Primary ion meets criteria at 250 fmol/mL

^I Primary ion meets criteria at 750 fmol/mL

Calibration and System Suitability

Prior to sample analysis, the instrument was tuned in both MS1 and MS2 modes to maximize transmission of ions using a commercially available tuning solution (NAIRB). Using the instrument’s automated tuning program, source and lens parameters were auto-adjusted to optimize peak intensity and shape, and the resolution and ion energy parameters were set for unit mass resolution on MS1 and MS2. A resolution check was also performed by the analyst to confirm the instrument met unit mass resolution. The instrument was also calibrated prior to each run using NAIRB. This was performed by infusing a calibrant solution of NAIRB into the mass spectrometer and allowing the software to auto-calibrate across the specified mass range (100-2000 m/z).

System suitability was determined by injecting a commercially available retention time synthetic peptide mixture (Pierce™ Peptide Retention Time Calibration Mixture, Thermo Fisher Scientific) and a solvent spike (31 fmol/mL peptide mixture in HPLC-grade water + 0.1% formic acid) followed by solvent blank(s) (HPLC-grade water) before each sample set. Experimental samples were bracketed by injections of the retention time peptide mixture and the solvent spike to ensure the instrument functioned as anticipated and to track changes in sensitivity during the analytical run.

Multiple Reaction Monitoring (MRM) Method Updates

A new column was purchased for use on the program. It was observed that peptide FdhA8 displayed poor retention on the trap and analytical columns. The solvent program (Table 3) was modified by increased the percentage of starting acetonitrile (organic phase), which improved retention of peptide FdhA8.

Table 8. Solvent Program for M-Class Chromatographic System

Time (min)	Flow Rate ($\mu\text{L}/\text{min}$)	% A	% B*
-	10	95	1
5	10	95	1
65	10	35	65
66	10	10	90
70	10	10	90
80	10	95	1
85	10	95	1

*Starting acetonitrile (organic phase) changed from 5% to 1%

The peptides monitored were updated to reflect most recent practices (Table 4).

Table 4. Mass Transitions Used for Updated IDL/MDL Experiments

ID	Peptide	Precursor m/z	Product m/z	Ion	Charge
FdhA2	SGSEIAFTGGLIK	644.4 [M+2H] ²⁺	814.5	y8	+1
			743.5	y7	+1
FdhA5	ALGIVYLDLSQAR	658.4 [M+2H] ²⁺	961.5	y8	+1
			862.4	y7	+1
FdhA8	NQAVSAPGEAK	540.3 [M+2H] ²⁺	837.5	y9	+1
			667.4	y7	+1
PceA4	IATQIPLLQDAAR	710.4 [M+2H] ²⁺	1006.6	y9	+1
			893.5	y8	+1
PceA5	LESGYVQNMVK	638.3 [M+2H] ²⁺	1033.5	y9	+1
			726.4	y6	+1
PceA7	DFWNNPEPIK	634.3 [M+2H] ²⁺	819.4	y7	+1
			591.4	y5	+1
PceA8	TSPSLISSATVGK	628.3 [M+2H] ²⁺	770.4	y8	+1
			657.4	y7	+1
TceA2	DVDDLLSAGK	520.8 [M+2H] ²⁺	826.4	y8	+1
			711.4	y7	+1
TceA3	VSSIIEPR	455.8 [M+2H] ²⁺	724.4	y6	+1
			524.3	y4	+1
TceA4	VNNPWWVTTR	706.4 [M+2H] ²⁺	1198.6	y9	+1
			955.5	y7	+1
TceA5	YFGASSVGAIK	554.3 [M+2H] ²⁺	797.5	y9	+1
			669.4	y7	+1
VcrA1	WGLYGPPHDSAPPDGSPVK	662.3 [M+3H] ³⁺	814.9	y16	+2
			733.4	y15	+2
VcrA2	YFGAGDVGALNLADPK	808.4	1177.6	y12	+1

		[M+2H] ²⁺	906.5	y9	+1
VcrA3	VPDHAVPINFK	415.6	573.3	y10	+2
		[M+3H] ³⁺	626.4	y5	+1
VcrA4	GVYEGPPDAPFTSWGNR	930.4	1411.7	y13	+1
		[M+2H] ²⁺	974.5	y8	+1
VcrA6	DQPWYVK	472.2	700.4	y5	+1
		[M+2H] ²⁺	417.3	y3	+1

Bovine serum albumin (Table 5) was used to monitor digestion efficiency for MDL sample preparation. This was performed by spiking in 10 µg of BSA into ammonium bicarbonate alongside the IS peptides prior to digestion and C18 desalting.

Table 5. Mass Transitions Used to Monitor Digestion Efficiency

ID	Peptide	Precursor <i>m/z</i>	Product <i>m/z</i>	Ion	Charge
BSA1	LVNELTEFAK	582.3 [M+2H] ²⁺	951.5	y8	+1
			708.4	y6	+1
BSA2	AEFVEVTKR	461.7 [M+2H] ²⁺	722.4	y6	+1
			575.3	y5	+1
BSA3	EYEATLEECCA K	751.8 [M+2H] ²⁺	909.4	y7	+1
			796.3	y6	+1
BSA4	QTALVELLK	507.8 [M+2H] ²⁺	785.5	y7	+1
			604.4	y5	+1

Digestion Efficiency

Digestion efficiencies for each BSA peptide were calculated by taking the ratio between the peak areas for the peptide in the MDL standard (containing IS peptides and BSA) and the digestion control (reference containing BSA only) (Table 6).

Table 6. Mass Transitions Used to Monitor Digestion Efficiency

Protein ¹	ID	Peptide	Digest Efficiency (%)			Avg Digest Efficiency (%)	Standard deviation (%)	RSD (%)
			MDL 1	MDL 2	MDL 3			
BSA1	BSA1	LVNELTEFAK	111	112	ND	111.5	0.7	0.6
	BSA2	AEFVEVTK	112	116	119	115.7	3.5	3.0
	BSA3	EYEATLEECCA K	ND	161	157	159.0	2.8	1.8
	BSA4	QTALVELLK	113	108	ND	110.5	3.5	3.2

¹ Level of BSA spiked was 10 µg

ND Peptide was not detected in the reference digest sample; calculation of efficiency could not be calculated
RSD (relative standard deviation)

Depending on the peptide, the digestion efficiency ranged from 111-159%. While BSA peptides 3 and 4 are diagnostic of efficiency, however these peptides should not be used as they are prone to cyclizing because of their N-terminal glutamine and glutamate amino acids. The replicates (n = 7 standards) were

precise with relative standard deviations $\leq 3\%$. While this data indicates that the digestion efficiency is well controlled, it is recommended that the level of BSA spiked be reduced by half to mitigate any detector saturation that could occur. While no saturation was observed in these sample sets, the intensities of BSA peptide in the samples ($>10^6$) are close to the level of detector saturation ($10^7 - 10^8$).

Instrument Detection Limit (IDL)

Isotopically labeled peptides were prepared as a mixture at 12.5 pmol/ μL in DMSO/Milli-Q water (50/50), aliquoted, and frozen at -80°C until use. Mixed IS peptide solution (12.5 pmol/ μL) was provided fresh to the analyst during each day of analysis. The analyst diluted the sample to 250 fmol/ μL in HPLC-grade water + 0.1% formic acid and serially diluted this solution 3-fold in water + 0.1% formic acid to prepare the following concentrations: 83, 27, 9, 3, 1, and 0.34 fmol/ μL . The lowest measurable concentration for each peptide, defined as signal-to-noise (S/N) ≥ 3 (as measured by MassLynx) for the primary and secondary ion, was assigned as the IDL for each peptide. Three trials were performed with the same dilution scheme; results generated are displayed in Table 7 below.

Table 7. IDL Replicate Experiment Results

Protein	ID	Peptide	IDL 1	IDL 2	IDL 3	Revised Average IDL
FdhA	FdhA2	SGSEIAFTGGLIK	0.3	0.3	0.3	0.3
	FdhA5	ALGIVYLDLSQAR	0.3	0.3	0.3	0.3
	FdhA8	NQAVSAPGEAK	9	1	1	9
PceA	PceA4	IATQIPLLQDAAR	3	1	0.3	3
	PceA5	LESGYVQNMVK	1	0.3	0.3	1
	PceA7	DFWNNPEPIK	3	1	0.3	3
	PceA8	TSPSLISSATVVK	0.3	0.3	0.3	0.3
TceA	TceA2	DVDDLLSAGK	0.3	0.3	0.3	0.3
	TceA3	VSSIIPEPR	0.3	0.3	0.3	0.3
	TceA4	VNNEPWWVTTR	9	3	3	9
	TceA5	YFGASSVGAIK	0.3	0.3	0.3	0.3
VcrA	VcrA1	WGLYGPPHDSAPPDGSVPK	27	83	27	83
	VcrA2	YFGAGDVGALNLADPK	27	9	3	27
	VcrA3	VPDHAVPINFK	0.3	0.3	0.3	0.3
	VcrA4	GVYEGPPDAPFTSWGNR	83	83	27	83
	VcrA6	DQPWYVK	1	0.3	0.3	1

Units are fmol/mL of sample
¹ Bolded letters denote heavy ^{13}C and ^{15}N labeled amino acid

The revised IDL was assigned as the largest IDL observed between the three replicate measurements. The revised IDL represents the lowest standard in diluent (water + 0.1% formic acid) that was observed on the instrument with signal to noise ≥ 3 .

Method Detection Limit (MDL)

To determine the MDL of peptide targets, the 12.5 pmol/ μL stock solution of IS peptides was diluted in ammonium bicarbonate to prepare the following final concentrations: 250, 83, 27, 9, 3, 1, and 0.3 fmol/ μL . Each sample was digested with trypsin overnight and desalted using C18 spin columns. This procedure mimics the matrix that is used during digestion and cleanup of field samples. The lowest measurable concentration for each peptide, defined as S/N ≥ 3 (as measured by MassLynx) for the primary and

secondary ion, was assigned as the MDL for each peptide. Three trials were performed with the same dilution scheme; results generated are displayed in Table 8.

Table 8. MDL Replicate Experiment Results

Protein	ID	Peptide ¹	MDL 1	MDL 2	MDL 3	Revised Average MDL
FdhA	FdhA2	SGSEIAFTGGLIK	3	3	3	3
	FdhA5	ALGIVYLDLSQAR	3	3	1	3
	FdhA8	NQAVSAPGEAK	3	3	3	3
PceA	PceA4	IATQIPLLQDAAR	9	9	9	9
	PceA5	LESGYVQNMVK	3	3	3	3
	PceA7	DFWNNPEPIK	1	1	1	1
	PceA8	TSPSLISSATVVK	0.3	0.3	1	1
TceA	TceA2	DVDDLLSAGK	0.3	3	3	3
	TceA3	VSSIIEPR	0.3	0.3	1	1
	TceA4	VNNEPWWVTTR	9	9	9	9
	TceA5	YFGASSVGAIK	0.3	0.3	1	1
VcrA	VcrA1	WGLYGPPHDSAPPDGSVVK	9	9	3	9
	VcrA2	YFGAGDVGALNLADPK	27	27	27	27
	VcrA3	VPDHAVPINFK	0.3	0.3	1	1
	VcrA4	GVYEGPPDAPFTSWGNR	83	27	27	83
	VcrA6	DQPWYVK	1	1	1	1

Units are fmol/mL of extract
¹ Bolded letters denote heavy ¹³C and ¹⁵N labeled amino acid

The revised MDL was assigned as the largest MDL observed between the three replicate measurements. The revised MDL represents the lowest standard in matrix (ammonium bicarbonate with subsequent C18 desalting) that was observed on the instrument with signal to noise ≥ 3 .

Comparison of Revised IDL and MDL Levels

The Battelle proteomics team expected the measured MDL to be larger than the measured IDL for two reasons: (1) matrix effects can result in suppression or enhancement of an analyte's response and, (2) sample handling and cleanup can result in signal losses. Note that for the majority of peptides, this expectation holds true: the measured MDL > measured IDL. For 3 peptides (FdhA8, PceA7, VcrA1, highlighted in red in Table 9), the relationship was reversed: measured MDL < measured IDL. This indicates that the analyte's response enhanced by addition of matrix (ammonium bicarbonate with C18 cleanup). For 4 peptides (TceA4, VcrA2, VcrA4, and VcrA6), the measured MDL = measured IDL. This suggests that the peptide response was not affected by the addition of matrix (ammonium bicarbonate with C18 cleanup).

While the measured IDL is useful for method development, the measured MDL is the more important in quantitative experiments as it represents the minimum quantifiable value for a given method. For subsequent quantitative experiments with microcosm samples, the MDL for each peptide will be used as the minimum quantifiable level. Samples with quantified peptide levels that are below the MDL are not reportable values but can be important for diagnostic purposes and for subsequent method development (Table 9).

Table 9. Comparison of Experimental Instrument and Method Detection Limits

Protein	ID	Peptide ¹	Experimental IDL	Experimental MDL
FdhA	FdhA2	SGSEIAFTGGLIK	0.3	3
	FdhA5	ALGIVYLD SQAR	0.3	3
	FdhA8	NQAVSAPGEAK	9	3
PceA	PceA4	IATQIPLLQDAAR	3	9
	PceA5	LESGYVQNMVK	1	3
	PceA7	DFWNNPEPIK	3	1
	PceA8	TSPSLISSATV GK	0.3	1
TceA	TceA2	DVDDLLSAGK	0.3	3
	TceA3	VSSII EPR	0.3	1
	TceA4	VNNEPWWVTTR	9	9
	TceA5	YFGASSVGAIK	0.3	1
VcrA	VcrA1	WGLYGPPHDSAPPDGSV PK	83	9
	VcrA2	YFGAGDVGALNLADPK	27	27
	VcrA3	VPDHAVPINFK	0.3	1
	VcrA4	GVYEGPPDAPFTSWG NR	83	83
	VcrA6	DQPWYVK	1	1

Units are fmol/mL of extract
¹ Bolded letters denote heavy ¹³C and ¹⁵N labeled amino acid

Comparison of Theoretical Detection Limits with IDL/MDL Results

A theoretical IDL for each peptide was also determined with existing IDL data (Table 10). Calculated (theoretical) IDL values are typically the IDL levels reported for publication. The theoretical IDLs were determined by measuring signal to noise for the secondary ions for each peptide at a concentration yielding signal to noise between 10 and 20. The proportionality between concentration and signal to noise was used to calculate the concentration of peptide that would theoretically yield a signal to noise = 3. This calculation assumes a linear relationship between concentration and signal to noise.

Table 10. Comparison of Detection Limits

Protein	ID	Peptide ¹	Theoretical IDL	Experimental IDL	Experimental MDL
FdhA	FdhA2	SGSEIAFTGGLIK	0.08	0.3	3
	FdhA5	ALGIVYLD SQAR	0.02	0.3	3
	FdhA8	NQAVSAPGEAK	0.84	9	3
PceA	PceA4	IATQIPLLQDAAR	0.58	3	9
	PceA5	LESGYVQNMVK	0.59	1	3
	PceA7	DFWNNPEPIK	0.21	3	1
	PceA8	TSPSLISSATV GK	0.09	0.3	1
TceA	TceA2	DVDDLLSAGK	0.04	0.3	3
	TceA3	VSSII EPR	0.02	0.3	1
	TceA4	VNNEPWWVTTR	2.17	9	9
	TceA5	YFGASSVGAIK	0.03	0.3	1
VcrA	VcrA1	WGLYGPPHDSAPPDGSV PK	19.15	83	9
	VcrA2	YFGAGDVGALNLADPK	9.68	27	27
	VcrA3	VPDHAVPINFK	0.33	0.3	1
	VcrA4	GVYEGPPDAPFTSWG NR	28.89	83	83
	VcrA6	DQPWYVK	0.25	1	1

Units are fmol/mL of extract
¹ Bolded letters denote heavy ¹³C and ¹⁵N labeled amino acid

APPENDIX B
VALIDATION STUDY OF QPROT ASSAY
QUANTITATION LIMITS

Study No. W912DW-17-P-0036

Determination of RDase Peptide Concentrations in the Validation Study

REPORT

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Table of Contents

<u>List of Acronyms</u>	ii
<u>1. Introduction</u>	3
<u>2. Dilution Study Setup, Protein Extraction and Quantification</u>	1
<u>2.1 Dilution Study Set up</u>	1
<u>2.2 RDase Isotopic Peptides</u>	1
<u>2.3 Protein Extraction and Quantification</u>	2
<u>2.4 Protein Concentrations in Dilution Study Samples</u>	2
<u>3. Summary</u>	3

List of Tables

<u>Table 1. Gene specific qPCR analysis of SDC-9 sample</u>	1
<u>Table 2. RDase peptides used in MRM assay</u>	2

List of Appendices

APPENDIX A: Protein Extraction, Quantification and Cleanup	A
APPENDIX B: List of all Isotopic Peptides and Their Transitions	B
APPENDIX C: Dilution Study I	C
APPENDIX D: Dilution Study II	D

List of Acronyms

CEs	collision energies
DHC	<i>Dehalococcoides</i>
IDL	instrument detection limit
IS	internal standards
LOD	limit of detection
LOQ	limit of quantification
MDL	method detection limit
MRM	multiple reaction monitoring-based
PCE	tetrachloroethene
RDase	reductive dehalogenase
VC	vinyl chloride

Introduction

In preparation for microcosm experiments planned for this project, the Battelle proteomics team, in collaboration with the University of Tennessee, has identified SDC-9 culture-specific reductive dehalogenase (RDase) peptides for quantification. These specific RDases are used in a multiple reaction monitoring-based (MRM) targeted proteomic assay to establish quantitative biomarker rate correlations, which are needed to generate in situ degradation rate estimates of chlorinated ethenes.

After the development of the MRM assay and determination of instrument detection limit (IDL) and method detection limit (MDL) for each RDase, dilution study was performed. The dilution study served to identify the lowest concentration of DHC cells that generate detectable and quantifiable concentrations of reductive dehalogenases (RDases) selected for quantification.

To maximize precision, stable isotope labeled internal standards (IS) were used to account for errors and losses that can occur during sample handling and variability in peptide ionization in the analysis of peptides. Because of the rigors of establishing these assays and successfully performing them in complex matrices, they tend to be implemented on only a selected number of analytes in parallel. With only a small number of analytes measured, it is common to expend considerable time optimizing tune parameters and collision energies (CEs) of each analyte individually to attain the highest sensitivity possible.

This report discusses set up of the dilution study and generated results. Data pertaining to system resolution check, calibration, and chromatograms of peptide detections are grouped per sample set in **Appendices C** and **D**.

Validation Study Setup, Protein Extraction and Quantification

Validation Study Set up

SDC-9 dechlorinating consortium was shipped to Battelle from Aptim in September 2017. The culture was aliquoted into 50 mL tubes and kept frozen in -80 °C until use. For the purposed of the dilution study, one 50 mL tube was shipped to the University of Tennessee for determination of DHC cell concentration and RDase genes (**Table 1**). Another tube was used for proteomic analysis. Starting SDC-9 culture of 2×10^7 DHC cells was diluted in triplicate to concentrations: 10^4 , 10^5 , 10^6 and 10^7 , and subject to protein extraction, tryptic digestion and quantification.

Table 1. Gene specific qPCR analysis of SDC-9 sample

Sample ID:	SDC-9 Culture
Analysis: qPCR	
Volume of sample filtered (mL)	30
Isolated DNA concentration (ng/ μ L)	67.5
Volume of purified DNA (μ L)	50
qPCR Assays (gene copies/mL)	
General <i>Bacteria</i> 16S rRNA gene	3.18E+8
<i>Dehalococcoides</i> 16S rRNA gene	2.26E+7
<i>Dehalococcoides vcrA</i> gene (cDCE→Ethene)	2.70E+7
<i>Dehalococcoides bvcA</i> gene (cDCE→Ethene)	ND
<i>Dehalococcoides tceA</i> gene (TCE→VC)	2.23E+7

RDase Isotopic Peptides

Dehalococcoides (DHC) comprise a genus-level group of bacteria within the phylum *Chloroflexi*, notable for their ability to respire halogenated compounds including recalcitrant groundwater contaminants. Their obligate use of halogenated organic compounds as an energy source has allowed successful development of DHC-containing enrichment cultures for bioaugmentation of chlorinated ethene-contaminated sites, such as SDC-9 consortium. Each DHC strain contains a unique complement of genes that are homologs of known RDases, the genes required for respiration of halogenated organic compounds. The SDC-9 consortium is a well-defined enrichment culture with a variety of robust tetrachloroethene (PCE) – vinyl chloride (VC) dechlorinators. A metagenome sequencing project for the SDC-9 consortium was completed by Battelle Memorial Institute and the University of Tennessee to determine specific RDase genes that could serve as targets in proteomic analyses.

Overall, 14 genes encoding RDases were identified, 10 of which best matched previously identified RDases from members of the genus *Dehalococcoides*. Of these 14 sequences, one *vcrA* and one *tceA* gene were identified, as well as two separate *pceA* genes. Peptide sequences of RDases and several sequences of FdhA protein that encodes for formate dehydrogenase were selected for targeted proteomic analysis. The detailed list of peptide sequences is presented in **Table 2**.

Table 2. RDase peptides used in MRM assay

Protein	Peptide ID	Peptide	MDL 1 (fmol/ μL)	MDL 2 (fmol/ μL)	MDL 3 (fmol/ μL)
FdhA	FdhA2	SGSEIAFTGGLIK	27	83	83
	FdhA5	ALGIVYLD SQAR	3	83	83
	FdhA8	NQAVSAPGEAK	>250	>250	>250
PceA	PceA4	IATQIPLLQDAAR	27 ^F	250 ^G	250
	PceA5	LESGYVQNMVK	27 ^D	250 ^C	>250
	PceA7	DFWNNPEPIK	27 ^D	250	250 ^C
	PceA8	TSPSLISSATVVGK	9	83	27
TceA	TceA2	DVDDLLSAGK	1	83	250 ^B
	TceA3	VSSIIIEPR	3	27	83
	TceA4	VNNEPWVVTTR	83	>250	>250
	TceA5	YFGASSVGAIK	9	83 ^A	250 ^G
VcrA	VcrA1	WGLYGPPHDSAPPDGSVPK	83	>250	>250
	VcrA2	YFGAGDVGALNLADPK	83	>250	>250
	VcrA3	VPDHA VPINFK	3	9	9
	VcrA4	GVYEGPPDAPFTSWGNR	250	>250	>250
	VcrA6	DQPWYVK	27	27	27
<p>MDL units are fmol/μL >250 fmol/μL denotes peptide was “not observed” ^A Primary and secondary ion pass at 3 fmol/μL with failure at 9 and 27 fmol/μL ^B Primary and secondary ion pass at 9 fmol/μL with failure at 27 and 83 fmol/μL ^C Primary ion meets criteria at 83 fmol/μL ^D Primary ion meets criteria at 9 fmol/μL ^F Primary ion meets criteria at 3 fmol/μL ^G Primary ion meets criteria at 27 fmol/μL</p>					

Protein Extraction and Quantification

For protein quantification, each sample was digested with trypsin and desalted using C18 spin columns. Protein extraction protocol, including protein detection with tryptophan assay and sample cleanup, has been developed for work with environmental samples and is located in **Appendix A** of this report.

Protein Concentrations in Dilution Study Samples

On average, 1 mg/mL protein was extracted in each sample. Peptide concentration data is tabulated in Excel file entitled “USACE ESTCP Dilution Study 11.15.17” attached to this report.

Only two out of three FdhA peptides, namely FdhA2 and FdhA 5, were observed in the dilution study. The FdhA8 peptide was not detected. FdhA 2 and FdhA 5 peptides were detected above method detection limit in **all** SDC-9 cell dilutions (10^4 to 10^7 DHC cells). However, their per cell concentrations were higher than reported in the literature. **Figure 1A** shows FdhA peptide concentrations per total cell number.

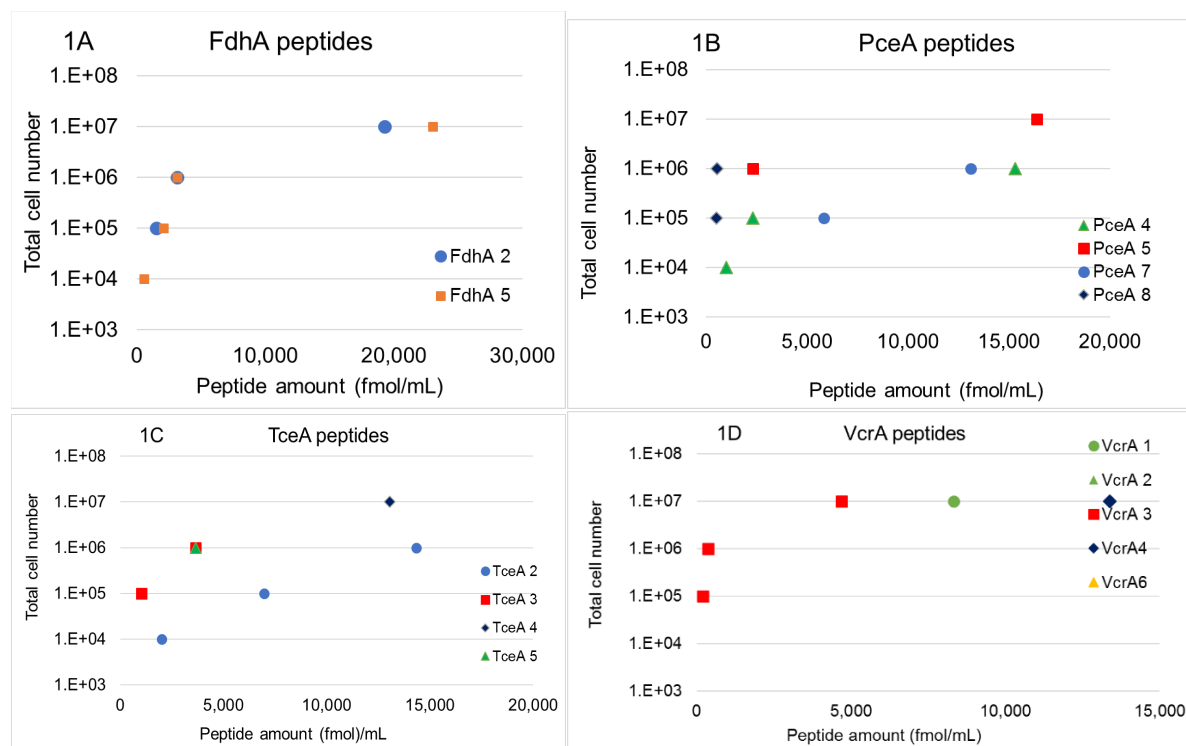


Figure 1. Concentration of (A) FdhA, (B) PceA, (C) TceA and (D) VcrA peptide in Dilution Study Samples

Figure 1B shows PceA peptide concentrations per total cell number. PceA5, PceA7 and PceA8 showed lower sensitivity than PceA4 peptide which was detected in 10^4 starting DHC cells. The lowest concentration of DHC cells that generated detectable and quantifiable concentrations of PceA5 and PceA7 was 10^5 , while 10^6 cells was needed to quantify PceA8 peptide. Thus, PceA4 peptide will be selected as a quantifier for the proceeding studies.

The TceA2 peptide, had highest sensitivity and was detected and quantified in samples containing 10^4 DHC cells (**Figure 1C**). This peptide will be selected as a quantifier for the proceeding studies. TceA3 peptide was detected in 10^5 DHC cell concentration and TceA4 and TceA5 were the least sensitive. These two peptides required 10^6 and 10^7 starting cell concentrations for quantification.

VcrA peptides were the least sensitive, with only VcrA3 peptide detected in 10^5 and 10^6 DHC cells (**Figure 1D**). The other two peptides, VcrA1 and VcrA4, were detected within their corresponding MDLs in 10^7 DHC cell concentration.

The analysis of the compiled data shows that the lowest concentration of DHC cells for quantification of FdhA, PceA and TceA peptides is 10^5 . However, quantification of VcrA peptides is possible when DHC concentrations of 10^6 or 10^7 are used.

Summary

Dilution study was set up to establish the minimum concentration of DHC cells required to detect FdhA, PceA, TceA and VcrA peptides using a targeted MRM assay. Prior to sample analysis, SDC-9 culture-specific RDase peptides were down selected and IDL and MDL were established for each peptide.

In this study, sample of SDC-9 culture was diluted in triplicate to 10^7 , 10^6 , 10^5 , 10^4 DHC cells and concentration of RDase and FdhA protein was analyzed using MRM proteomics.

Overall, the most sensitive peptides for quantification were FdhA5, PceA4, TceA2 and VcrA3 and required between 10^4 and 10^5 DHC cells to be detected. These peptides will serve as quantifiers in the next set of experiments. The required lowest concentration of DHC cells for detection of the remainder of peptides varied per protein, for example, to detect other TceA peptides a minimum of 10^5 cells need to be provided, but to detect VcrA specific peptides the cell concentrations need to be couple orders of magnitude higher. Thus, the total recommended DHC concentration for targeted proteomics is 10^6 cells, regardless of sample volume.

APPENDIX C
METHOD STANDARD OPERATING PROCEDURES
AND CALIBRATION OF ANALYTICAL EQUIPMENT

Primer and probe design

The amino acid sequences of RDases determined in SDC-9™ metagenomic analysis were compared to other published sequences in NCBI using BLASTP (<http://www.ncbi.nlm.nih.gov/BLAST>), and sequences were aligned with Geneious R11.0.2 (<http://www.geneious.com>, Kearsse et al., 2012). The specificity of primers and probes targeting regions of the target genes met the criteria of the Geneious R11.0.2 and the specificity of the primers and probes was also verified using BLAST analysis.

To validate each assay, primers were tested first with SYBR Green chemistry using QuantStudio 12K Flex Real-Time PCR System (Life Technologies, Grand Island, NY). The 10 µL qPCR mixture was composed of 5 µL of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 300 nM of each primer, 2.0 µL of template DNA and the remaining volume sterile nuclease-free water. The PCR cycle parameters applied were as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. After amplification, a melting curve analysis was carried out to confirm that the signal obtained in SYBR Green qPCR originated from specific target PCR products, not from primer dimers or non-specific amplifications. SYBR-Green qPCR assay criteria were used to validate each assay (i.e., the efficiency of the reaction should be 90-110%; the slope of standard curve should be between -3.1 to -3.6, and R² should >0.99, respectively). Amplification efficiencies were calculated by the method of Pfaffl (2001).

Following validation and optimization of each assay with SYBR Green qPCR, the primers and probe specific to each target assay were used for TaqMan qPCR. Each 10 µL mixture contained 5 µL of TaqMan Universal PCR Master Mix No AmpErase UNG (Applied Biosystems, Foster City, CA), 300 nM of each primer, 300 nM of probe and 2.0 µL of template DNA. TaqMan qPCR assays were run using QuantStudio 12K Flex Real-Time PCR System under the same PCR cycle conditions as described for SYBR Green qPCR. The target assays had amplification efficiencies between 90-110%, a standard curve slope between -3.3 to -3.6 and a standard curve with R² > 0.99. These values met the parameters suggested in literature (Holmes et al., 2006; Karlen et al., 2007; Ritalahti et al., 2009). The default instrument settings were used and LOD and LOQ values were determined as 1-10 and 10-50 copies per µL for each assay.

Standard curve preparation: Plasmid DNA was served as templates for standard curve preparation. Template plasmid DNA (pDNA) was synthesized utilizing the pMK-RQ vector and incorporated into *E. coli* by Life Technologies (Grand Island, NY) or the target gene fragment was inserted into the pCR™ 2.1 Vector using the Invitrogen TA Cloning™ kit (Life Technologies, Grand Island, NY) according to manufacturer's instructions. The *E. coli* transformant was grown in Luria Broth with ampicillin (100 mg/L) or kanamycin (50 mg/L) at 37°C overnight. pDNA was isolated using the Zymo Research Zippy™ Plasmid Miniprep Kit (Zymo Research Corp., Irvine, CA) and quantified using a NanoDrop and the Qubit 2.0 Fluorometer.

Standard curves were included with every qPCR plate using 10-fold serial dilutions of plasmid DNA over a 7 orders of magnitude range beginning at a 1 ng µL⁻¹ concentration (~8 log gene copies) and decreasing to 10⁻⁷ ng µL⁻¹. All standard curves had a total of eight calibration points

and were run in triplicate. To calculate the number of gene copies in a known amount of DNA and gene copies per sample, the equations given in Ritalahti et al. (2006) were applied.

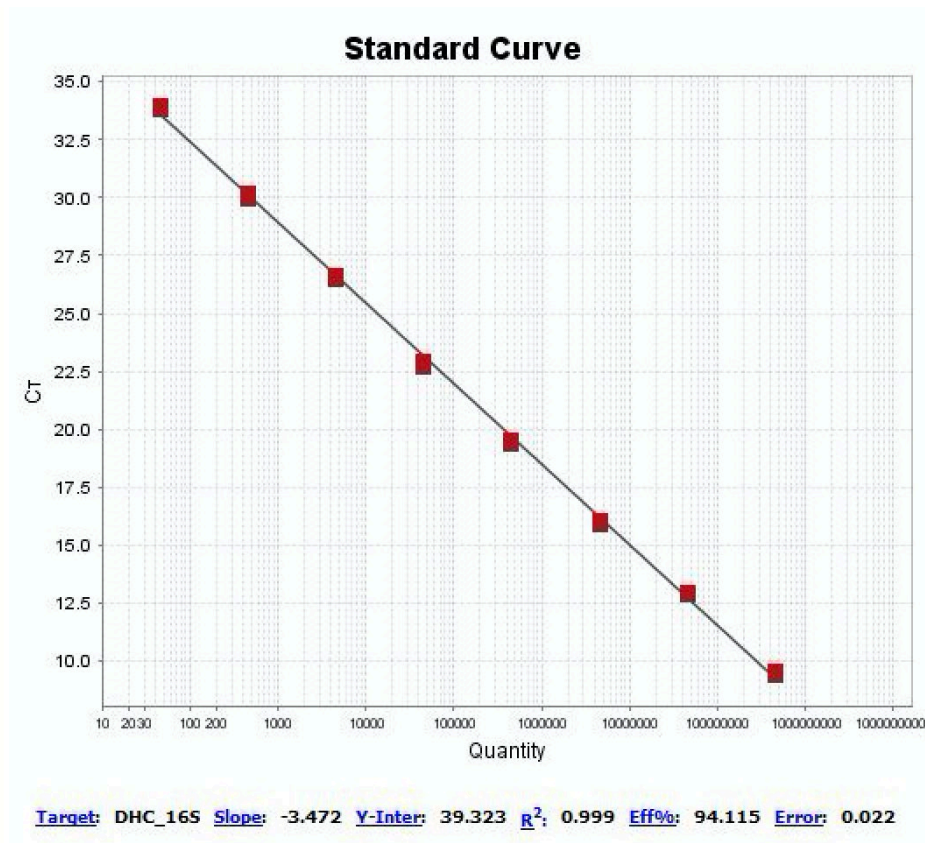


Figure 1. Example of standard curve for DHC_16S assay

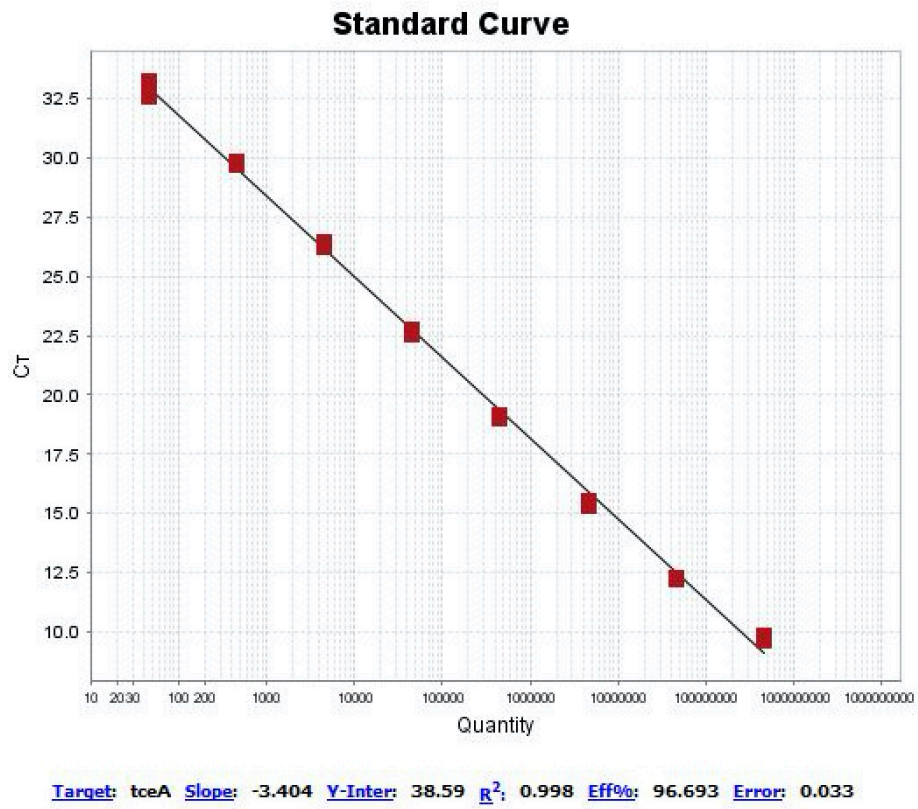


Figure 2. Example of standard curve for *tceA* assay

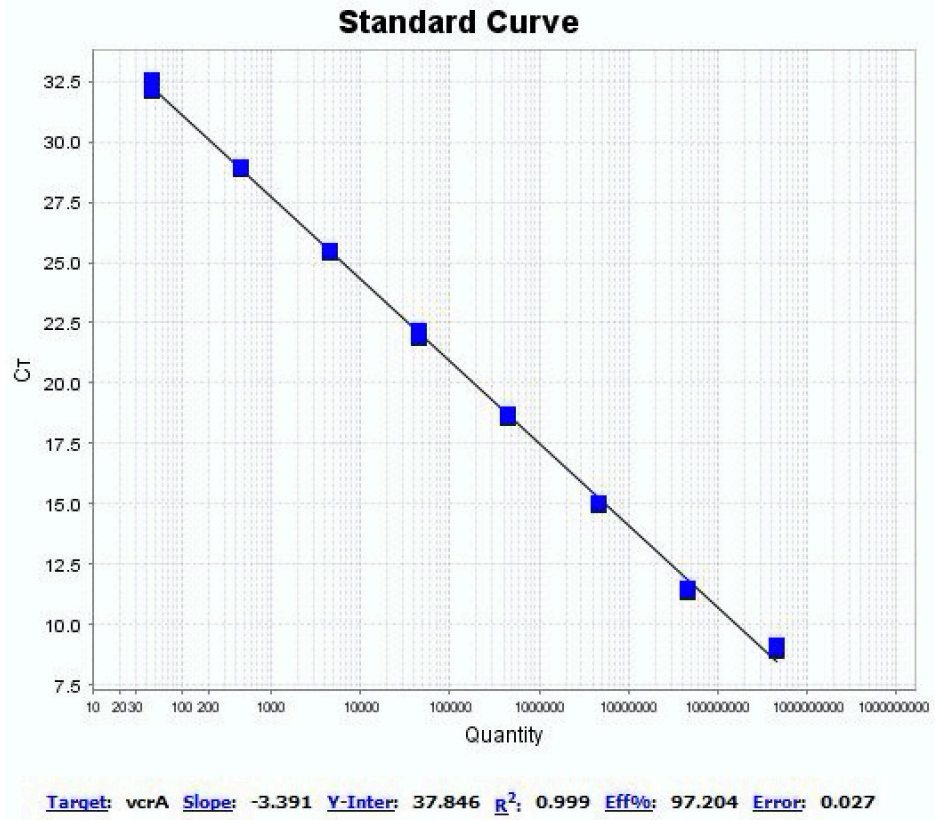


Figure 3. Example of standard curve for *vcrA* assay

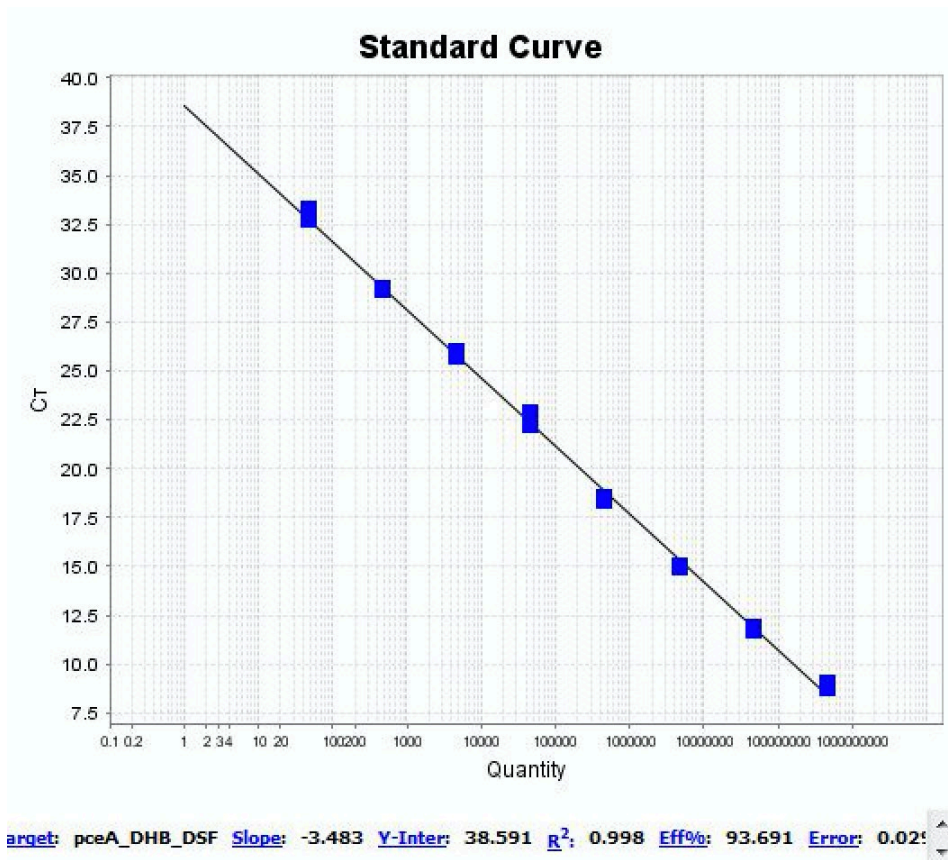
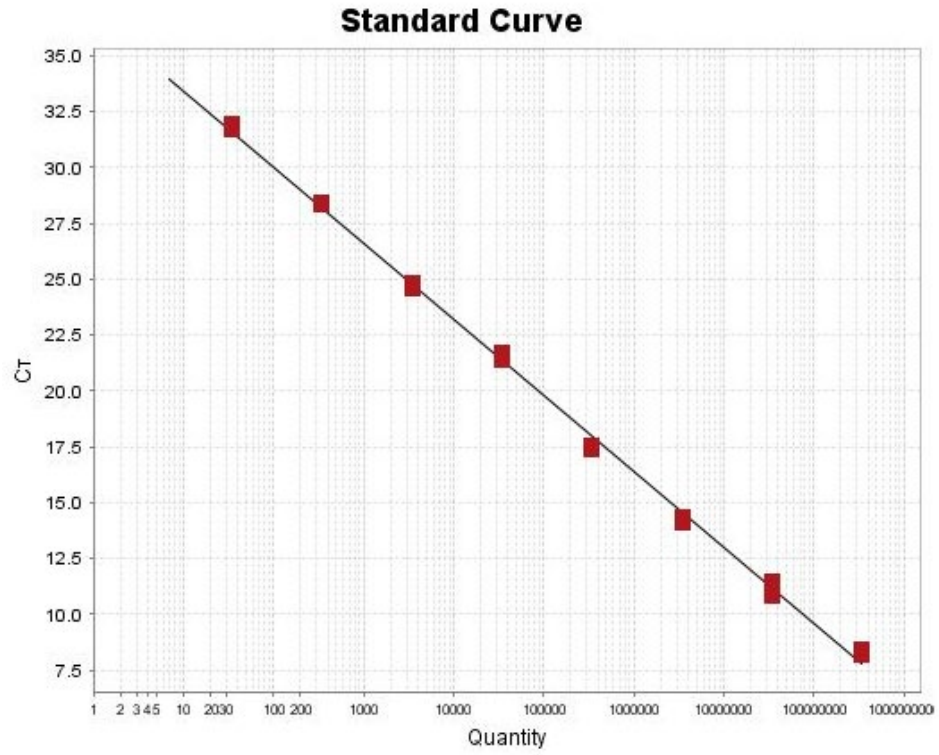


Figure 4. Example of standard curve for SDC9_24 *pceA* assay



Target: *fdhA*_DHC_All Slope: -3.403 Y-Inter: 36.858 R²: 0.998 Eff%: 96.721 Error: 0.03

Figure 5. Example of standard curve for *fdhA* assay

SHOTGUN AND TARGETED PROTEOMICS

The procedure described below has been developed and used by the Battelle Memorial Proteomics lab and is protected under the provisional patent.

Initial Discovery - Shotgun (bottom-up) Proteomics

1. Samples are analyzed by reverse-phase microflow HPLC-ESI-MS/MS using an Eksigent Nano 415 liquid chromatograph system (Sciex, Concord, CAN) which is directly connected to a quadrupole time-of-flight (QqTOF) TripleTOF 5600 mass spectrometer (Sciex, Concord, CAN).
2. The instrumentation is controlled using Analyst TF 1.6 and the Eksigent control software.
3. A total of 100 μL of sample is injected onto the analytical column (Eksigent 3C18-CL-120, 3 μm particle size, 120 \AA pore size, 0.3 x 150 mm) using a trap-and-elute method. In order to achieve 100 μL on column, 10 full loop volumes (10 μL loop) are injected and trapped onto a Pepmap 300 cartridge (C18, 5 μm particle size, 0.3 x 5 mm, Thermo Scientific, Rockwood, TN). Each loop injection is washed with mobile phase A.
4. Once fully loaded, the samples are eluted from the trap and separated. All solvent concentration changes are linear with respect to time. Mobile phase solutions are purchased from Burdick and Jackson and are as follows: mobile phase A: 0.1% formic acid (v/v) in water (LC-MS grade); and mobile phase B: 0.1% formic acid (v/v) in acetonitrile (LC-MS grade).
5. Continuing mass calibration of the TOF MS and TOF MS/MS is performed throughout the analysis sequence by analyzing a digested beta-galactosidase standard (Sciex, Concord, CAN). Mass spectrometric analysis is performed using data dependent acquisition (referred to as information dependent acquisitions, or IDA). Full scan spectra are acquired from 400 to 1250 m/z with a 250 millisecond acquisition time.
6. For collision induced dissociation tandem mass spectrometry (CID MS/MS) in IDA mode, the mass window for precursor ion selection of the quadrupole mass analyzer is set to unit resolution (± 0.5 m/z). For MS/MS analysis, precursor ions were fragmented in a collision cell using nitrogen as the collision gas. For IDA analysis, the instrument is set to trigger product ion scans (from 100 – 1500 m/z) only after specific criteria are met by the precursor ions. The Rolling Collision Energy algorithm is used to determine the appropriate collision energy for each precursor mass.

Additional Discovery plus Select Targets - Shotgun (bottom-up) Proteomics

Based on the results of the initial shotgun analysis, specific targets are identified for further investigation. These targets are divided into two groups and samples are analyzed in two separate analyses. The instrument is set up with an inclusion list of these specific targets that would automatically trigger a product ion scan if the specified precursor was detected. The instrumentation, mobile phase, LC method, and injection volume were the same as the initial discovery analysis.

Labeled Peptide Analysis

1. Isotopically labeled peptides are purchased from Thermo Fisher Scientific based on the results of the targeted analysis.
2. Individual stocks of each peptide are prepared in 0.1% formic acid. From these stocks, multiple mixed solutions are prepared at 0.1, 1.0, 10, 100, and 1000 Xmol/ μ L in order to determine the approximate detection limit of each peptide and also the instrumental linearity.
3. Two test samples that were previously analyzed are split and spiked at a final on-column concentration of 200 fmol. Using these samples, 90 minute is used for all further analysis. All instrumentation, mobile phase, and injection volumes were the same as the initial discovery analysis

Shotgun with Peptide Targets

Test samples are split and spiked with each labeled peptide. These samples are analyzed using the 90 minute gradient for specific CVOC targets plus any additional discovery data that may have been missed on the first initial analyses. The instrumentation, mobile phase, LC method, and injection volume were the same as the labeled peptide analysis.

MRMHR for MTBE Quantification

1. A mass spectrometric method will be built to perform product ion scans of the specific targets plus associated labeled peptides that were previously purchased. The analysis is performed using a 15 minute gradient, with a total runtime of 16 minutes, including mobile phase equilibration. The samples will be spiked with both labeled peptides for a final concentration of X mol on column. The instrumentation, mobile phase, and LC method are the same as the labeled peptide analysis.
2. Integrated reconstructed ion chromatograms of precursor-product ion transitions of both the labeled and un-labeled peptide targets are produced using MultiQuant software version 2.1 (Sciex, Concord, CAN). Multiple precursor-product ion transitions are plotted to add confidence to the tentative target detections.

3. Using the response of each labeled peptide and its spiked concentration, a response factor is calculated. These calculated response factors are used for quantification of the unlabeled peptide targets.

CALLIBRATION OF AB SCIEX TripleTOF® 5600/5600+ INSTRUMENT

For tuning the system, use the following solutions that come with the installation kit:

For positive mode:

- For optimizing TOF MS - MSMS high resolution or MSMS High Sensitivity, use the Tuning Solution.
- For Q1 calibration, use the PPG POS solution.

In negative mode:

- For optimizing TOF MS - MSMS High Resolution or MSMS High Sensitivity, use Taurocholic acid.
- For Q1 calibration, use the PPG 3000 solution.

Required material

- Tuning solutions that are supplied in the Standards Chemical Kit shipped with the system. If needed, a new Kit can be ordered from AB SCIEX.
- Gas-tight syringes (1.0 ml is recommended)
- PEEK (red) sample tubing

Prerequisites

- Make sure that a printer is configured.
- Make sure that the spray is stable and that the proper tuning solution is being used.

Optimize the Instrument

The following procedure shows how to verify the performance of the instrument.

1. In the Navigation bar, under **Tune and Calibrate**, double-click **Manual Tuning**.
2. Run a TOF MS or Product ion scan type and confirm that there is a stable TIC and that the peaks of interest are present in the spectrum.
3. In the Navigation bar, under **Tune and Calibrate**, double-click **Instrument**

Optimization.

Note: AB SCIEX recommends that after using the Taurocholic acid, repeat the channel alignment using the PPG 3000 solution.

4. Select a tuning solution. Make sure that the tuning solution matches the reference table.
5. The **Verify Performance Only** check box is preselected. Click **Next**.

For this example, leave this option selected. If the report indicates that the instrument needs tuning, then run Instrument Optimization again and select one or more scan modes to optimize. Make sure that the ion source and syringe parameters are suitable.

7. Click **GO**.

The Verifying Performance screen appears. After the process has completed, the Results Summary appears showing the resolution and intensity for each scan mode.

Example of continuing calibration method with beta galactosidase is detailed in Table A1. Figures A1 and A2 show reference table editor for the opening calibration with tuning solution. And beta-galactosidase. The editor references the compounds and masses used during calibration.

Table H1. Continuing calibration method with beta-galactosidase.

HPLC Mass Spectrometer	Eksigent Nano 415 AB Sciex 5600+ Triple ToF																								
Mass Spec Source	Electrospray, positive ion mode																								
Mass Spec Parameters	Experiment 1: Scan Type: ToF MS ToF Mass Range: 400 - 1500 Da Accumulation Time: 0.250 seconds Experiment 2: Scan Type: Product Ion Products of: 729.37 Da ToF Mass Range: 100 - 1500 Da Accumulation Time: 0.500 seconds																								
HPLC Column	Eksigent 3C18-CL-120, 3 μm, 120 A, 0.3 x 150 mm																								
Column Temperature	30° c																								
Mobile Phase Components	A= 0.1% formic acid in water B= 0.1% formic acid in methanol																								
Gradient Profile	All changes are linear with respect to time: <table border="1" data-bbox="667 1377 1088 1692"> <thead> <tr> <th>Time, min</th> <th>%B</th> <th>Flow rate, μL/min</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>5</td> <td>5</td> </tr> <tr> <td>1</td> <td>5</td> <td>5</td> </tr> <tr> <td>8</td> <td>35</td> <td>5</td> </tr> <tr> <td>9</td> <td>90</td> <td>5</td> </tr> <tr> <td>11</td> <td>90</td> <td>5</td> </tr> <tr> <td>12</td> <td>5</td> <td>5</td> </tr> <tr> <td>16</td> <td>5</td> <td>5</td> </tr> </tbody> </table>	Time, min	%B	Flow rate, μL/min	0	5	5	1	5	5	8	35	5	9	90	5	11	90	5	12	5	5	16	5	5
Time, min	%B	Flow rate, μL/min																							
0	5	5																							
1	5	5																							
8	35	5																							
9	90	5																							
11	90	5																							
12	5	5																							
16	5	5																							
Injection Volume	1μL (Trap and Elute, 5 minute wash with Mobile Phase A at 5μL/min)																								
Run Time	16 min																								

Reference Table Editor

Name: APCI Positive Calibration Solution New Copy Delete Positive Negative Calibration Valve Position: A

Reference Ions for TOF MS Calibration:

	Use	Compound Name	Precursor m/z (Da)	Use for MS/MS	CE for MS/MS	DP for MS/MS	Retention Time (min)
1	<input checked="" type="checkbox"/>	aminoheptanoic acid	146.11756	<input type="checkbox"/>	30.000	80.000	0.00
2	<input checked="" type="checkbox"/>	amino-dPEG 4-acid	266.15981	<input type="checkbox"/>	30.000	80.000	0.00
3	<input checked="" type="checkbox"/>	clomipramine	315.16225	<input type="checkbox"/>	27.000	80.000	0.00
4	<input checked="" type="checkbox"/>	amino-dPEG 6-acid	354.21224	<input type="checkbox"/>	30.000	80.000	0.00
5	<input checked="" type="checkbox"/>	amino-dPEG 8-acid	442.26467	<input type="checkbox"/>	30.000	80.000	0.00
6	<input checked="" type="checkbox"/>	reserpine	609.28066	<input checked="" type="checkbox"/>	45.000	80.000	0.00
7	<input checked="" type="checkbox"/>	amino-dPEG 12-acid	618.36953	<input type="checkbox"/>	30.000	80.000	0.00
8	<input checked="" type="checkbox"/>	Hexakis(2,2,3,3-tetra	922.00980	<input type="checkbox"/>	30.000	80.000	0.00
9	<input type="checkbox"/>	Hexakis(1,1,5-octafl	1521.97148	<input type="checkbox"/>	30.000	80.000	0.00
10	<input type="checkbox"/>			<input type="checkbox"/>			
11	<input type="checkbox"/>			<input type="checkbox"/>			
12	<input type="checkbox"/>			<input type="checkbox"/>			
13	<input type="checkbox"/>			<input type="checkbox"/>			
14	<input type="checkbox"/>			<input type="checkbox"/>			

Reference Ions for MS/MS Calibration:
(Product of 609.28066 Da)

	Use	Fragment Name	Fragment m/z (Da)
1	<input checked="" type="checkbox"/>	C11H12NO	174.09130
2	<input checked="" type="checkbox"/>	C10H11O4	195.06520
3	<input checked="" type="checkbox"/>	C13H18NO3	236.12810
4	<input checked="" type="checkbox"/>	C22H25N2O3	365.18600
5	<input checked="" type="checkbox"/>	C23H29N2O4	397.21220
6	<input checked="" type="checkbox"/>	C23H30NO8	448.19660
7	<input checked="" type="checkbox"/>	C33H40N2O9	609.28066
8	<input type="checkbox"/>		
9	<input type="checkbox"/>		
10	<input type="checkbox"/>		
11	<input type="checkbox"/>		
12	<input type="checkbox"/>		
13	<input type="checkbox"/>		
14	<input type="checkbox"/>		

Retention time is only used for non-CDS configuration. Retention Time Tolerance: +/- 0.000 sec

OK Cancel Help

Figure H1. Reference table editor for the opening calibration with tuning solution. The editor references the compounds and masses used during calibration. Not all compounds are used and have a check mark if the mass is used for calibration.

Reference Table Editor

Name: Beta Galactosidase Digest New Copy Delete Positive Negative Calibration Valve Position: A

Reference Ions for TOF MS Calibration:

	Use	Compound Name	Precursor m/z (Da)	Use for MS/MS	CE for MS/MS	DP for MS/MS	Retention Time (min)
1	<input type="checkbox"/>	WLPAMSER	495.24730	<input type="checkbox"/>	24.000	100.000	6.75
2	<input type="checkbox"/>	YSQQQLMETSHR	503.23680	<input type="checkbox"/>	27.000	100.000	9.11
3	<input type="checkbox"/>	RDWENPGVTQLNR	528.93410	<input type="checkbox"/>	25.000	100.000	13.40
4	<input type="checkbox"/>	WVGYGQDSR	534.24890	<input type="checkbox"/>	23.000	100.000	9.65
5	<input checked="" type="checkbox"/>	GDFQFNISR	542.26450	<input type="checkbox"/>	26.000	100.000	8.00
6	<input checked="" type="checkbox"/>	IDPNAWVER	550.28020	<input type="checkbox"/>	27.000	100.000	7.67
7	<input type="checkbox"/>	DVSLHLKPTTQISDF	567.05510	<input type="checkbox"/>	30.000	100.000	13.31
8	<input checked="" type="checkbox"/>	VDEDQPPFAVPK	671.33790	<input type="checkbox"/>	33.000	100.000	7.50
9	<input checked="" type="checkbox"/>	WENPGVTQLNR	714.84690	<input type="checkbox"/>	32.000	100.000	7.50
10	<input checked="" type="checkbox"/>	APLDNDIGVSEATR	729.36520	<input checked="" type="checkbox"/>	48.000	100.000	6.99
11	<input type="checkbox"/>			<input type="checkbox"/>			
12	<input type="checkbox"/>			<input type="checkbox"/>			
13	<input type="checkbox"/>			<input type="checkbox"/>			
14	<input type="checkbox"/>			<input type="checkbox"/>			

Reference Ions for MS/MS Calibration:
(Product of 729.36520 Da)

	Use	Fragment Name	Fragment m/z (Da)
1	<input type="checkbox"/>	y1	175.11900
2	<input checked="" type="checkbox"/>	y3	347.20370
3	<input checked="" type="checkbox"/>	y5	563.27840
4	<input type="checkbox"/>		729.36520
5	<input checked="" type="checkbox"/>	y8	832.45230
6	<input checked="" type="checkbox"/>	y10	1061.52220
7	<input checked="" type="checkbox"/>	y12	1289.63320
8	<input type="checkbox"/>		
9	<input type="checkbox"/>		
10	<input type="checkbox"/>		
11	<input type="checkbox"/>		
12	<input type="checkbox"/>		
13	<input type="checkbox"/>		
14	<input type="checkbox"/>		

Retention time is only used for non-CDS configuration. Retention Time Tolerance: +/- 180.000 sec

OK Cancel Help

Figure H2. Reference table editor for the opening calibration with betagalactosidase. The editor references the compounds and masses used during calibration. Not all compounds are used and have a check mark if the mass is used for calibration.

APPENDIX D
MICROCOSM ANALYTICAL AND
BIOMARKER ABUNDANCE DATA

JBLM1, #3

SET 3			Fort Lewis JBML			START			7/18/17 10:55																							
10 ⁶ DHC/mL			ATL ID			VOC's ug/L						Methane/Ethene ug/L						Anions ng/L						VFA mg/L								
DATE	TIME(days)	Bottle	pH	mean	ATL ID	VOC's ug/L			Methane			Ethene			Bromide			Lactic			Acetic			Propionic								
						VC	Q	mean	eDCE	Q	mean	Q	mean	Q	mean	Q	mean	Q	mean	Q	mean	Q	mean	Q	mean							
7/18/17 10:55	0	3A	7.62		9721	53	U		4560	D		3.03		0.44	U		9.08		152	D	0.93	U		3.7	U	3.70						
		3B	7.77	7.74		53	U	53.00	4680	D	4427	3.45		2.97	U	0.44	8.98	180	D	180.67	0.93	U	0.93	3.7	U	3.70						
		3C	7.84	0.11		53	U	0	4040	D	340	2.44		0.51	U	0.00	9.44		210	D	29.01	0.93	U	0.00	3.7	U	0.00					
7/24/17 9:00	5.9	3A	7.17		9742	159	JD		4040	D		13.3		0.44	U		9.47		196	D	45.4	D		100	D							
		3B	7.50	7.31		67.6	JD	124.87	4020	D	3963.33	10.8		12.20	U	0.44	8.77		296	D	219.00	22.9	D	33.07	13.7	JD	57.80					
		3C	7.25	0.17		148	D	49.89843	3830	D	115.9023	12.5		1.28	U	0.00	8.57		165	D	68.46	30.9	D	11.41	59.7	D	43.18					
7/27/17 9:00	8.9	3A	6.97		9726	64.8	JD		2900	D		63.3		0.44	U		8.07		223	U		114	D		220	D						
		3B	6.92	7.00		61.2	JD	84.67	2050	D	2996.67	63.6		64.93	U	0.44	8.69		8.48	223	U	2.23	121	D	119.67	226	D	225.00				
		3C	7.10	0.09		128	JD	32.52095	3120	D	223.0385	67.9		2.57	U	0.00	8.69		0.36	223	U	0.00	124	D	5.13	229	D	4.58				
7/31/17 9:00	12.9	3A	7.00		9727	577	D		3180	D		145		2.42			9.46		187	D			170	D								
		3B	7.06	7.04		323	D	474.33	3430	D	3260.00	271		200.00	1.08	J	1.99	9.34		9.25	116	U	1.52	109	D	129.67	120	D	158.33			
		3C	7.07	0.04		523	D	133.8108	3170	D	147.3092	184		64.51	2.47		8.96		0.26	116	U	0.62	93	D	50.29	185	D	34.03				
8/3/17 9:00	15.9	3A	6.97		9728	695	D		3050	D		227		4.61			8.60		116	U		108	D		195	D						
		3B	6.98	6.99		433	D	595.67	3320	D	3073.33	300		262.00	1.73	J	3.39	8.33		8.35	116	U	1.16	101	D	101.60	186	D	188.67			
		3C	7.02	0.03		659	D	142.0188	2850	D	235.8672	259		36.59	3.84		8.12		0.24	116	U	0.00	95.8	D	6.12	185	D	5.51				
8/7/17 9:00	19.9	3A	7.12		9730	932	D		2810	D		273		9.9			8.50		112	U		76	D		138	D						
		3B	7.10	7.13		529	D	774.00	3120	D	2793.33	334		297.00	2.97	J	6.78	8.23		8.30	112	U	1.12	100	D	91.00	184	D	166.67			
		3C	7.16	0.03		861	D	215.1255	2450	D	335.3108	284		32.51	7.46		8.18		0.17	112	U	0.00	97	D	13.08	178	D	25.01				
8/10/17 9:00	22.9	3A	7.05		9734	977	D		2410	D		225		11.1			7.52		112	U		90.6	D		164	D						
		3B	7.05	7.06		586	D	849.00	2840	D	2490.00	268		234.33	3.30		7.97		7.76	112	U	1.12	102	D	93.60	187	D	176.00				
		3C	7.07	0.01		984	D	227.7916	2220	D	317.6476	210		30.11	9.29		7.78		0.23	112	U	0.00	88.2	D	7.37	177	D	11.53				
8/14/17 9:40	26.9	3A	7.06		9735	1110	D		2210	D		228		17.5			6.94		112	U		91.5	D		164	D						
		3B	7.12	7.12		740	D	1000.00	2750	D	2290.00	154		201.00	2.88	J	12.49	6.98		6.99	112	U	1.12	101	D	97.50	183	D	175.67			
		3C	7.18	0.06		1150	D	226.0531	1910	D	425.6759	221		40.85	17.1		7.04		0.05	112	U	0.00	100	D	5.22	180	D	10.21				
8/23/17 9:00	35.9	3A	7.06		9738	1820	D		1760	D		199		0.37	U		7.25		112	U		108	D		208	D						
		3B	7.08	7.08		1250	D	1566.67	2250	D	1733.33	210		199.33	0.37	U	0.37	7.42		7.44	112	U	1.12	115	D	113.33	221	D	219.00			
		3C	7.09	0.02		1630	D	290.2298	1190	D	530.5029	189		10.50	0.37	U	0.00	7.66		0.21	112	U	0.00	117	D	4.73	228	D	10.15			
9/11/17 9:00	54.9	3A	7.09		9739	1770	D		600	D		146		75.5			7.04		112	U		78	D		170	D						
		3B	7.00	7.05		1390	D	1576.67	1250	D	776.00	248		209.67	33.1		7.13		7.05	112	U	1.12	74.1	D	98.03	177	D	221.00				
		3C	7.06	0.05		1570	D	190.0877	478	D	415.0036	235		55.52	169		6.99		0.07	112	U	0.00	142	D	38.13	316	D	82.35				
10/9/17 10:00	83.0	3A	7.48		9752	1820	D		41	U		241		168			5.35		116	U		103	D		213	D						
		3B	7.44	7.46		1690	D	1663.33	340	D	140.67	188		195.33	100		5.25		5.34	116	U	1.16	96.7	D	102.23	208	D	214.33				
		3C	7.46	0.02		1480	D	171.5615	41	U	172.6277	157		42.48	212		5.42		0.09	116	U	0.00	107	D	5.19	222	D	7.09				

JBLM1, #6

SET 6		Fort Lewis JBML			START	7/18/17 14:00																										
Killed Control							VOC's ug/L					Methane/Ethane ug/L					Anions mg/L					VFA mg/L										
DATE	TIME(days)	Bottle	pH	mean	ATL ID	VC	Q	mean	cDCE	Q	mean	Methane	Q	mean	Ethane	Q	mean	Bromide	Q	mean	Lactic	Q	mean	Acetic	Q	mean	Propionic	Q	mean			
7/18/17 14:00	0	6A	6.60		9721	53	U		3450	D		2.95			0.44	U		8.13			331	D		33.4	D		3.7	U				
		6B	6.59	6.59		53	U	53.00	2810	D	3293.33	3.23		3.28	0.44	U	0.44	6.61		7.85	317	D	334.00	63.1	D	38.13	3.7	U	3.70			
		6C	6.58	0.01		53	U	0.00	3620	D	427.12	3.66		0.36	0.44	U	0.00	8.81		1.13	354	D	18.68	17.9	D	22.97	3.7	U	0.00			
		6A	6.93		9742	53	U		4570	D		1.74			0.44	U		7.84			298	D		14.9	JD	3.7	U					
		6B	6.94	6.93		53	U	53.00	4250	D	4433.33	1.69		1.66	0.44	U	0.44	8.22		8.09	359	D	343.00	0.93	U	5.59	3.7	U	3.70			
		6C	6.93	0.01		53	U	0.00	4480	D	165.03	1.55		0.10	0.44	U	0.00	8.20		0.21	372	D	39.51	0.93	U	8.07	3.7	U	0.00			
		6A	6.92		9726	52.8	U		3700	D		1.73			0.44	U		8.43			384	D		0.93	U	3.7	U					
		6B	6.94	6.94		52.8	U	75.50	1520	D	2633.33	1.09		1.49	0.44	U	0.44	8.97		8.70	357	D	343.67	0.47	U	0.78	1.85	U	3.08			
		6C	6.96	0.02		52.8	U	0.00	7680	D	1098.75	1.66		0.35	0.44	U	0.00	8.70		0.27	290	D	48.40	0.93	U	0.27	3.7	U	1.07			
		6A	6.98		9727	52.8	U		4230	D		1.71			0.44	U		9.87			357	D		130	D	1.85	U					
		6B	6.96	6.97		52.8	U	52.80	4080	D	4113.33	1.34		1.49	0.44	U	0.44	9.17		9.26	321	D	346.67	27.9	D	56.90	3.7	U	3.08			
		6C	6.98	0.01		52.8	U	0.00	4030	D	104.08	1.43		0.19	0.44	U	0.00	8.73		0.57	362	D	22.37	12.8	D	63.76	3.7	U	1.07			
		6A	6.96		9728	52.8	U		4290	D		2.21			0.44	U		8.92			317	D		103	D	1.85	U					
		6B	6.98	6.97		52.8	U	52.80	4170	D	4066.67	0.19	U	1.30	0.44	U	0.44	8.31		8.47	356	D	344.33	24.1	D	48.87	1.85	U	1.85			
		6C	6.98	0.01		52.8	U	0.00	3740	D	289.19	1.51		1.03	0.44	U	0.00	8.18		0.40	360	D	23.76	19.5	D	46.94	1.85	U	0.00			
		6A	6.99		9730	52.8	U		4200	D		1.59			0.44	U		7.29			338	D		0.47	U	1.85	U					
		6B	7.01	7.01		52.8	U	52.80	4130	D	4010.00	1.64		1.49	0.44	U	0.44	7.64		7.55	342	D	330.00	0.47	U	0.47	1.85	U	1.85			
		6C	7.02	0.02		52.8	U	0.00	3700	D	270.74	1.24		0.22	0.44	U	0.00	7.71		0.23	310	D	17.44	0.47	U	0.00	1.85	U	0.00			
		6A	7.00		9734	52.8	U		4160	D		1.68			0.44	U		7.22			341	D		0.47	U	1.85	U					
		6B	7.05	7.04		52.8	U	52.80	3950	D	3963.33	1.37		1.47	0.44	U	0.44	7.45		7.38	345	D	347.67	0.47	U	0.47	1.85	U	1.85			
		6C	7.06	0.03		52.8	U	0.00	3780	D	190.35	1.36		0.18	0.44	U	0.00	7.46		0.14	357	D	8.33	0.47	U	0.00	1.85	U	0.00			
		6A	6.88		9735	52.8	U		3940	D		1.35			0.44	U		7.82			343	D		0.47	U	1.85	U					
		6B	6.97	6.95		52.8	U	52.80	3800	D	3920.00	1.70		1.49	0.44	U	0.44	6.97		7.29	356	D	355.33	0.47	U	0.47	1.85	U	1.85			
		6C	7.00	0.06		52.8	U	0.00	4020	D	111.36	1.43		0.18	0.44	U	0.00	7.09		0.46	367	D	12.01	0.47	U	0.00	1.85	U	0.00			
		6A	6.98		9739	52.8	U		3640	D		2.37			0.44	U		6.05			380	D		0.47	U	1.85	U					
		6B	6.99	6.99		52.8	U	52.80	3620	D	3600.00	2.72		2.49	0.44	U	0.44	7.09		6.84	376	D	387.67	0.47	U	0.47	1.85	U	1.85			
		6C	7.00	0.01		52.8	U	0.00	3540	D	52.92	2.39		0.20	0.44	U	0.00	7.38		0.70	407	D	16.86	0.47	U	0.00	1.85	U	0.00			
		6A	7.23		9752	52.8	U		2980	D		1.48			0.44	U		5.48			529	D		0.47	U	1.85	U					
		6B	7.10	7.17		52.8	U	52.80	3160	D	2936.67	1.62		1.58	0.44	U	0.44	5.97		5.81	538	D	510.00	0.47	U	0.47	1.85	U	1.85			
		6C	7.19	0.07		52.8	U	0.00	2670	D	247.86	1.64		0.09	0.44	U	0.00	5.97		0.28	463	D	40.95	0.47	U	0.00	1.85	U	0.00			

APPENDIX E
KEY POINTS OF CONTACT

KEY POINTS OF CONTACT

The key personnel involved in this project and their contact information is summarized in Table 8-1 below. The Principal Investigator (PI) and all co-PIs share responsibility for the overall execution and delivery of this project, including data quality, analysis, interpretation and preparation of deliverables. Mandy Michalsen, PI, is responsible for the overall execution of this project and identification/coordination with cVOC-contaminated DoD field sites. Ember Korver, USACE Project Manager, is responsible for contractual oversight and general project support. Paul Hatzinger, co-PI, is responsible for microcosm testing, SDC-9™ culture growth and application, chemical analyses for microcosms, and data interpretation. Frank Löffler, co-PI, is responsible for qPCR, gene-transcript-protein correlation factors, data interpretation. Kate Kucharzyk, co-PI, is responsible for proteomic and genomic analysis and data interpretation. John Wilson and Jack Istok, co-PIs, are responsible for technical reviews and data interpretation.

Table 8-1. Project points of contact

POINT OF CONTACT Name	ORGANIZATION Name Address	Phone Fax E-mail	Role in Project
Mandy Michalsen	U.S. Army Corps of Engineers, Seattle	(p) 206-764-3324 mandy.m.michalsen@usace.army.mil	Principal Investigator, Field Support
Ember Korver	U.S. Army Corps of Engineers, Seattle	(p) 206-764-6792 ember.e.korver@usace.army.mil	Project Management, Contract Administration
Paul Hatzinger	CB&I	(p) 267-337-4003 paul.hatzinger@cbifederaleservices.com	Co-Principal Investigator
Frank Löffler	University of Tennessee	(p) 865-974-4933 frank.loeffler@utk.edu	Co-Principal Investigator
Kate Kucharzyk	Battelle Memorial Institute	(p) 614-424-5489 kucharzyk@battelle.org	Co-Principal Investigator
John Wilson	Scissortail Environmental Solutions	(p) 580-421-3551 john@sissortailenv.com	Co-Principal Investigator
Jack Istok	Oregon State University	(p) 541-619-3996 jack.istok@oregonstate.edu	Co-Principal Investigator