

FINAL REPORT

Advanced Environmental Molecular Diagnostics to Assess, Monitor, and Predict Microbial Activities at Complicated Chlorinated Solvent Sites

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14. ABSTRACT Bioremediation plays a crucial role in transforming and detoxifying chlorinated solvents and has been implemented as a stand-alone technology at sites undergoing monitored natural attenuation (MNA), biostimulation with or without bioaugmentation treatment, or as a polishing step when physical/chemical treatment serves as the primary remedy. Molecular Biological Tools (MBTs), foremost quantitative PCR (qPCR), provide information about the presence and abundance of keystone dechlorinating bacteria, and are instrumental for site assessment, bioremediation monitoring, and the implementation of adaptive site management strategies. The overarching objective of this project was to advance MBTs and their application to minimize biases and to more effectively assess, predict, monitor, optimize, and manage reductive dechlorination processes at DoD sites impacted with chlorinated solvents. To further assist in the interpretation of MBT data, additional aims were to assess measureable parameters that correlate with the detoxification or incomplete (stalled) degradation of chlorinated ethenes, and to identify knowledge gaps that currently limit the efficient application of MBTs for decision-making at chlorinated solvent sites.					
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Acronyms and Abbreviations

aa	Amino acid
ASM	Adaptive site management
1,1-DCA	1,1-Dichloroethane
1,1,1-TCA	1,1,1-Trichloroethane
1,2-D	1,2-Dichloropropane
BDI	Bio-Dechlor INOCULUM
BES	2-Bromoethanesulfonate
BN-PAGE	Blue native polyacrylamide gel electrophoresis
<i>bvcA</i>	RDase gene implicated in DCE and VC reductive dechlorination
CA	Chloroethane
CART	Classification and regression tree
cDCE	<i>cis</i> -1,2-Dichloroethene
cDFE	<i>cis</i> -1,2-Difluoroethene
cDNA	Complementary deoxyribonucleic acid
<i>cerA</i>	RDase gene implicated in DCE and VC reductive dechlorination
CF	Chloroform
CFC-113	1,1,2-Trichloro-1,2,2-trifluoroethane
<i>cfrA</i>	RDase gene implicated in CF and 1,1,1-TCA reductive dechlorination
CISM	Complex iron-sulfur molybdoenzyme
Cl ⁻	Inorganic chloride
Crt	Relative threshold cycle
CTC	Cost to completion
CTFE	Chlorotrifluoroethene
DCE	Dichloroethene
DCM	Dichloromethane
DEFO	<i>Dehalobacterium formicoaceticum</i>
DIEL	‘ <i>Candidatus</i> Dichloromethanomonas elyunquensis’
DMB	Dimethylbenzimidazole
DNQ	Detectable but non-quantifiable
<i>dcpA</i>	RDase gene implicated in 1,2-D reductive dechlorination
<i>dcrA</i>	RDase gene implicated in 1,1-DCA reductive dechlorination
ddH ₂ O	Double-distilled water (ultra pure and sterile water)
2D-LC-MS/MS	Two-dimensional liquid chromatography tandem mass spectrometry
<i>Dhc</i>	<i>Dehalococcoides mccartyi</i>
<i>Dhgm</i>	<i>Dehalogenimonas</i>
<i>Dhb</i>	<i>Dehalobacter</i>
<i>Dsf</i>	<i>Desulfitobacterium</i>
DNAPL	Dense non-aqueous-phase liquid
DP	Detoxification potential
dPCR	Digital polymerase chain reaction
DTT	Dithiothreitol
EMBL	European Molecular Biology Laboratory
ESI	Electrospray ionization
ETC	Electron Transfer Components
FDR	False discovery rate

FWHM	Full width at half maximum
<i>Geo</i>	<i>Geobacter lovleyi</i>
IAA	Iodoacetamide
ISCO	<i>In situ</i> chemical oxidation
ISCR	<i>In situ</i> chemical reduction
KB-1	Commercial bioaugmentation consortium
LB	Lysogeny Broth
LC-MRM-MS	Liquid chromatography, multiple reaction-monitoring-mass spectrometry
LDF	Linear dsDNA fragments
LOD	Limit of detection
LOQ	Limit of quantification
LTQ Orbitrap	Linear trap quadrupole mass spectrometer
MBTs	Molecular biological tools
MCL	Maximum concentration level
MDL	Minimum detection limit
MGB	Minor Groove Binder
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
MNA	Monitored natural attenuation
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
NASNI	Naval Air Station North Island
N ₂ O	Nitrous oxide
NCBI	National Center for Biotechnology Information
NSAF	Normalized spectral abundance factor
nt	Nucleotide
OTU	Operational taxonomic unit
PCE	Tetrachloroethene
<i>pceA</i>	RDase gene implicated in PCE and/or TCE reductive dechlorination
PDL	Practical detection limit
pDNA	Plasmid deoxyribonucleic acid
PSM	Peptide spectrum match
<i>pteA</i>	RDase gene implicated in PCE reductive dechlorination
qPCR	Quantitative real-time polymerase chain reaction
RDase	Reductive dehalogenase
RD-qChip	An array plate for high-throughput qPCR targeting reductive dechlorination biomarker genes/transcripts
ROC	Receiver operating characteristic
<i>rpoB</i>	Gene that encodes the β subunit of bacterial RNA polymerase
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
Q-Exactive Plus	High mass accuracy and resolution mass spectrometer
QqQ-MS	Triple quadrupole mass spectrometry
SDC-9	<i>Dhc</i> -containing bioaugmentation consortium (SDC-9 mixed culture)
SDS	Sodium dodecyl sulfate
Std. dev.	Standard deviation
TC	Third Creek (a Tennessee River tributary in Knoxville, TN)

TCA	Trichloroacetic acid
tDCE	<i>trans</i> -1,2-Dichloroethene
TCE	Trichloroethene
<i>tceA</i>	RDase gene implicated in TCE reductive dechlorination
TCA	Trichloroacetic acid
T3C	A chlorinated solvent-dechlorinating consortium derived from Third Creek sediment
TFE	Trifluoroethene
TGR	Transcript-to-gene ratio
T_m	Melting temperature
VC	Vinyl chloride
<i>vcrA</i>	RDase gene implicated in DCE and VC reductive dechlorination
XIC	Extracted ion chromatogram

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Abstract

A.1 Objectives.

Bioremediation plays a crucial role in transforming and detoxifying chlorinated solvents and has been implemented as a stand-alone technology at sites undergoing monitored natural attenuation (MNA), biostimulation with or without bioaugmentation treatment, or as a polishing step when physical/chemical treatment serves as the primary remedy. Molecular Biological Tools (MBTs), foremost quantitative PCR (qPCR), provide information about the presence and abundance of keystone dechlorinating bacteria, and are instrumental for site assessment, bioremediation monitoring, and the implementation of adaptive site management strategies. Although the benefits of microbial analyses are indisputable, uncertainty about the interpretation of MBT data exists, and a consensus has not been reached regarding the value of MBTs for improved decision-making to accelerate paths towards site closure. The overarching objective of this project was to advance MBTs and their application to minimize biases and to more effectively assess, predict, monitor, optimize, and manage reductive dechlorination processes at DoD sites impacted with chlorinated solvents. To further assist in the interpretation of MBT data, additional aims were to assess measureable parameters that correlate with the detoxification or incomplete (stalled) degradation of chlorinated ethenes, and to identify knowledge gaps that currently limit the efficient application of MBTs for decision-making at chlorinated solvent sites. Specifically, the following interrelated technical research objectives (tasks) were addressed:

1. Advance understanding of the diversity and ecophysiology of organohalide-respiring bacteria contributing to chlorinated solvent detoxification.
2. Identify additional reductive dechlorination biomarkers.
3. Develop a high-throughput pPCR tool for monitoring reductive dechlorination biomarker genes.
4. Explore the utility of environmental proteomics workflows for bioremediation monitoring.
5. Extract information from existing databases and integrate MBT data with site geochemical information to predict reductive dechlorination performance.
6. Apply new tools to DoD sites impacted with chlorinated solvents.
7. Disseminate and explain the value of MBT information for site assessment, bioremediation monitoring and optimization, adaptive site management, and performance prediction to RPMs, industry and government for general acceptance and broad implementation

A.2 Technical Approach.

Available pure cultures and consortia capable of using chlorinated solvents, including chlorinated ethenes, as electron acceptors were used to unravel specific nutritional requirements and the response to inhibitors that impact dechlorination activity. Enrichment cultures were used to discover microbes with novel biomarkers for detoxification of chlorinated solvents. To expand the qPCR approach to a broader suite of biomarker genes, an open array plate targeting 112 reductive dechlorination biomarker genes was designed and validated. Further, an environmental proteomics pipeline was developed to allow the measurement of biomarker proteins in laboratory cultures and contaminated groundwater. To develop predictive understanding of detoxification (i.e., ethene formation) in groundwater aquifers impacted with chlorinated solvents, existing databases were mined for microbial (i.e., qPCR) and geochemical data, including contaminant and ethene concentrations. The outcomes of these research efforts were published in peer-reviewed journals, presented at technical conferences and webinars, and communicated to practitioners, including RPMs.

A.3 Results.

The project made a series of discoveries that have already impacted bioremediation practice at DoD sites. The major accomplishments include:

- A new dechlorinating bacterium, '*Candidatus Dehalogenimonas etheniformans*' was discovered that dechlorinates TCE to ethene. This finding indicates that detoxification of chlorinated ethenes is not limited to *Dehalococcoides mccartyi* (*Dhc*) strains.
- Strain GP harbors 52 putative reductive dehalogenase genes, including *cerA*, which serves as a new biomarker for vinyl chloride reductive dechlorination.
- Additional novel biomarkers for the degradation of chlorinated solvents were discovered.
- The RD-qChip targeting 102 reductive dechlorination biomarkers and enabling high-throughput qPCR applications was designed and validated.
- The analysis of 859 groundwater representing 62 sites impacted with chlorinated ethenes corroborated the value of quantitative DNA biomarker analysis. Normalized qPCR measurements predict ethene formation, which is likely when *Dhc* 16S rRNA gene and VC RDase gene abundances exceed 10^7 and 10^6 copies L^{-1} , respectively, and when the 16S rRNA- and *bvcA* + *vcrA*-to-total bacterial 16S rRNA gene ratios exceed 0.1%.
- Detailed study of streambed sediment linked qPCR data with *in situ* contaminant attenuation and identified the fractured bedrock-sediment interface as a critical zone for microbial activity.
- Organohalide-respiring Dehalococcoidia are corrinoid auxotrophs and rely on the microbial community to supply this essential nutrient.
- The corrinoid lower base controls *Dhc* reductive dechlorination rates and extents (i.e., detoxification), emphasizing the role of the microbial community for synthesizing the corrinoid and lower base structures.
- Targeted proteomic workflows can be applied to groundwater samples and provide protein level information about *Dhc* dechlorination activity.
- Machine learning-based data mining applied to geochemical and microbial (i.e., qPCR) data sets collected from sites impacted with chlorinated ethenes can predict ethene formation. A major need for realizing the predictive capabilities is a curated, open-access, up-to-date and comprehensive collection of biogeochemical groundwater monitoring data.
- In plumes with co-mingled chlorofluorocarbons such as 1,1,2-trichloro-1,2,2-trifluoroethane (CFC-113) inhibition of *Dhc* reductive dechlorination must be expected.
- N_2O is a potent inhibitor of bacterial reductive dechlorination and the presence of micromolar concentrations can lead to stalled dechlorination at sites impacted with chlorinated ethenes.
- Biostimulation with ammonium can enhance *Dhc* reductive dechlorination rates; however, a “do nothing” approach that relies on indigenous diazotrophs can achieve similar dechlorination end points and avoids the potential for stalled dechlorination due to inhibitory levels of N_2O .

A.4 Benefits.

High-throughput qPCR technology and environmental proteomics assist in the identification of parameters determining the feasibility and potential success of microbial remedies, so that non-productive investments can be avoided, realistic performance predictions can be established, and bioremediation sites can be efficiently managed to achieve cleanup goals and early site closures. More robust and comprehensive information about the microbiology and its activity will prevent and overcome suboptimal bioremediation performance (e.g., inhibition, nutritional limitations), and long-term performance predictions of bioremediation systems with and without intervention become feasible.

Project Objectives and Tasks

Bioremediation plays a crucial role in removal of chlorinated solvents when implemented as a stand-alone technology at sites undergoing MNA, biostimulation with and without bioaugmentation treatment, or as a polishing step at sites where physical/chemical treatment has been implemented. MBTs provide information about the presence/absence and abundance of keystone dechlorinating bacteria (i.e., organohalide-respiring *Dehalococcoidia*) during site assessment to guide remedy selection. At bioremediation sites, monitoring regimes rely on MBT data to link contaminant degradation with treatment and to implement and site management strategies that promise productive contaminant degradation. A number of case studies have demonstrated the value of microbial analysis using MBTs; however, uncertainty about the interpretation of MBT results exists, and a consensus has not been reached regarding the value of MBTs for enhanced decision-making to accelerate paths towards site closure. The overarching project objective was to advance MBTs and their application to minimize biases and to more effectively assess, predict, monitor, optimize, and manage reductive dechlorination processes at contaminated DoD sites. Specifically, innovative high-throughput qPCR technology and environmental proteomic workflows were explored to characterize dechlorinating microbial communities and activities. To further assist in the interpretation of MBT data, additional aims were to assess measurable parameters that correlate with the detoxification or incomplete (stalled) degradation of chlorinated ethenes, and to identify knowledge gaps that currently limit the efficient application of MBTs for decision-making at chlorinated solvent sites. Figure 1 shows an overview of the workflow to advance the existing MBTs.

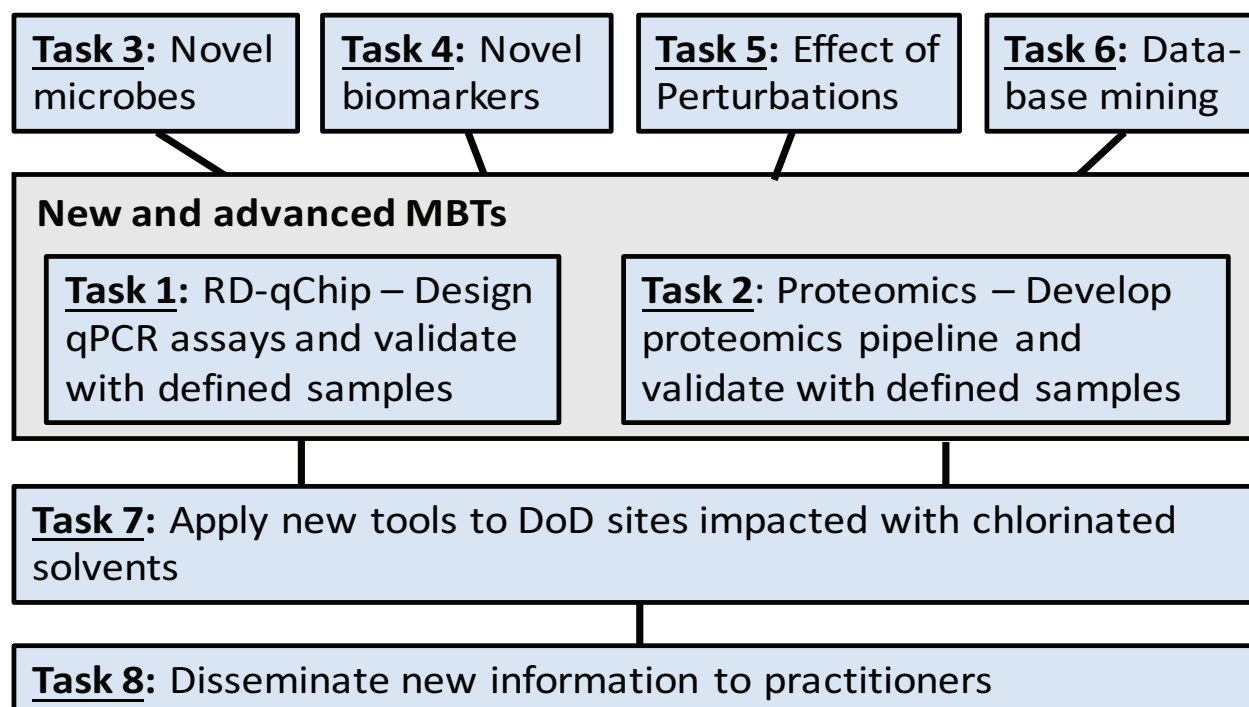


Figure 1. Overview of tasks.

The successful completion of these tasks advanced understanding of the reductive dechlorination process culminating in enhanced, application-ready site assessment and monitoring technology (i.e., the RD-qChip and a proteomics pipeline).

To achieve the overall goal to advance MBT application in support of bioremediation efforts at sites impacted with chlorinated solvents, this research project addressed the following interrelated tasks.

1. Advance understanding of the diversity and ecophysiology of organohalide-respiring bacteria contributing to chlorinated solvent detoxification.
2. Identify additional reductive dechlorination biomarkers.
3. Develop a high-throughput qPCR approach for monitoring reductive dechlorination biomarker genes.
4. Explore the utility of environmental proteomics workflows for bioremediation monitoring.
5. Extract information from existing databases and integrate MBT data with site geochemical information to predict reductive dechlorination performance.
6. Apply new tools to DoD sites impacted with chlorinated solvents.
7. Disseminate and explain the value of MBT information for site assessment, bioremediation monitoring and optimization, adaptive site management, and performance prediction to RPMs, industry and government for general acceptance and broad implementation

This final report summarizes the activities conducted to address Tasks 1-6, summarizes and discusses the results and outcomes, and brings the findings into context with contaminated site remediation. The completion of Tasks 1-6 addresses the Statement of Need (ERSON-13-02) by (i) providing information about critical parameters that inform about the feasibility of bioremediation at sites impacted with chlorinated ethenes, (ii) introducing new solutions to overcome stalled dechlorination or impractical dechlorination rates to meet performance objectives, (iii) generating tools to recognize situations where the current technology is insufficient to meet remedial goals (i.e., allows adaptive site management [ASM] strategies), and (iv) developing new methodologies with predictive capability of remediation performance.

Background

Contaminated groundwater is a major cost driver at many military facilities, and chlorinated solvents, in particular chlorinated ethenes, are the most prevalent groundwater contaminants found at over 3,000 DoD sites. The DoD's updated Defense Environmental Restoration Program calls for achieving Response Complete (RC) at 95% by FY 2021 with an estimated Cost to Complete (CTC) of \$12.8 billion (Leeson et al., 2011).

Bioremediation plays a major role in the transformation and detoxification of chlorinated solvents, and has had documented successes when implemented as a stand-alone technology at monitored natural attenuation (MNA), biostimulation, and bioaugmentation field sites, or as a polishing step at sites where physical-chemical treatment was chosen as the primary remedy (Ellis et al., 2000; Lendvay et al., 2003; Major et al., 2002). At locations where physical-chemical treatment options have been implemented, cleanup goals often are not met because complete contaminant mass removal is generally not achieved. Since dechlorinating bacteria are commonly present in the contaminated subsurface, they will by default contribute to contaminant transformation independent of the physical-chemical remedy being implemented. This has been an important realization and led to the concept of a combined remedy or treatment train approaches that integrate aggressive physical-chemical treatment (e.g., surfactant flushing, ISCO, ISCR, thermal) with microbial activity as a polishing step (Christ et al., 2005; Munakata-Marr et al., 2011). These observations indicate that knowledge of the microbiology is relevant for all chlorinated solvent-contaminated sites, including those where physical-chemical treatment options have been chosen as the primary remedy.

Improved MBTs to assess, monitor and predict microbial activity will be of value to the DoD to meet remediation goals and reduce the CTC burden. Organism- and pathway-specific biomarker genes have been identified and MBTs are available to measure the presence and abundance of dechlorination biomarker genes in environmental samples. MBTs provide information about keystone dechlorinating bacteria (e.g., *Dhc*, *Dhgm*), and are used for site assessment, bioremediation monitoring, and implementing ASM approaches. Although the benefits of microbial analyses are indisputable, uncertainty about MBT application at contaminated sites exists, and a consensus on the value of MBTs to make better-informed decisions and accelerate paths towards site closure has not been reached. Key shortcomings are that most current MBTs examine a single target gene at a time, cannot provide direct information about actual activity, and ambiguity exists regarding the interpretation of MBT data, which is partly caused by data inconsistencies between analytical laboratories. These shortcomings are a reason for the reluctance on the part of the regulatory community to fully embrace the value of MBT data. Without regulatory endorsement, practitioners will remain hesitant to use MBTs to make better-informed site management decisions. To ensure that DoD RPMs capitalize on the MBT approach to the extent possible and efficiently achieve the goals of the Defense Environmental Restoration Program, this project developed high-throughput qPCR technology for the analysis of reductive dechlorination biomarker genes. This approach increases the informational content of MBT data while reducing the uncertainty associated with qPCR measurements and their interpretation, so that contaminant fate and longevity can be more accurately predicted, and site management decisions can be made with greater confidence.

Relevant information about the microbiology and pathways contributing to chlorinated solvent detoxification has accrued over the last 20 years, which is a prerequisite for the successful

implementation of bioremediation at chlorinated solvent sites. Keystone bacteria involved in the transformation and detoxification of chlorinated propanes (Löffler et al., 1997a; Ritalahti et al., 2004; Yan et al., 2008), chlorinated ethanes (Grostern et al., 2006a; Grostern et al., 2006b; Zhao et al., 2015), chlorinated ethenes (He et al., 2003b; Maymó-Gatell et al., 1997; Yang et al., 2017c), and chlorinated methanes (Grostern et al., 2010; Justicia-Leon et al., 2012; Lee et al., 2011c; Zhao et al., 2017b) have been identified. In addition, genome sequencing, reverse genetics in combination with enzyme assays, and the application of microarray technology have identified a suite of reductive dechlorination biomarker genes. Efforts to identify more functional biomarker genes are ongoing and are supported by genome and metagenome sequencing projects. It is highly probable that these continued efforts will identify additional biomarker genes and complement the suite of pathway- and organism-specific biomarker genes.

In addition to RDase genes with assigned function, several hundred putative RDase genes with unknown function have been identified (Clark et al., 2018; Hug et al., 2013a). Other genes that can serve as potential reductive dechlorination biomarkers include genes encoding proteins directly involved in electron transfer to the RDase proteins, as well as proteins involved in supplying corrinoid to the organohalide-respiring organism.

Functional RDases possess a corrinoid prosthetic group (also referred to as co-factor) that fulfills essential functions for catalysis. Recent studies revealed that some organisms depend upon specific corrin ring biosynthesis to successfully degrade toxic chlorinated compounds to benign ethene. *Dhc* is dependent on exogenous cyanocobalamin (Löffler et al., 2013b) as *Dhc* contains the requisite genes to scavenge corrinoid from other organisms but lacks the full *de novo* corrin ring biosynthesis pathway (Yan et al., 2016).

Several biomarker genes have been identified for monitoring reductive dechlorination of chlorinated ethenes. Tetrachloroethene (PCE) can undergo stepwise reductive dechlorination to trichloroethene (TCE), the dichloroethenes *cis*-1,2-dichloroethene (*cDCE*), *trans*-1,2-dichloroethene (*tDCE*) and 1,1-dichloroethene (1,1-DCE), vinyl chloride (VC) and ultimately environmentally benign ethene (Figure 2).

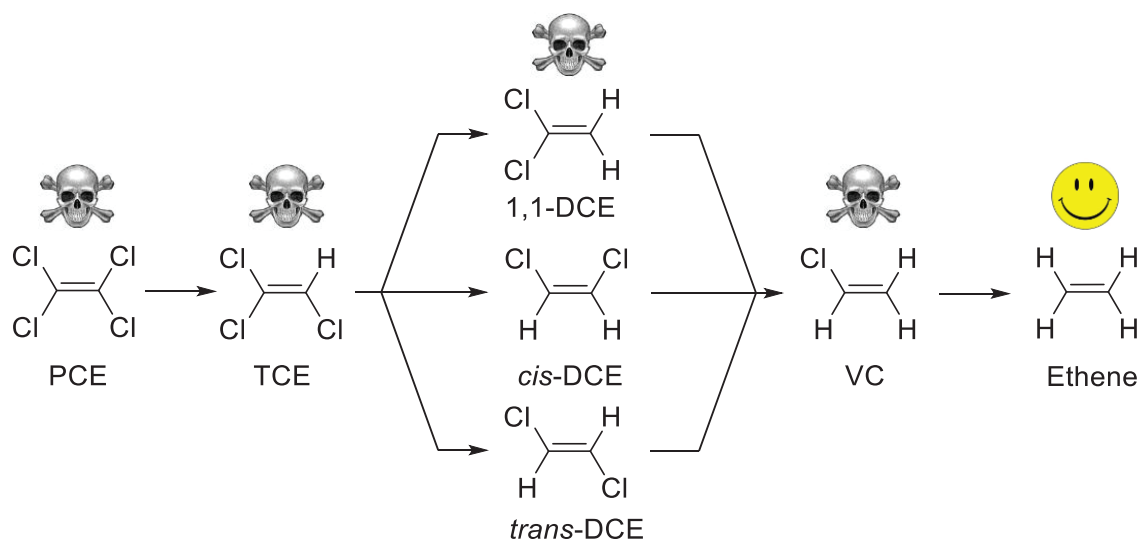


Figure 2. Degradation pathway for microbial reductive dechlorination of chlorinated ethenes.

The most commonly observed DCE intermediate is *cDCE* but some culture were reported to dechlorinate PCE/TCE to 1,1-DCE, *tDCE*, or a mixture of *cDCE* and *tDCE* (Cheng et al., 2009; Futamata et al., 2007; Griffin et al., 2004; Kittelmann et al., 2008; Zhang et al., 2006).

The reductive dechlorination sequence from PCE to ethene typically involves two taxonomically distinct bacterial groups (Flynn et al., 2000). Diverse bacterial taxa dechlorinate PCE to TCE and cDCE, whereas the dechlorination of cDCE to VC and ethene is limited to certain *Dhc* strains (He et al., 2003b; Löffler et al., 2013b; Maymó-Gatell et al., 1997). More recent work demonstrated that members of the *Dhc*-related genus *Dhgm* also contribute to the reductive dechlorination of chlorinated ethenes. *Dhgm* sp. strain WBC-2 was identified in a mixed culture enriched with 1,1,2,2-tetrachloroethane and is responsible for transforming *trans*-1,2-dichloroethene (tDCE) to VC (Molenda et al., 2016). In this research project, we discovered ‘*Candidatus* Dehalogenimonas etheniformans’ strain GP in an enrichment culture derived from a pristine source material (Yang et al., 2017c). Strain GP dechlorinates TCE to ethene and uses VC as a growth-supporting electron acceptor indicating that the ability to grow with VC as electron acceptor is not limited to members of the *Dhc* genus (Yang et al., 2017c). Of note, a few *Dhc* strains have RDase genes that allow them to dechlorinate PCE to ethene under laboratory conditions, and arguments have been made that those *Dhc* strains are relevant for bioremediation (Maymó-Gatell et al., 1997; Nijenhuis et al., 2005; Pöritz et al., 2013; Zhao et al., 2019a). Considering that PCE-to-cDCE dechlorinating bacteria generally exhibit higher PCE and TCE dechlorination rates than organohalide-respiring Dehalococcoidia, are common members of aquifer microbiomes, and are present in commercial bioaugmentation inocula, the benefit of *Dhc/Dhgm* strains capable of PCE and TCE reductive dechlorination for achieving detoxification is uncertain. Under typical *in situ* conditions, the dechlorination of PCE/TCE to non-toxic ethene is most likely catalyzed by more than one organohalide-respiring population (Duhamel et al., 2004; Duhamel et al., 2002; Lendvay et al., 2003; Schaefer et al., 2009; Sung et al., 2006a; Sung et al., 2003; Vainberg et al., 2009).

A considerable number of organism- and process-specific reductive dechlorination biomarker genes have been identified for site assessment and bioremediation monitoring (Table 15), and the quantitative measurement of these biomarkers is the basis for informed site management decision-making. Measuring biomarker gene presence and abundance provides crucial information about the feasibility and potential success of a biological remedy at contaminated DoD sites. While the benefits of MBTs for site assessment, monitoring and decision-making are indisputable, the technology has not achieved its full potential and has not been fully embraced by practitioners and regulatory agencies. Current limitations that prevent a broader use of MBT data to support decision-making circle around the following issues:

- (i) Contemporary MBTs provide an incomplete snapshot of the dechlorinating community, and it is not clear how MBT data correlate with current observations in terms of contaminant degradation and detoxification and serve as predictors for future system performance.
- (ii) Contemporary MBTs are not linked with rate information, a fact that limits their value to engineers interested in developing reactive transport models and predicting contaminant fate.
- (iii) Uncertainty exists whether MBTs provide meaningful information at difficult (“complex”) sites (i.e., large dilute plumes; fractured matrices; DNAPL source zones; mixed contaminant plumes).
- (iv) Data inconsistencies have been observed, which has compromised confidence in MBTs.
- (v) Practicing and regulating communities are hesitant to promote widespread MBT application, mainly because MBT data acquisition and interpretation does not follow standardized procedures.

- (vi) The (flawed) argument that MBT analyses do not add value for implementing bioremediation and ASM approaches.

A SERDP and ESTCP Workshop Report (Leeson et al., 2011) highlighted that insufficient site characterization prior to technology implementation often results in flawed design, implementation, and performance monitoring strategies, thus increasing costs and extending schedules. With inadequate upfront site characterization, management decisions remain an empirical and subjective practice rather than being based on scientific principles with predictable outcomes. To overcome these limitations and more accurately assess, predict, monitor, optimize, and manage reductive dechlorination processes at contaminated DoD sites, this research effort capitalized on prior accomplishments in identifying keystone bacteria and reductive dechlorination biomarkers, and employed state-of-the-art analytical methodologies including high-throughput qPCR and environmental proteomics workflows. The application of these tools to laboratory cultures advanced our understanding of the requirements and constraints of keystone bacteria contributing to chlorinated solvent detoxification; and, provided a more complete and more refined picture of the dechlorinating microbial community, its activity, and response to perturbations. High-throughput qPCR provides a comprehensive picture of the biodegrading bacteria community based on the complement of known organism- and process-specific biomarker genes, enables the design of site-tailored measurements to facilitate data interpretation and further reduce costs, and eliminates ambiguity associated with data acquisition and interpretation.

High-throughput OpenArray® qPCR

The QuantStudio® 12K Flex Real-Time PCR System (OpenArray® system) is a small volume (nanoliter) fluidics platform that offers flexibility and scalability and enables high-throughput sample processing to obtain over 12,000 data points in a single 2-hr instrument run (http://tools.invitrogen.com/content/sfs/brochures/cms_098456.pdf). The OpenArray® system addresses many limitations of the current qPCR technology, and the OpenArray® system has set the standard for monitoring multiple nucleic acid biomarkers simultaneously. The OpenArray® system offers the flexibility to accommodate a variety of qPCR assay plates, including standard 96-well plates for assay optimization and routine single gene analysis of a few samples, 384-well plates for additional target genes and samples, and up to four OpenArray® qPCR plates each yielding 3,072 data points (>12,000 data total) for high-throughput applications. The flexibility in plate types allows appropriate scaling to achieve stepwise progression to larger numbers of biomarker targets. Each OpenArray® qPCR plate can accommodate from 56 assays and 48 samples, up to 224 assays and 12 different samples. Biomarker targets can be added first in sets of 56, then 112, 168 and eventually 224 as relevant and validated qPCR assays are developed. In contrast to manual plate setup in conventional qPCR, the OpenArray® system uses simple, automated workflows with integrated data analysis software and quality control built-in, thus effectively mitigating inherent human biases, and generating data that are comparable between samples (i.e., sites) and analytical laboratories. Of note, the OpenArray® system completely avoids manual pipetting, which has been recognized as a major source of qPCR data variability and error (SERDP project ER-1561). The majority of variation would arise from sample collection and DNA extraction procedures, but those errors can be accounted for by adding appropriate internal standards ((Hatt et al., 2013); SERDP project ER-1561).

A limitation of DNA-targeted measurements is that gene presence informs about the potential, but not the actual activity (i.e., gene expression). For example, the detection and quantification of a bacterial population that can degrade the contaminant of interest, but can also grow with other

substrates, does not imply that the bacterium is actively degrading the contaminant. Temporal gene-targeted monitoring can provide evidence of gene activity as long as growth of the microorganisms is tightly linked with contaminant degradation, as is the case with strictly organohalide-respiring bacteria of the genera *Dehalococcoides*, *Dehalobacter*, and *Dehalogenimonas*. For example, an increase in *Dhc* biomarker gene copy numbers over time can only be explained with growth-linked reductive dechlorination, and thus gene expression. Still, this information is insufficient to extract meaningful information about dechlorination activity and rates of contaminant removal, and the measurement of gene transcripts (mRNA) was suggested as a more direct measure of gene activity (Fung et al., 2007; Johnson et al., 2005a; Lee et al., 2008a; Lee et al., 2006; Lee et al., 2008b; Rahm et al., 2006; Rahm et al., 2008a). While mRNA measurements are promising, this approach has not led to a better understanding how MBT data can be used to extrapolate *in situ* contaminant transformation rates. A number of problems complicate transcript analysis (Amos et al., 2008; Fletcher et al., 2011), and key issues that limit the utility of the approach include the rapid turnover of mRNA in cells and the difficulty of quantitatively extracting RNA from environmental sample matrices due to the inherent instability of RNA. To complicate matters further, the transcriptional regulation of RDase genes is poorly understood. For example, even under inducing conditions (i.e. in the presence of a chlorinated electron acceptor), the transcript numbers per cell remain very low (e.g., < 1 transcript per cell) (Amos et al., 2008; Heavner et al., 2018). Further, a single chlorinated electron acceptor can trigger the expression of multiple RDase genes (Müller et al., 2004; Wagner et al., 2013; Waller et al., 2005) and the upregulation of RDase gene expression was observed when the chlorinated electron acceptor has been exhausted (Fletcher et al., 2011; Johnson et al., 2008). An alternative and presumably even more direct measure of gene expression and actual activity is the measurement of protein (enzymes) a cell produces as a consequence of gene expression. This research effort explored the feasibility of proteomic workflows to detect and quantify the *Dhc* biomarker proteins in axenic *Dhc* cultures, dechlorinating consortia, and groundwater samples.

Environmental proteomics

The quantitative analysis of a protein (enzyme) is a direct measure of microbial (in)activity. In contrast to nucleic acids, protein cannot be amplified, and the analysis is confined by the amount of target protein present in the sample, which has been the major limitation for measuring specific proteins, especially in environmental samples.

Global or comprehensive proteomics studies are usually conducted via a bottom-up strategy where proteins from a biological sample are extracted and then enzymatically digested to yield peptide mixtures that are analyzed with LC-MS/MS (Zhang et al., 2013). These types of experiments maximize protein identification information and some endpoints are, for example, to map large-scale proteomes or to potentially discover proteins with different abundances in a variety of organisms or conditions (Schiffmann et al., 2014b). One mode of achieving the comprehensive analysis of proteins in a sample is through two-dimensional liquid chromatography-tandem mass spectrometry (2D-LC-MS/MS), which provides the sensitivity and the dynamic range needed to comprehensively measure proteins at a remarkably deep level in environmental matrices (Aebbersold et al., 2003; Blackler et al., 2006; Lin et al., 2003; VerBerkmoes et al., 2009; Washburn et al., 2001). Computational tools such as Sequest (Eng et al., 1994) and DTASelect (Tabb et al., 2002) have been developed for automated peptide and protein identifications.

The 2D-LC-MS/MS approach has been applied to dechlorinating consortia and could discriminate RDases in different *Dhc* strains (Morris et al., 2007; Morris et al., 2006), and additional studies

reported effects of nitrogen limitation on the proteome profile in *Dhc* strain 195 (Lee et al., 2012). These examples demonstrate the feasibility of measuring *Dhc* proteins with proteomic workflows in dechlorinating pure cultures and enrichment cultures. What has yet to be accomplished is the design and application of a proteomics pipeline that measures reductive dechlorination biomarker proteins in environmental matrices (e.g., groundwater) with sufficient sensitivity, specificity and sample throughput, which was a focus of this research effort.

Different than in global proteomics, in a targeted proteomics experiment via liquid chromatography-multiple reaction monitoring-mass spectrometry (LC-MRM-MS), a specific set of peptides that serve as surrogates for proteins of interest are detected and quantified (Lange et al., 2008), while ignoring the rest of the proteins that could be present in a sample. The focused nature of targeted proteomics approaches can provide lower protein detection limits (with some studies quantifying proteins in the attomole range) and higher specificities compared to untargeted global proteomics approaches (Liebler et al., 2013; Schiffmann et al., 2014a). Both of these characteristics are important, especially considering that in groundwater samples *Dhc* bacteria are members of diverse microbial communities and *Dhc* may not be the most predominant microorganisms (Löffler et al., 2013a). A targeted proteomics experiment requires about 1.5 hours to collect MRM spectral data, whereas a global proteomics experiment requires 4-24 hours (depending on the LC separation) to collect MS/MS spectral data. In addition, the reduced datasets generated in the targeted approach can be analyzed in shorter time frames, and the results can be communicated faster to the end user. Previous work has employed targeted proteomics approaches to quantify RDase expression in axenic *Dhc* cultures and *Dhc*-containing mixed culture (Rowe et al., 2012; Schiffmann et al., 2014a; Werner et al., 2009); however, the utility of a targeted proteomics approach for the analysis of groundwater samples from aquifers that vary in their hydrological, chemical, geological and biological compositions still needs to be demonstrated.

In this project, protein and peptide selection for targeted proteomics was developed by first analyzing the expression profiles of dechlorination biomarkers expressed in axenic cultures of *Dhc* strains using bottom-up global proteomics. Additionally, knowing what peptides are being produced from the enzymatic digestion of the protein biomarkers was essential to select an initial list of peptide candidates representing the selected biomarkers. The initial list of peptides was then evaluated by monitoring the signals of such peptides but this time in targeted proteomics mode using a different mass spectrometer. A total of 37 peptides representing six proteins were selected after signal quality evaluation of each initial peptide candidate in targeted proteomics mode and after *in silico* evaluation verifying uniqueness of each peptide to the respective target proteins. These peptides were then monitored in targeted proteomics mode in a bacterial consortium containing *Dhc* and groundwater samples collected from sites impacted with chlorinated ethenes. An overview of this workflow is presented in

Figure 3.

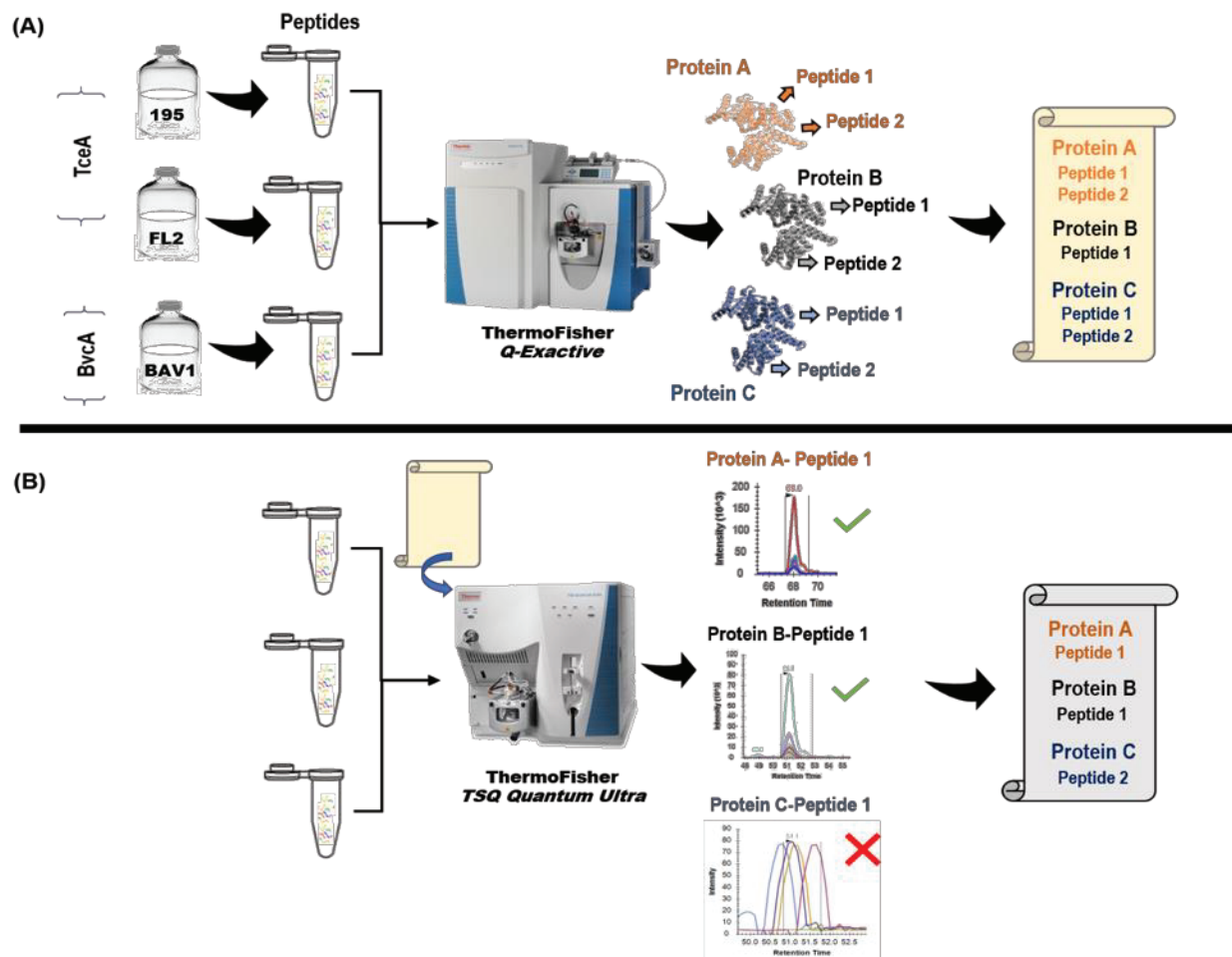


Figure 3. Overview of the proteomics techniques used to detect reductive dechlorination biomarker proteins in environmental samples.

(A) Axenic cultures of *Dhc* strains 195 and FL2, known to express the TceA RDase (TCE→VC and ethene), as well as strain BAV1, which expresses the BvcA RDase (DCEs→VC→ethene), were processed for global proteomics analysis. After protein extraction and enzymatic digestion, each peptide mixture was analyzed independently on a Q Exactive mass spectrometer. From these analyses, the abundance of protein biomarkers (represented by proteins A, B, and C) and their peptides was evaluated (i.e., peptide 2 of protein B was not selected). Based on intensities and empirical rules, an initial list of peptides from these proteins were selected. (B) The same peptide mixtures were then analyzed on a TSQ Quantum Ultra mass spectrometer to evaluate the signal of the selected peptides on targeted mode. After signal quality evaluation and other *in silico* analyses, a reduced set of peptides, representative of the proteins of interest, was defined. These peptides were then monitored in a bacterial consortium and six contaminated groundwater samples in targeted mode using the same TSQ instrument. More details can be found in the Methods section, Task 2.

Novel microorganisms involved in the degradation of chlorinated solvents

A diverse group of organohalide-respiring bacteria has been discovered and a number of isolates have been studied. In the case of **chlorinated ethenes**, the sequential reductive dechlorination of PCE to ethene (Figure 2) is typically carried out by two distinct bacterial guilds under *in situ* conditions (Flynn et al., 2000). Members of the genera *Geobacter*, *Sulfurospirillum*,

Desulfitobacterium, and *Dehalobacter* are often found at contaminated sites and dechlorinate PCE and/or TCE to cDCE. While a diversity of bacteria can dechlorinate PCE and/or TCE to cDCE, the utilization of cDCE has been limited to a single bacterial group: *Dehalococcoides mccartyi* (Löffler et al., 2013a; Löffler et al., 2013b). A more recent finding suggested that a *Dehalogenimonas* sp. identified in a mixed culture was able to use tDCE as a growth-supporting electron acceptor to produce VC as end product (Molenda et al., 2016). This *Dehalogenimonas* sp. could not grow with VC as electron acceptor. Based on the current knowledge, the formation of ethene at sites impacted with chlorinated ethenes is firmly linked to the presence and activity of *Dhc*. Therefore, the absence of *Dhc* leads to the conclusion that bacterial dechlorination to ethene cannot be expected, which impacts site management decision making. We have obtained an enrichment culture that dechlorinates TCE to ethene, but all efforts to detect *Dhc* 16S rRNA genes or the known VC RDase of *Dhc* (i.e., *bvcA*, *vcrA*) have failed. The specific aim was to characterize this culture and identify the dechlorinating population(s). The demonstration that microbial detoxification of chlorinated ethenes can occur in the absence of *Dhc* would expand our understanding of the microbiology contributing to the degradation and detoxification of chlorinated ethenes, impact the interpretation of qPCR data, and impact the implementation of bioremediation as a cleanup strategy at chlorinated solvent sites.

In addition to chlorinated ethenes, **chlorinated methanes** are common contaminants at many military installations. Of particular concern is chloroform (CF), which was shown to inhibit the activity of *Dhc* and reductive dechlorination of chlorinated ethenes (Duhamel et al., 2002) (Futagami et al., 2013; Maymó-Gatell et al., 2001). Some organohalide-respiring bacteria utilize CF as respiratory electron acceptor and produce dichloromethane (DCM) as end product (Groster et al., 2010) (Ding et al., 2014). While this process alleviates the inhibitory effect of CF, it generates DCM, a regulated compound with an MCL of 5 ppb. While the degradation of DCM under oxic conditions is fairly well understood (Kohler-Staub et al., 1985; Leisinger et al., 1995; McDonald et al., 2002; Nikolausz et al., 2006; Vannelli et al., 1999), the reductive dechlorination of CF is a strictly anaerobic process that generates DCM and inorganic chloride as products, and can occur in anoxic groundwater aquifers. To date, *Dehalobacterium formicoaceticum* (DEFO) is the only known isolate capable of utilizing DCM as growth substrate in the absence of oxygen (Mägli et al., 1996). In contrast to chlorinated ethenes, chlorinated ethanes and CF, DCM does not undergo direct hydrogenolysis (i.e., reductive dechlorination). Instead, based on physiological and biochemical evidence, DCM is funnelled into the Wood-Ljungdahl pathway (reductive acetyl-CoA pathway) after chlorine removal and broken down to acetate, formate, carbon dioxide and inorganic chloride (Mägli et al., 1998; Meßmer et al., 1993). To expand our knowledge of the microbiology involved in the anaerobic degradation of DCM, an enrichment culture that used DCM as the sole source of energy under anoxic conditions was characterized.

Organism- and process-specific biomarkers

Knowledge about organism- and process-specific biomarkers is the basis for the development of prognostic and diagnostic tools used for site assessment and bioremediation monitoring. Information about the presence and abundance of biomarkers can guide site management decisions to effectively achieve site-specific remediation goals. A suite of biomarkers to assess the potential for chlorinated solvent degradation and implement site monitoring regimes is available. Table 1 summarizes the knowledge of organism-specific and process-specific biomarker genes for anaerobic dechlorination of chlorinated solvents available in 2012.

Table 1. Organism-specific (i.e., 16S rRNA genes) and pathway-specific (i.e., functional) biomarker genes involved in the transformation of chlorinated solvents (knowledge base in 2012).

Dechlorination step	RDase gene	Organism	Organism-specific assay		Functional gene assay	Reference
			Genus ^a	Species		
PCE → TCE	-	<i>Desulfitobacterium</i> sp.	☐		-	(Bouchard et al., 1996; Gerritse et al., 1996; Löffler et al., 1997b)
PCE/TCE → cDCE	<i>pceA</i>	<i>Sulfurospirillum</i> spp.	☐		- ^b	(Neumann et al., 1996) (John et al., 2009)
PCE/TCE → cDCE	<i>pceA</i>	<i>Desulfitobacterium</i> spp.	☐		- ^b	(Reinhold et al., 2012)
PCE/TCE → cDCE	<i>pceA</i>	<i>Geobacter lovleyi</i>		☐	- ^b	(Amos et al., 2007c; Wagner et al., 2012)
PCE/TCE → cDCE	<i>pceA</i>	<i>Desulfuromonas</i> spp.		☐	- ^b	(Krumholz et al., 1996; Lee et al., 2011b; Löffler et al., 2000)
PCE/TCE → cDCE	<i>pceA</i>	<i>Dehalobacter</i> sp.	☐		- ^b	(Maillard et al., 2003)
PCE → TCE	<i>pceA</i>	<i>Dhc</i>	☐		- ^b	(Fung et al., 2007; Magnuson et al., 1998)
TCE → VC	<i>tceA</i>	<i>Dhc</i>	☐		☐	(Ritalahti et al., 2006) (Holmes et al., 2006)
cDCE → ethene	<i>vcrA</i>	<i>Dhc</i>	☐		☐	(Holmes et al., 2006) (Ritalahti et al., 2006) (Ritalahti et al., 2006) (Sung et al., 2006b)
cDCE → ethene	<i>bvcA</i>	<i>Dhc</i>	☐		☐	(Holmes et al., 2006)
1,2-D → propene	<i>dcpA</i>	<i>Dhc</i>	☐		-	(Moe et al., 2009)
DCM → CO ₂	-	<i>Dehalobacter</i>		☐	-	(Justicia-Leon et al., 2012)

PCE, tetrachloroethene; TCE, trichloroethene; cDCE, *cis*-1,2-dichloroethene; VC, vinyl chloride; 1,2-D, 1,2-dichloropropane; DCM, dichloromethane

^a Genus-specific MBTs are available; however, not all members of the genus are capable of catalyzing the respective dechlorination step.

^b A specific assay has not been designed but is possible with the available information.

A suite of useful biomarkers for site assessment and bioremediation monitoring has been discovered; however, comprehensive understanding of the phylogentic diversity of microorganisms contributing to chlorinated solvent degradation has not been attained. The diversity of relevant functional genes for monitoring chlorinated solvent degradation processes has also not been captured. For example, crucial biomarkers for monitoring detoxification at sites impacted with chlorinated ethenes are genes encoding VC RDase, and the *bvcA* and *vcrA* genes of *Dhc* are used for this purpose. At a number of sites, ethene formation has been observed but these VC RDase genes were not detected, suggesting that other, yet to be identified VC reductive dechlorination biomarker genes exist. This project took advantage of an available enrichment culture that dechlorinated TCE to ethene in the absence of *Dhc* to reveal new biomarkers for ethene formation. This enrichment culture tested negative for the known VC RDase genes *bvcA* and *vcrA* suggesting that a novel VC RDase was involved in the reductive dechlorination process. To date, biomarkers to assess anaerobic DCM degradation are not available, and we obtained the only known DCM-degrading pure culture, *Dehalobacterium formicoaceticum* (Defo) from a type culture collection (ATCC). In addition, we obtained a stable DCM-degrading consortium harboring the DCM degrader '*Candidatus* Dichloromethanomonas elyunquensis' (DIEL) (Kleindienst et al., 2017). In this project, we characterized these cultures with the goal of identifying new biomarkers for anaerobic DCM degradation.

Response of organohalide-respiring bacteria to perturbations

The discovery of bacteria that grow with chlorinated compounds as electron acceptors and benefit from this process (i.e., conserve energy for growth) was a milestone for bioremediation at sites impacted with chloroorganic contaminants. Extensive research characterized the physiology and nutritional requirements of organohalide-respiring bacteria, initially focused on PCE-to-cDCE-dechlorinating bacteria (Gerritse et al., 1996; Holliger et al., 1998a; Krumholz et al., 1996; Löffler et al., 2000; Neumann et al., 1996; Wild et al., 1996). The focus then shifted to *Dhc* because members of this group converted toxic DCEs and VC to environmentally benign ethene (Löffler et al., 2013a). *Dhc* are difficult to grow and experimental work has focused on elucidating the nutritional requirements of *Dhc*. These efforts improved *Dhc* cell yields, increased reductive dechlorination rates, and resulted in more robust procedures for cultivating *Dhc*-containing consortia and axenic *Dhc* cultures.

Dhc strictly require corrinoid for the assembly of functional RDase enzyme systems. Remarkably, the available *Dhc* isolates cannot synthesize the corrin ring system and are corrinoid auxotrophs, indicating that they depend on the external supply of corrinoid produced by corrinoid prototrophs (i.e., community members with the ability for de novo corrinoid biosynthesis) (Hug et al., 2013b; Men et al., 2012; Yan et al., 2016; Yi et al., 2012). To better predict and potentially manage *Dhc* reductive dechlorination activity, this research explored the specific coabamide requirement of *Dhc*.

Prior research was primarily focused on optimizing conditions to improve reductive dechlorination activity; however, comparably few studies have determined factors that negatively impact organohalide-respiring bacteria involved in the degradation of chlorinated ethenes. Chlorofluorocarbons are common co-contaminants in chlorinated solvent plumes, and this research assessed the impact of 1,1,2-trichloro-1,2,2-trifluoroethane (CFC-113) on *Dhc* activity toward chlorinated ethenes.

The addition of fermentable organic materials (e.g. emulsified vegetable oil) to contaminated groundwater aquifers will lower the redox potential and increase the flux of hydrogen, and the benefits of electron donor biostimulation on reductive dechlorination activity are well documented

(Adrian et al., 2016; Löffler et al., 2013a). In contrast, the need for nitrogen biostimulation is not understood and this research explored the impact of nitrogenous compounds on organohalide-respiring bacteria.

Database mining

At the majority of contaminated sites, groundwater monitoring data have been collected for decades, but have not been systematically analyzed (Ronen et al., 2012; Timmerman et al., 2010; Ward et al., 1986). Systematic processing (e.g., data mining) of the available data, combined with knowledge about the effects of biogeochemical parameters on *in situ* reductive dechlorination, can rank individual environmental parameters in terms of relative relevance and lead to the development of predictive models. The term “data mining” refers to an analytic process designed to explore data (usually large data sets) and to identify unrecognized patterns and trends (Kim et al., 2009; Thuraishingham, 1998). Since the 1990s, machine learning-based data mining approaches have been applied to solve complex, dynamic environmental problems in various disciplines (Cortés et al., 2000; Kim et al., 2009). For example, data mining approaches adopting various machine learning algorithms were used to predict influent quality in wastewater treatment plants (Kusiak et al., 2013), to determine groundwater pollution vulnerability using hydrogeological parameters (Yoo et al., 2016), to assess heavy metal sorption to soil (Vega et al., 2009), to classify land cover and uses (Pal et al., 2003), and to estimate soil bacterial diversity (Kim et al., 2011). A robust model with predictive capabilities to support decision-making and effective technology implementation to cleanup contaminated aquifers would represent a major advance.

The reductive dechlorination potential of a site has been considered to be a relevant criterion to decide on the most promising *in situ* remediation strategy. (Fennell et al., 2001; Maphosa et al., 2010; Wiedemeier et al., 1996). Attempts, therefore, have been made to apply PCR-based tools to assess and monitor dechlorinating bacteria and to predict the *in situ* reductive dechlorination potential. A number of studies demonstrated significant correlations between the abundance of *Dhc* and observed *in situ* reductive dechlorination activities (Imfeld et al., 2008; Lebron, 2011; Lu et al., 2006a; Lu et al., 2006b; Ritalahti et al., 2010b). Thus, the abundance of *Dhc* biomarkers, i.e., 16S rRNA and RDase genes, has been considered to provide diagnostic and prognostic information about *in situ* dechlorination activity and potential (Clark et al., 2018; Lebron, 2011; Ritalahti et al., 2010b). However, site-specific dechlorination potential under complex *in situ* conditions cannot be predicted solely based on *Dhc* biomarker information.

Geochemical factors including nutrient and electron donor (H_2) availability, dissolved oxygen (DO), nitrate (NO_3^-), nitrite (NO_2^-), ferrous iron (Fe^{2+}), sulfate (SO_4^{2-}), sulfide (S^{2-}), and methane (CH_4) concentrations, total organic carbon (TOC) content, as well as pH and oxidation-reduction potential (ORP) are known to affect reductive dechlorination activity in subsurface environments (Löffler et al., 2006; Stroo et al., 2013; Wiedemeier et al., 1998). For example, pH values below 5.5 or $DO > 2\text{--}5\text{ mg/L}$ inhibit reductive dechlorination, and thus, pH and DO have been considered important geochemical factors (Stroo et al., 2013; Wiedemeier et al., 1998; Yang et al., 2017a; Yang et al., 2017b). Although *Dhc* can use hydrogen at low concentrations, competition for electron donor and other nutrients with NO_3^- , Fe^{3+} , SO_4^{2-} , and CO_2 -reducing populations have been reported to affect reductive dechlorination activity (Aulenta et al., 2006; Berggren et al., 2013; Boopathy et al., 2001).

Guidelines based on measurable biogeochemical parameters including *Dhc* 16S rRNA gene copies, pH, DO, ORP, NO_3^- , Fe^{2+} , SO_4^{2-} , S^{2-} , CH_4 , TOC, and H_2 (Table 2) have been developed to assist practitioners in deciding on the most promising site remedy (Lebron, 2011; Stroo et al.,

2013; Wiedemeier et al., 1998).

Table 2. A scientific consensus of conditions affecting the reductive dechlorination process mediated by organohalide-respiring bacteria in subsurface environments. ^a

Parameter	Condition, abundance, or concentration	Description
<i>Dhc</i> 16S rRNA gene copies	<10 ⁴ <i>Dhc</i> cells/L	Low <i>Dhc</i> ; dechlorination activity limited and ethene formation unlikely
pH	pH < 5.5 or > 9	Unfavorable pH range for reductive dechlorination
DO	> 2 mg/L	<i>Dhc</i> not tolerant to oxygen
ORP	> -100 mV	Organohalide-respiring bacteria require reducing conditions
Nitrate	> 1 mg/L	Unfavorable redox conditions and competition for electron donor affects organohalide respiring bacteria
Ferrous iron	< 1 mg/L	Fe ³⁺ -reducing activity ongoing and iron-reducers may compete for electron donors
Sulfate	> 1,000 mg/L	Sulfate-reducers compete for electron donors and produce potentially toxic sulfide
Sulfide	> 5 mM	Potentially toxic to organohalide-respiring bacteria
Methane	< 0.5 mg/L	Oxygen likely present
TOC	< 20 mg/L	Electron donor availability limits reductive dechlorination activity
Hydrogen	< 1 nM	Electron donor availability limits reductive dechlorination activity

^a Based on information provided in the following references: (He et al., 2005; Stroo et al., 2013; Wiedemeier et al., 1998).

Wiedemeier *et al.* suggested a scoring system based on measurable geochemical parameters and contaminant information to aid in decision-making (Wiedemeier et al., 1998). Although the popular scoring system has been applied to many sites, the system does not consider biological parameters (e.g., *Dhc* biomarkers), which is a major limitation. Stroo *et al.* applied a flowchart approach and proposed a decision guide for site managers to determine if bioaugmentation treatment will have benefits for reaching cleanup goals (Stroo et al., 2013). These guidelines generally rely on the existing knowledge and experience, but decision-making heavily depends on a limited number of observations, generally a single parameter (e.g., presence/absence of *Dhc* 16S rRNA genes, concentrations of a single inhibitory geochemical parameter, etc.) that correlates with dechlorination activity. Also, the scoring system relies on empirical judgment rather than comprehensive quantitative analysis reflecting the synergetic (i.e., interrelated) effects of multiple parameters.

At the majority of contaminated sites, groundwater monitoring data have been collected for decades, but have not been systematically analyzed (Ronen et al., 2012; Timmerman et al., 2010; Ward et al., 1986). Systematic processing (e.g., data mining) of the available data, combined with knowledge about the effects of biogeochemical parameters on in situ reductive dechlorination, can rank individual environmental parameters in terms of relative relevance and lead to the development of predictive models. The term “data mining” refers to an analytic process designed

to explore data (usually large datasets) and to identify unrecognized patterns and trends (Kim et al., 2009; Thuraisingham, 1998). Since the 1990s, machine learning-based data mining approaches have been applied to solve complex, dynamic environmental problems in various disciplines (Cortés et al., 2000; Kim et al., 2009). For example, data mining approaches adopting various machine learning algorithms were used to predict influent quality in wastewater treatment plants, (Kusiak et al., 2013) to determine groundwater pollution vulnerability using hydrogeological parameters, (Yoo et al., 2016) to assess heavy metal sorption to soil, (Vega et al., 2009) to classify land cover and uses, (Pal et al., 2003) and to estimate soil bacterial diversity (Kim et al., 2011). A robust model with predictive capabilities to support decision-making and effective technology implementation to cleanup contaminated aquifers would represent a major advance.

Methods

Task 1: RD-qChip – Design qPCR assays and validate with defined samples

Selection of gene targets

Organism- and process-specific biomarker genes were identified based on the available peer-reviewed literature. The focus was on 16S rRNA genes of organohalide-respiring bacteria and functional genes directly or indirectly involved in reductive dechlorination of chlorinated solvents, in particular chlorinated ethenes. In addition, the sequences of genes that provide general information about the microbial community (e.g., total bacterial and total archaeal 16S rRNA genes) and its functional attributes were collected. The selection of functional genes included those encoding corrinoid uptake and salvage, hydrogenases, nitrogenases, VC oxidases, and *Dhc* electron transport proteins. These target genes are listed in Table 3 and Table 4.

Primer and probe design for qPCR

TaqMan[®] chemistry and SYBR[™] chemistry design criteria were used for our primer and probe design. SYBR[™] chemistry uses SYBR Green I dye, which specifically binds to double-stranded DNA and detects all amplified double-stranded DNA molecules, including any double-stranded non-specific amplification products and primer dimers. TaqMan[®] chemistry uses a fluorogenic probe to enable the detection of the specific PCR product with higher specificity and sensitivity (TaqMan vs SYBR Chemistry for Real-Time PCR, Thermo Fisher Scientific, Grand Island, NY) (Arya et al., 2005). A number of qPCR assays targeting the genes of interest are available in literature but upon reevaluation of these assays for use for the OpenArray[®] system, numerous assays had to be redesigned to meet the stringent criteria of the OpenArray[®] platform. The OpenArray[®] approach requires all assays to hybridize to their targets at a uniform temperature. In order to run OpenArray[®] plates at the selected annealing temperature of 60°C, primers and probes were designed to meet this criterion. To provide some flexibility in designing probes that meet the uniform temperature hybridization constraint, all probes were synthesized with the Minor Groove Binder (MGB) modification (Thermo Fisher Scientific, Grand Island, NY). Design parameters for the target assays included annealing temperature ranges of 45-65°C for primers and 48-65°C for probes (MGB increases probe melting temperature therefore shorter probes with low melting temperature can be used); primer and probe lengths ranged between 15-30 bp and 13-25 bp, respectively; and the total amplicon length did generally not exceed 250 bp with only a few exceptions (i.e., the Total Bacteria_16S, DHC_VcrA2 and DHC_BvcA2 assays in RD-qChip version 1; the Total Bacteria_16S and *cbiZ_VS195* qPCR assays in RD-qChip version 2, which produced longer (i.e., > 250 bp) amplicons (see Table A-49). Following primer design for each target gene, qPCR with SYBR Green detection chemistry was performed to verify primer

performance at an annealing temperature of 60°C. All qPCR primers were obtained from IDT Technologies (Coralville, IA).

Sequences for each target gene of interest were obtained from public databases (e.g., NCBI, EMBL) and imported into bioinformatics software (Geneious R11.0.2 (<http://www.geneious.com>, (Kearse et al., 2012)). Geneious was used to align target genes that share sequence similarity and identify conserved regions. A 13 to 25 base pair region was selected for the probe in the conserved region with no mismatched nucleotides (i.e., 100% sequence match). The specificity of the primers and probes was also verified using primer-BLAST analysis (Ye et al., 2012) against the NCBI nucleotide collection (nr) database. Once specificity for a target gene was verified for the probe, a similar process was used for designing the primers with an amplicon length of 50 to 250 bp. The designed primers and probes were checked for possible hairpins, self-dimerization and hetero-dimerization using the OligoAnalyzer tool available from IDT Technologies (Coralville, IA) and the Primer 3 tool available in Geneious. All primers and probes were designed to specifically meet the Taqman detection chemistry parameters.

Theoretically, different primer/probe combinations targeting the same gene should result in comparable qPCR data. Multiple primer/probe sets targeting genes with the same function can reveal allelic differences and possibly provide information about the host organism(s) of the target gene(s). Therefore, multiple assays for the same gene target were included to demonstrate the consistency across the array plate for the same sample. For both RD-qChip v1 and RD-qChip v2, several assays targeting the same gene were designed with different primer/probe combinations and located on the respective plates. For RD-qChip v1, these assays included the *Dhc* 16S rRNA gene, *Dhb* 16S rRNA gene, and several other 16S rRNA gene-targeted assays as well as two assays for *cbiZ* (*Dhc* Pinellas subgroup), *mbrA*, *bvcA*, *vcrA* (all *Dhc*), and *hymC* (*Dhc* Pinellas group). For RD-qChip v2, *hycE*, *hymC*, *echE*, *hupL*, *fdhA* (CISM) and *cobS* (*Dhc* Pinella subgroup) assays were selected for two primer/probe combinations. Also, in order to verify the consistency of the assays and to ensure that the results are not affected by the physical location of the assays across the same OpenArray plate, four target assays (*Dhc* 16S rRNA gene, *vcrA*, *cobT* and *fdhA* [CISM]) specific to *Dhc* were selected and placed on the RD-qChip v2 in duplicate at physically separate locations. Theoretically, the same assays in different locations of the RD-qChip v2 plate should produce similar results.

Reverse transcription qPCR (RT-qPCR)

The quantification of biomarker transcripts with RT-qPCR can be compromised by mRNA loss during sample handling, the entire process of RNA extraction, and by inefficiencies in reverse transcription steps (Johnson et al., 2005b; Ritalahti et al., 2010a). Two approaches have been applied to increase the accuracy of RT-qPCR measurements. Biomarker transcripts can be normalized to the transcript abundance of a housekeeping gene (e.g., *rpoB*), for which a constant expression level is assumed. For this purpose, a TaqMan® qPCR assay was designed for the *rpoB* gene of *Dhc* and included on the RD-qChip v2. Another approach used for normalization of biomarker transcripts is the addition of a known number of reference mRNA molecules (i.e., firefly luciferase mRNA; Promega, 2 µL (2.0E+10 copies)) into each sample prior to RNA extraction. Quantification of the reference mRNA informs about RNA losses during the extraction and purification process and inefficiencies during the reverse transcription step (Amos et al., 2008; Johnson et al., 2005b; Ritalahti et al., 2010a). Normalization assumes that the loss of biomarker mRNA equals the loss of luciferase mRNA. To implement the luciferase normalization approach as an internal standard for quantification of mRNA loss, an existing primer pair (Johnson et al., 2005b) was combined with a new MGB probe to specifically target the firefly luciferase gene and

placed on RD qChipv1 and v2. The abundance of target transcripts quantified as cDNA were then divided by the recovery (%) value of luciferase transcripts as cDNA for each sample to determine the number of target transcripts in the sample (Amos et al., 2008; Johnson et al., 2005b; Ritalahti et al., 2010a).

qPCR assay validation

To validate each assay, SYBR[™] Green qPCR was performed in singleplex qPCR using either a 96-well or a 384-well block (Applied Biosystem 7500 Real-Time PCR System, Life Technologies ViiA 7 Real-Time PCR System, and Life Technologies QuantStudio[®] 12K Flex Real-Time PCR System, Life Technologies, Grand Island, NY). The 10- μ L qPCR mixture was composed of 5 μ L Power SYBR[™] Green PCR Master Mix (Applied Biosystems, Foster City, CA), 0.3 μ M of each primer, 2.0 μ L of template DNA and the remaining volume sterile ddH₂O. 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 qPCR originated from the specific target PCR product and not from primer dimers or non-specific amplification. qPCR amplifications were performed in triplicate for each DNA sample and the corresponding standard (Ritalahti et al., 2006). Assays with extensive primer dimer formation and/or non-specific amplification product formation were eliminated, and new primers were designed for these assays.

The assays that exhibited the desired target specificity, amplification efficiency values ranging between 90 and 110%, and R^2 values >0.985 were further tested for their sensitivity and specificity using the TaqMan[®] qPCR chemistry. TaqMan[®] probes with MGB modification and a nonfluorescent quencher were ordered from Thermo Fisher Scientific. Assays for TaqMan[®] qPCR were prepared as follows for a 10- μ L reaction volume: 5 μ L of TaqMan[®] Universal PCR Master Mix No AmpErase UNG (Applied Biosystems, Foster City, CA), 0.3 μ M of each primer, 0.3 μ M of probe and 2.0 μ L of template DNA. TaqMan[®] qPCR assays were run under the same PCR cycling conditions as described for SYBR[™] Green qPCR. Assays with observed amplification efficiency (E) % of 90 to 110% (except for the total bacterial 16S rRNA gene-targeted assay, which uses degenerate primers: E% = 80%), standard curve slopes ranging from -3.1 to -3.6, and standard curves with $R^2 > 0.985$ met the selection criteria, and were included in the OpenArray[®] plate design (i.e., RD-qChip v1 and v2).

Standard curve preparation

Standard curves for RD-qChip v1 were prepared using plasmid DNA (pDNA) with synthetic target gene fragments or PCR-amplified DNA fragments. Synthetic oligobucleotides were incorporated into *E. coli* by Life Technologies (Grand Island, NY). Target gene amplicons were cloned into the pCR[™]2.1 vector and introduced into *E. coli* using the Invitrogen TA Cloning[™] kit (Life Technologies, Grand Island, NY). For validation of RD-qChip v2 assays with traditional qPCR, standard curves were prepared with either template pDNA or synthetic linear DNA fragments obtained from IDT (Coralville, IA) or GeneArt (Life Technologies, Grand Island, NY). For validation of RD-qChip v2, standard curves were generated using synthetic linear DNA fragments obtained from GeneArt (Life Technologies, Grand Island, NY).

The *E. coli* transformants were grown in Lysogeny Broth (LB) with ampicillin (100 μ g/L) or kanamycin (50 μ g/L) at 37°C overnight. pDNA was isolated using the Zymo Research Zyppy[™] Plasmid Miniprep Kit (Zymo Research Corp., Irvine, CA) and quantified using a NanoDrop and the Qubit 2.0 Fluorometer. The synthetic linear DNA fragment standard curves span a concentration range of approximately 10E+1 to 10E+8 target gene copies/ μ L and were prepared

using a 10-fold serial dilution series of template DNA. All standard curves had a total of eight calibration points.

OpenArray® plate layout and design

The initial RD-qChip v1 design included 56 assays and was the basis for the improved RD-qChip v2 design with 112 assays. Following primer and probe validation, target gene assays were arranged on the OpenArray® plate according to their functionality (Table 3 and Table 4).

RD-qChip v1: Custom OpenArray® plates for the QuantStudio® 12K Flex Real-Time PCR System (Life Technologies, Grand Island, NY) were ordered with primers and probes targeting the selected 42 genes (total 56 assays) are listed in Table 3 and shown in Figure 4. Detailed information about the primer/probe sequences, target genes, etc. that were incorporated in the design of RD-qChip v1 are available in Table A-48. Included in the plate design was the gene expression control gene (*luciferase*) for use as internal standard to aid in quantification and increase accuracy of RT-qPCR measurements of mRNA targets.

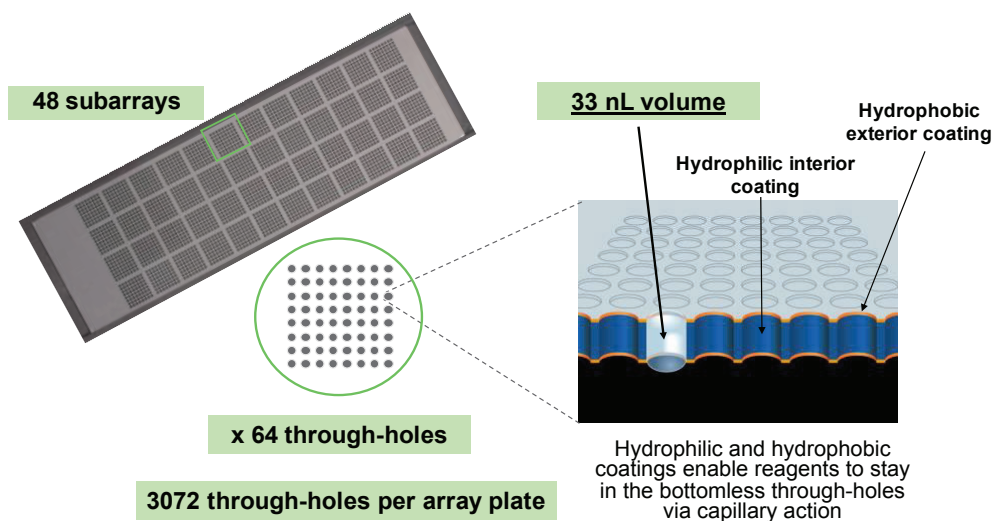


Figure 4. Schematic of an OpenArray® plate. An array plate consists of 48 subarrays each with 64 through-holes. The primers and the probe for individual assays are present in individual 33-nL through-holes. Each 33-nL compartment represents a qOCR assay and 3,072 assays can be run on one OpenArray® plate. The QuantStudio® 12K Flex Real-Time PCR System can run four plates simultaneously for a theoretical maximum of 12,288 assays.

Table 3. Target genes included in the design of RD-qChip v1.

Target Organism/Group	Category	Specific Target
Total Bacteria (general)	Phylogenetic	16S rRNA gene
Archaea (Methanogens)	Phylogenetic	16S rRNA gene
<i>Dehalococcoides mccartyi</i>	Phylogenetic	16S rRNA gene
<i>Dehalobacter</i>	Phylogenetic	16S rRNA gene
<i>Dehalobacterium formicoaceticum</i>	Phylogenetic	16S rRNA gene
<i>Dehalogenimonas</i>	Phylogenetic	16S rRNA gene
<i>Geobacter</i>	Phylogenetic	16S rRNA gene
<i>Anaeromyxobacter</i>	Phylogenetic	16S rRNA gene
<i>Dehalobacterium formicoaceticum</i>	Phylogenetic	16S rRNA gene
<i>Spirochaetes</i>	Phylogenetic	16S rRNA gene
<i>Acidaminobacter</i>	Phylogenetic	16S rRNA gene
<i>Methanospirillum</i>	Phylogenetic	16S rRNA gene
<i>Sphingobacteria</i>	Phylogenetic	16S rRNA gene
<i>Desulfovibrio</i>	Phylogenetic	16S rRNA gene
<i>Nitrospira</i>	Phylogenetic	16S rRNA gene
<i>VadinBC27</i>	Phylogenetic	16S rRNA gene
<i>Dehalococcoides mccartyi</i>	RDase	<i>bvcA, vcrA, pceA, dcpA, cbrA, mbrA</i>
<i>Geobacter lovleyi</i>	RDase	<i>pceA</i>
<i>Sulfurospirillum</i>	RDase	<i>pceA</i>
<i>Desulfitobacterium</i>	RDase	<i>pceA</i>
<i>Dehalococcoides mccartyi</i>	Hydrogenase	<i>hycE, fdhA, vhuA, hycE, fehymC, hupL</i>
<i>Dehalococcoides mccartyi</i>	Corrinoid metabolism	<i>cbiZ, cobU</i>
<i>Desulfovibrio</i>	Sulfate reductase	<i>aprA</i>
<i>Pseudomonas</i>	Sulfate reductase	<i>aprA</i>
<i>Mycobacterium</i>	VC oxidation	<i>etnC, etnE</i>
<i>Anaeromyxobacter</i>	Mn (IV) reduction	Gene encoding a c-type cytochrome implicated in Mn (IV) reduction
Firefly <i>Photinus pyralis</i>	Expression control	<i>luciferase</i>

The RD-qChip v2 was designed based on the experiences obtained with RD-qChip v1. Specifically, unproductive assays were replaced (29 assays were kept in the RD-qChip v2 design), and the total number of assays increased from 56 to 112 (112 assays targeting 102 genes) to accommodate the inclusion of additional target genes of interest. The list of the target genes included in RD-qChip v2 is given in Table 4. Custom OpenArray® plates with 112 assays for the QuantStudio® 12K Flex Real-Time PCR System were ordered from Life Technologies (Grand Island, NY). The OpenArray® plate RD-qChip v2 design comprises 112 assays, including 102 target biomarker genes. With this design, each OpenArray® plate can run 24 samples in parallel. The list of the assays on the RD-qChip v2 OpenArray® plate is given in Table 4.

Table 4. Target genes included in the RD-qChip v2 design.

Target Organism/Group	Category	Specific Target
Total Bacteria (general)	Phylogenetic	16S rRNA gene
Archaea (Methanogens)	Phylogenetic	16S rRNA gene
<i>Dhc</i>	Phylogenetic	16S rRNA gene
<i>Dhb</i>	Phylogenetic	16S rRNA gene
DEFO	Phylogenetic	16S rRNA gene
<i>Dhgm</i>	Phylogenetic	16S rRNA gene
DIEL	Phylogenetic	16S rRNA gene
<i>Geobacter</i>	Phylogenetic	16S rRNA gene
<i>Dehalobium chlorocoercia</i> DF1	Phylogenetic	16S rRNA gene
<i>Sulfurospirillum</i>	Phylogenetic	16S rRNA gene
<i>Desulfitobacterium</i>	Phylogenetic	16S rRNA gene
<i>Anaeromyxobacter</i>	Phylogenetic	16S rRNA gene
<i>Sphaerochaeta</i> (FLiPS)	Phylogenetic	16S rRNA gene
<i>Acidaminobacter</i>	Phylogenetic	16S rRNA gene
<i>Nitrospira</i>	Phylogenetic	16S rRNA gene
<i>Spirochaetes</i>	Phylogenetic	16S rRNA gene
<i>Desulfovibrio</i>	Phylogenetic	16S rRNA gene
<i>Sphingobacteria</i>	Phylogenetic	16S rRNA gene
<i>Methanospirillum</i>	Phylogenetic	16S rRNA gene
<i>Rikenellaceae</i>	Phylogenetic	16S rRNA gene
<i>Dhc</i>	RDase	<i>tceA, bvcA, vcrA, pceA, dcpA, cbrA, mbrA, dcmb_1041, dcmb_1339, dcmb_1434, pcbA1, pcbA4/pcbA5</i> , other putative RDase genes
<i>Dhgm</i>	RDase	<i>dcpA, cerA, fdhA</i> , other putative RDase genes
<i>Dhb</i>	RDase	<i>pceA, cfrA, thmA, tmrA</i>
<i>Desulfitobacterium</i>	RDase	<i>pceA, ctrA, dcaA</i>
Consortium RM	RDase	<i>rdhA-AWM53_01188-RM, rdhA-AWM53_01801-RM, rdhA-AWM53_00864-RM</i>
Consortium SDC-9	RDase	<i>SDC9_45582-rdhA, SDC9_09280-rdhA, SDC9_23241-rdhA, SDC9_07142-rdhA, SDC9_07143-rdhA, SDC9_23243-rdhA, SDC9_48350-rdhA</i>
<i>Mycobacterium, Nocardioide</i> s	VC oxidation	<i>etnC</i>
<i>Mycobacterium, Nocardioide</i> s, <i>Pseudomonas, Ochrobactrum</i>	VC oxidation	<i>etnE</i>
<i>Dhc</i>	Hydrogenase, electron transport	<i>fdhA, hymB, hymC, hycE, echE, vhuA, hupL</i>
<i>Dhci</i>	Corrinoid metabolism	<i>cbiZ, cobA, cobC, cobS, cobT, cobU, btuC, btuD, btuF</i>
<i>Dhb</i>	Corrinoid metabolism	<i>cobT</i>
<i>Desulfitobacterium</i>	Corrinoid metabolism	<i>cobT</i>
<i>Dhc</i>	Nitrogenase	<i>nifD, nifH, nifK</i>
<i>Desulfovibrio</i>	Nitrogenase	<i>nifD</i>
<i>Dhc</i>	Housekeeping gene (Internal Control)	<i>rpoB</i>
Firefly <i>Photinus pyralis</i>	Expression Control	<i>luciferase</i>

Sample preparation for OpenArray® plate

The robotic liquid handling system OpenArray® AccuFill™ (Life Technologies) was used to load samples onto the OpenArray® plates. The AccuFill™ requires a 384-well plate for sample preparation and to subsequent transfer to the OpenArray® plate. The OpenArray 384-well sample plate was prepared by using the robotic QIAgility System (Qiagen, Valencia, CA, USA) which loaded the PCR mix and DNA (cDNA) into the wells (PCR mix = 2.5 µL of 2X OpenArray® Real Time PCR Master Mix + nuclease-free water (1.3 µL for RD-qChip v1 or 0.5 µL for RD-qChip v2); 1.2 µL DNA (cDNA) for RD-qChip v1 or 2 µL of DNA (or cDNA) sample for RD-qChip v2). The loaded OpenArray 384-well sample plate was sealed (Quant Studio 12K Flex OpenArray® 384-well sample plate seals, Life Technologies) and then centrifuged for 1 min at 2,000 rpm to ensure all liquid is at the bottom of the wells and to eliminate any air bubbles.

OpenArray® qPCR

The robotic liquid handling system OpenArray® AccuFill™ transferred the samples from the OpenArray 384-well sample preparation plate to the OpenArray® plate. Once the OpenArray® plate was loaded, the plate was sealed with the provided Case Lid within 90 seconds to prevent any evaporative losses using the QuantStudio® 12K Flex OpenArray® Plate Press 2.0. Then the OpenArray® plate case was slowly loaded with OpenArray® Immersion Fluid in one gentle continuous motion to avoid entrapping of air bubbles. The loading port of the OpenArray® plate was sealed by inserting the OpenArray® Plug before the downward portion of the OpenArray® plate was wiped with 95% ethanol. The sealed OpenArray® plate was loaded into the QuantStudio® 12K Flex Real-Time PCR System and run using the standard OpenArray® parameters.

Validation of RD-qChip v1

The custom OpenArray® plates were validated using DNA standards containing mixed target pDNAs. Two different mixtures of pDNA were prepared to use in the validation of the RD-qChip v1. Mixture 1 included phylogentic targeted genes (i.e. *Dhc*, *Dhb*, *Dhgm*, *Geo*, etc...) and mixture 2 included other process-specific targeted genes (i.e. *vrA*, *bvcA*, *tceA*, *cerA*, *cbrA*, etc...) on the RD-qChip v1. The mixtures were prepared by normalizing all pDNA to 10 ng/µL in one 1.5 ml Eppendorf tube. A serial 10-fold dilution of the DNA mixtures yielded a DNA concentration range for each DNA target from 2.02 E+00 to 2.02 E+08 gene copies/µL (1.58E+06 to 1.58E-02 gene copies/33 nL reaction volume).

Validation of RD-qChip v2

To evaluate specificity, sensitivity and reproducibility of RD-qChip v2, two RD-qChip v2 plates were run using ten-fold serial dilution series of the mixture of 14 linear DNA fragments in the range of 1.22 E+01 to 1.22 E+08 gene copies/µL (1.61E-01 copies to 1.61E+06 gene copies/33 nL-well). The standards were prepared in two different days and RD-qChip v2 runs were performed in the same day with standard preparation. The standards were run in triplicate on RD qChip v2 using QuantStudio 12K Flex Real-Time PCR System.

Task 2: Proteomics – develop proteomics pipeline and validate with defined samples

In silico analysis to evaluate the uniqueness of peptidomes of *Dhc* strains

To check how similar the peptidomes of known *Dhc* strains (i.e., strains 195, VS, GT, BAV1, FL2, and CBDB1) are versus those from bacteria commonly found in groundwater aquifers and sediment/subsurface environments, an *in silico* tryptic digestion of their proteomes was conducted

using the Peptidome Clustering tool of the web application Unipept 3.2 (<https://unipept.ugent.be/>) (Mesuere et al., 2016). Peptidome similarity percentages were calculated based on the minimum similarity method and then clustered by the UPGMA algorithm (Bouguettaya, 1996).

RDase phylogenetic tree construction

Sequence relatedness of RDases proteins in the proteomes of *Dhc* strains 195, FL2 and BAV1 was inferred by phylogenetic analysis using 52 protein sequences that were aligned with the MUSCLE algorithm (Edgar, 2004). All positions in the sequences containing gaps and missing data were eliminated, leaving a total of 56 amino acids positions in the final dataset. A phylogenetic tree in MEGA 7 using the Maximum Likelihood algorithm based on the JTT matrix-based model was then constructed (Jones et al., 1992; Kumar et al., 2016). Estimation of the relative confidence scores in phylogenetic groups were determined by using 100 bootstrap replications of the data set (Felsenstein, 1985).

***Dehalococcoides mccartyi* (*Dhc*) cultures and growth conditions**

Biological duplicates of actively dechlorinating axenic cultures of *Dhc* strains 195 and FL2, known to express the RDase TceA (TCE→VC/ethene), as well as strain BAV1, which expresses the BvcA RDase (DCEs→VC→ethene), were prepared and used to monitor the abundances of the targeted *Dhc* proteins in both global and targeted proteomics measurements. Demonstration of the targeted proteomics approach also used 100 mL (~1E+10 cells) of the PCE-to-ethene-dechlorinating consortium Bio-Dechlor INOCULUM (BDI) known to contain several *Dhc* strains and a PCE-to-cDCE-dechlorinating *Geobacter* species and multiple *Dhc* strains (Fletcher et al., 2011). The BDI consortium was grown with PCE as electron acceptor and lactate as electron donor. Cultures were grown in completely synthetic, defined mineral salts medium as previously described (Yan et al., 2016). Approximately 100 mL of culture suspensions (~1E+10 cells) were passed through Sterivex 0.22 µm filter units (EMD Millipore Corporation, Billerica, MA, USA) to collect the biomass. Filters were stored at -80 °C prior to protein extraction and digestion. *Dhc* cell numbers were calculated by qPCR enumeration of 16S rRNA genes (Ritalahti et al., 2006; Ritalahti et al., 2010b). The known *Dhc* genomes contain single copies of the 16S rRNA gene and the RDase genes, and the gene copies measured with qPCR equal the *Dhc* cell number (Löffler et al., 2013b).

Task 3: Novel microbes

The goal was to identify and characterize novel microbes involved in the reductive dechlorination of chlorinated ethenes. The focus was on a methanogenic enrichment culture that dechlorinated TCE to ethene. Despite repeated efforts, no *Dhc* biomarkers could be detected, implying that the mixed culture harbored one or more non-*Dhc* populations responsible for reductive dechlorination to non-toxic ethene.

Source material and growth conditions

The enrichment culture was derived from PCE-dechlorinating microcosms that had been established with grape pomace compost collected in the wine-growing area of Rotenberg near Stuttgart, Germany. Defined, completely synthetic, reduced and bicarbonate-buffered mineral salts medium with 5 mM lactate was prepared in 160-mL glass serum bottles following established protocols (Löffler et al., 2005). After autoclaving, Wolin vitamins were added from an anoxic, filter-sterilized (0.22 µm) stock solution (Wolin et al., 1963). Other additions included 10 mL of hydrogen gas and 5 µL neat PCE (360 µM aqueous concentration). The bottles were incubated at 30°C in the dark without agitation. Prior to use, all plastic syringes were flushed with sterile,

oxygen-free nitrogen gas to remove any residual air. Repeated transfers (3%, vol/vol) to 160-mL glass serum bottles with 100 mL of medium amended with lactate, hydrogen and PCE yielded solid-free, ethene-producing enrichment cultures. In subcultures, 2 mL (83.0 μ mol) of VC replaced PCE as electron acceptor. To inhibit methanogenesis, 1.2 mM 2-bromoethanesulfonate (BES) was added. Live cultures were set up in at least duplicate, and negative controls included autoclaved replicates of live incubations and vessels that received no electron acceptor.

DNA extraction, PCR and amplicon sequencing

DNA extraction and PCR assays targeting *Dhc* and *Dhgm* 16S rRNA genes and the *tceA*, *bvcA* and *vcrA* RDase genes followed established procedures (Ritalahti et al., 2006; Yan et al., 2009). Briefly, cells were collected from 2 mL culture suspensions by vacuum filtration onto 0.22 μ m membrane filters (Millipore GVWP025000, EMD Millipore Corp., Billerica, MA, USA). Trapped cells were disrupted by bead beating at a speed of 3.25 m/s for 5 minutes at room temperature (Omni Bead Ruptor Homogenizer, Kennesaw, GA, USA). Genomic DNA was extracted with the PowerSoil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's manual. DNA extracted from PCE- and VC-fed culture GP (referring to the culture source, grape pomace) biomass was treated with the Genomic DNA Clean and Concentrator Kit (Zymo Research, Irvine, CA, USA). DNA quantity and quality were estimated with a NanoDrop 1000 (Thermo Fisher Scientific, NanoDrop Wilmington, DE, USA). Genomic DNA samples from two replicate cultures were pooled for Illumina sequencing. Purified DNA samples were PCR-amplified using barcoded-primers F515/R806 targeting the V4 region of the 16S rRNA gene. The amplicon sequencing approach followed established protocols (Caporaso et al., 2012; Caporaso et al., 2011). Raw sequences were paired and analyzed using the mothur software package (www.mothur.org) following MiSeq standard operating procedures (Schloss et al., 2009).

Quantitative real-time PCR (qPCR)

A TaqMan chemistry-based quantitative PCR (qPCR) assay was developed to specifically target and enumerate *Dhgm* 16S rRNA genes. The forward primer Dhgm478F (5'-AGCAGCCGCGGTAATACG -3'), the reverse primer Dhgm536R (5'-CCACTTTACGCCCAATAAATCC-3') and the TaqMan probe Dhgm500Probe (5'-6FAM-AGGCGAGCGTTAT-MGB -3') matching *Dhgm* 16S rRNA gene sequences, which were retrieved from NCBI database, were designed using the Primer3 plug-in in Geneious 8.1.7. The assay specificity was verified by in silico analysis using the Primer3 plug-in in Geneious 8.1.7 and the Primer-BLAST tool provided by NCBI, and was further experimentally confirmed using pure culture genomic DNA of *Dhc* strain 195 and strain FL2 and 171 bp-long synthesized DNA oligos containing the corresponding amplicon sequence of the *Dhc* 16S rRNA gene (Integrated DNA Technologies, Coralville, IA, USA) (Figure 5). All qPCR assays consisted of two-fold concentrated TaqMan Universal PCR master mix (Applied Biosystems, Waltham, MA, USA), UltraPure nuclease-free water (Invitrogen, Carlsbad, CA, USA), 300 nM probe, 300 nM of each primer, and a DNA template and were performed using an Applied Biosystems ViiA 7 system (Applied Biosystems, Waltham, MA, USA). The qPCR cycle conditions were as follows: 50 °C for 2 min and then held at 95 °C for 10 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. The standard curves were generated using three independent dilution series of plasmid DNA carrying a nearly complete sequence (1,492 bp) of the *Dhgm* strain BL-DC-9 16S rRNA gene (NCBI accession NR_074337.1) or a 171 bp-long synthesized DNA oligo (Integrated DNA Technologies, Coralville, IA, USA) covering the 59 bp amplicon region. This *Dhgm* qPCR assay standard curve had a slope of -3.676, a y-intercept of 40.97, a R² value of 0.997, and a PCR amplification efficiency of 87.10% (Figure 5). The detection

limit was 11.4 gene copies per reaction, and the linear range spanned $1.14\text{E}+02$ to $1.14\text{E}+08$ gene copies per reaction.

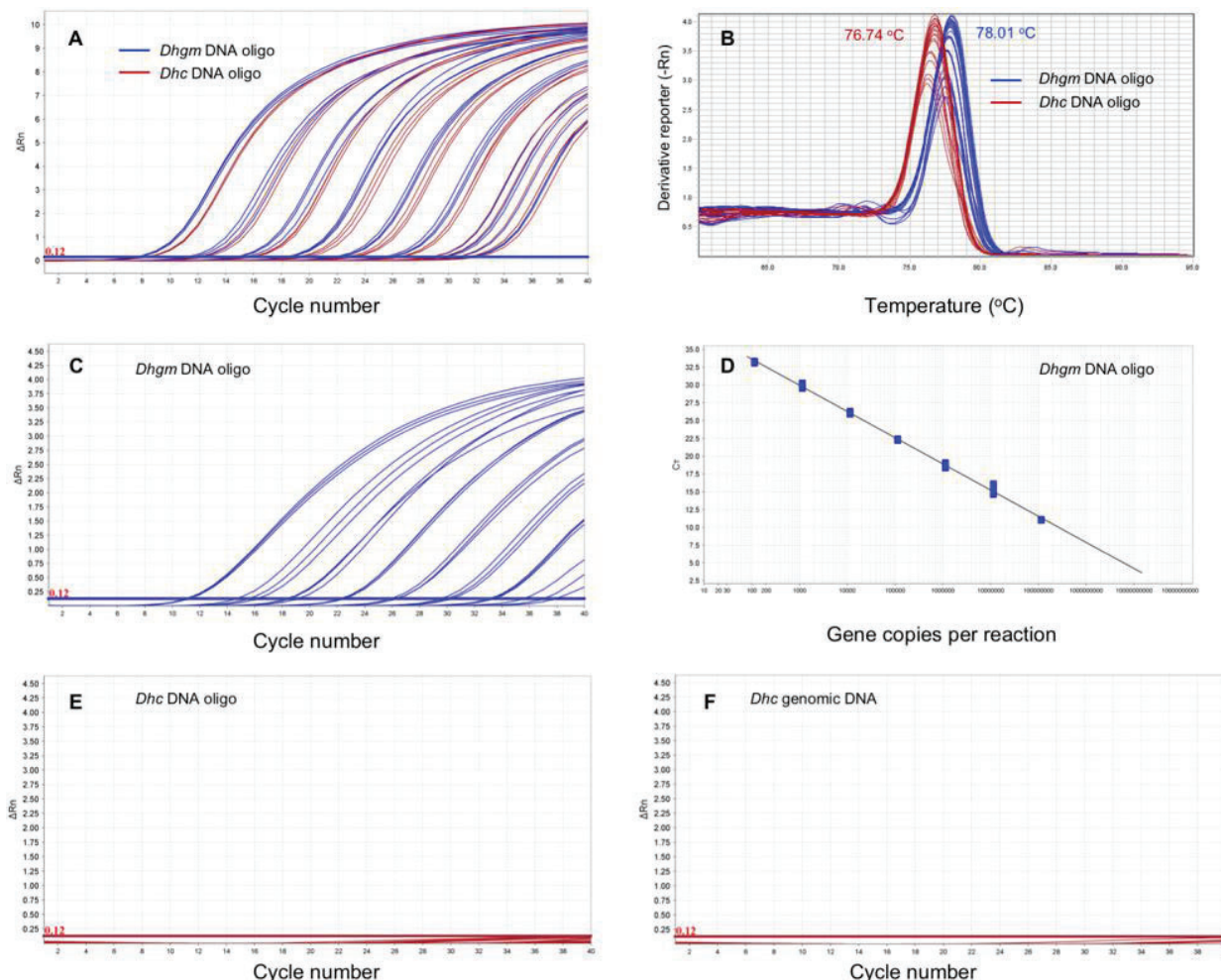


Figure 5. Validation of the *Dhgm* qPCR assay specificity. (A) qPCR with SYBR Green detection chemistry amplified both the *Dhgm* 16S rRNA gene and *Dhc* 16S rRNA gene. (B) Melting curve analysis differentiated the 59-bp PCR products amplified from DNA oligos carrying a partial *Dhgm* or *Dhc* 16S rRNA gene. Amplification curves (C) and standard curve (D) of the TaqMan chemistry *Dhgm* qPCR assay with 10-fold dilutions of *Dhgm* DNA oligo templates as standards, from $1.14\text{E}+08$ to $1.14\text{E}+01$ gene copies per reaction. No fluorescence signals were generated in TaqMan assays with a *Dhc* DNA oligo (10-fold dilutions from $1.14\text{E}+08$ to $1.14\text{E}+01$ gene copies per reaction) (E) or *Dhc* strain 195 genomic DNA ($1.6\text{E}+06$ 16S rRNA gene copies per reaction) or strain FL2 genomic DNA ($0.8\text{E}+06$ 16S rRNA gene copies per reaction) (F) as templates. Note: the threshold line is set at 0.12 for the determination of the C_T (threshold cycle) number.

***Dhc* and *Dhgm* abundances in groundwater impacted with chlorinated solvents**

DNA was extracted from groundwater (1,173 samples) or biomass collected on site onto Sterivex cartridges (Ritalahti et al., 2010b). *Dhc* 16S rRNA gene-targeted qPCR followed established procedures (Ritalahti et al., 2006; Yan et al., 2009) and *Dhgm* 16S rRNA genes were enumerated with the Dhgm478F-Dhgm500Probe-Dhgm536R assay.

Task 4: Identification of novel biomarkers for the degradation of chlorinated solvents

Several organism- and process-specific biomarker genes have been discovered and their utility for monitoring reductive dechlorination processes has been demonstrated. The specific goals of Task 4 efforts were to discover novel biomarkers for reductive dechlorination of 1,2-D to environmentally benign propene, additional biomarkers for VC-to-ethene reductive dechlorination, and biomarkers for DCM degradation. The discovery of biomarkers for 1,2-D reductive dechlorination (dichloroelimination) focused on the *Dhc*-containing enrichment cultures KS and RC obtained in prior work (Löffler et al., 1997a; Ritalahti et al., 2004). The discovery of novel VC RDase genes focused on the TCE-dechlorinating enrichment culture GP capable of growing with VC as electron acceptor. These enrichment cultures were grown and maintained as described under Task 3.

Discovery of a novel biomarker for VC reductive dechlorination in mixed culture GP

Metagenome sequencing and comparative analyses

Metagenomic sequencing of the VC-grown enrichment culture GP was performed using the MiSeq platform provided through Center for Environmental Biotechnology (University of Tennessee Knoxville, Knoxville, TN, USA). The Nextera-prepped metagenome library with an average fragment size of 450 bp was sequenced using the v3 reagent kit for a 2x300 run. After being trimmed and filtered, the resulting 13,667,850 paired-end reads were assembled following established procedures (Oh et al., 2011). Additional information about data processing and analyses are available in the peer-reviewed literature (Yang et al., 2017c).

For a comparative metagenome analysis, the sequences of VC-fed culture GP (MG-RAST ID: 4625853.3) were uploaded to the MG-RAST server and compared with three PCE-/TCE-dechlorinating consortia KB-1 (MG-RAST ID: 4450840.3), ANAS (MG-RAST ID: 4451655.3) and Donna II (MG-RAST ID: 4451259.3). Metagenomic data sets from non-dechlorinating communities of an acid mine drainage site (Richmond Mine, Iron Mountain, CA; MG-RAST ID: 4441137.3 and 4441138.3) and a pristine freshwater lake in Antarctica (Ace Lake; MG-RAST ID: 4443683.3) were included in the analysis due to their well-documented meta information, good quality sequence reads, and their distinct origins. Taxonomic and functional annotation results from MG-RAST were imported into STAMP for principal component analysis (PCA) and visualization (Parks et al., 2014).

Genome binning and annotation

Binning of metagenomic contigs was conducted with MetaWatt v1.7 (Strous et al., 2012) and VizBin (Laczny et al., 2015), using GC content, tetranucleotide frequency and coverage as quality metrics to assess consistency of contigs within the genomic bin (Laczny et al., 2015). Contigs belonging to the *Dhgm* bin were further assessed with CheckM (Parks et al., 2015) using default settings to further assess genome bin completeness, contamination and taxonomic affiliation. The draft genome bin was uploaded to RAST (Overbeek et al., 2014) for annotation (Accession ID: 1536648.4).

Phylogenetic analyses. Maximum likelihood tree estimation was performed using RAxML v8.2.5 (Stamatakis, 2014) on individual and concatenated 5S, 16S and 23S rRNA gene alignments with 1000 bootstrap replicates. Evolutionary model selection was evaluated with jModelTest v2.1.5 (Darriba et al., 2012). In all cases, the generalized time-reversible model (Tavaré, 1986) with estimations of the proportion of invariable sites and rate heterogeneity among sites (eight substitution rate categories) was implemented in RAxML. For concatenated alignments, a partition

file was provided to RAxML and rate heterogeneity among partitions was estimated individually. The phylogenetic tree of RDase proteins was built using Geneious software with default settings of the MAFFT Alignment and Geneious Tree Builder tools.

Proteomics analysis

Proteins were extracted from culture GP grown with TCE, 1,1-DCE, cDCE and VC as electron acceptors and subjected to LC-MS/MS analysis following established procedures (Chourey et al., 2010; Chourey et al., 2013). Biomass harvest times are described in Table 5.

Table 5. Dechlorination activity in quintuplicate cultivation vessels inoculated with enrichment culture GP and amended with different chlorinated ethenes as electron acceptors. The concentrations of chlorinated ethenes and ethene were measured on Day 60. One of the replicate vessels was sacrificed on Day 60 for proteomics analysis. Highlighted in bold font are the initial amounts of chlorinated solvents added to the cultures.

Time	Chlorinated ethenes and ethene (μmol)				
	TCE	cDCE	1,1-DCE	VC	Ethene
Day 0	77.4	0.00	0.00	0.00	0.00
Day 60	27.9	20.8	14.3	12.6	0.5
Day 0		126.1		0.00	0.00
Day 60		62.0		61.1	2.9
Day 0			97.0	0.00	0.00
Day 60			57.0	38.4	1.6
Day 0				165.0	0.00
Day 60				147.1	10.6

Discovery of a biomarker for reductive dechlorination of 1,2-D

1,2-D-dechlorinating cultures

The 1,2-D-dechlorinating mixed cultures RC and KS were derived from the Red Cedar River, near Okemos, MI, and the King Salmon River, AK, respectively. The Red Cedar River has no known anthropogenic sources of chlorinated solvents but is located in an agricultural area. The King Salmon River sediment had reported hydro- carbon contamination. Both cultures are nonmethanogenic and have been maintained for more than 15 years in reduced mineral salts medium (Ritalahti et al., 2004). *Dhgm* lykanthroporepellens strain BL-DC-9 was kindly provided by William M. Moe and maintained as described (Moe et al., 2009; Yan et al., 2012).

Microcosms and enrichment cultures

Microcosms were established with materials from the sources listed in Table 6. Sediment and aquifer solids were collected in sterile glass or plastic containers from the Third Creek in Knoxville, TN (three locations), the Neckar River and an agricultural site near Stuttgart, Germany. The Third Creek site has a history of PCE, TCE, and 1,1,1-TCA contamination. The Neckar River site has no history of chlorinated solvent contamination but is located near urban and industrial

areas. Grape pomace was collected from a pile associated with a winemaking facility near Stuttgart, Germany. The samples from the municipality of Barra Mansa Brazil were from a contaminated mixed-waste site that contained up to 7,860 µg/L of CF and 125 µg/L of CT and 6.6 µg/L 1,1,1-TCA. Additional groundwater samples from a contaminated industrial site in Waynesboro (GA, USA) were collected. The Waynesboro site was a former facility that produced pesticides and herbicides and is primarily contaminated with 1,2-D up to 30,000 µg/L, 1,2-DCA, and alpha-, beta-, delta-, and gamma-hexachlorocyclohexane but other contaminants such as CF and CT were also present. Additional samples included aquifer material from a chlorinated solvent-contaminated site at Fort Pierce (FL, USA), which was contaminated with mainly 1,2-D (up to 24,000 µg/L), 1,2-DCA and VC; however, the solids provided for microcosm set up were collected outside of the plume. Bio-Sep® beads from Bio-Trap samplers (www.microbe.com/index.php/Bio-Trap-Samplers/bio-trap-samplers.html) that were deployed in monitoring wells inside the 1,2-D plume at the Ft. Pierce site were obtained for DNA extraction and analysis. All samples were delivered to the laboratory with overnight carrier and immediately processed or stored at 4°C for no more than 1 month. In addition, DNA was obtained from samples collected from an *in situ* uranium bioreduction pilot test plot in area 3 (wells FW104, FW103, FW100-2, and FW100-3) at the Integrated Field Research Challenge site at the Oak Ridge National Laboratory (Amos et al., 2007c; Wu et al., 2006).

Microcosms were prepared inside an anoxic chamber (N₂/H₂, 97/3%, vol/vol) using established procedures (He et al., 2002) with the following modifications: One g (wet weight) of solids were transferred to sterile 60-mL (nominal capacity) glass serum bottles containing 40 mL of defined, completely synthetic reduced mineral salts basal salts medium (Löffler et al., 1996) amended with lactate (5 mM) 30 mM bicarbonate (pH 7.2), 0.2 mM Na₂S x 9H₂O, resazurin (0.25 mg/L), vitamins (Wolin et al., 1963) and 0.2 mM 1,2-D. Microcosms established with groundwater were initiated with 20 mL of groundwater plus 20 mL of medium. All microcosms were prepared in duplicates and incubated statically, in the dark, at room temperature. After all of the 1,2-D was dechlorinated to propene, 3% inocula [vol/vol] were transferred to glass vessels containing fresh medium. After four consecutive transfers, all solids had been removed. Propene and 1,2-D concentrations were monitored by manually injecting 0.1 mL headspace samples into a HP 7890 gas chromatograph (GC) equipped with a DB-624 column (60 m length, 0.32 mm diameter; 1.8 µm film thickness) and a flame ionization detector (FID) as described (Amos et al., 2007a). To verify propene formation, additional GC measurements were made with an Agilent HP-PLOT/Q column (30 m length, 0.53 mm diameter; 40 µm of film thickness), which resolves propene from other C₁-C₃ alkanes and alkenes.

Table 6. Site materials used for microcosm setup to evaluate 1,2-D reductive dechlorination activity and analyzed for the presence of *Dhc* and *Dhgm* 16S rRNA gene and the *dcpA* gene.

Sample designation	Sample location	Sample type	Major reported contaminants	Date of collection	Dechlorination end products	<i>Dhc</i>	<i>Dhgm</i>	<i>dcpA</i>
Microcosms								
Third Creek, TRS1	Third Creek, Knoxville, TN, USA	Sediment	PCE, TCE, 1,1,1-TCA	Feb. 2011	Propene	+	+	+
Third Creek, TRS2	Third Creek, Knoxville, TN, USA	Sediment	PCE, TCE, 1,1,1-TCA	Feb. 2011	Propene	+	+	+
Third Creek, TRS3	Third Creek, Knoxville, TN, USA	Sediment	PCE, TCE, 1,1,1-TCA	March, 2011	Propene	+	+	+
Neckar River	Stuttgart, Germany	Sediment	None	May, 2011	Propene	+	+	+
Grape Pomace	Stuttgart, Germany	Solids ^a	None	May, 2011	Propene	+	+	+
001-ST-SO, 2.7-2.9 m	Barra Mansa, Brazil	Sediment	Chloroform, CCl ₄	Aug. 2010	- ^b	+	ND	+
002-ST-SO, 5.7-5.8 m	Barra Mansa, Brazil	Sediment	Chloroform, CCl ₄	Aug. 2010	- ^b	+	ND	+
Way-MW13D-12J811	Waynesboro, GA, USA	Groundwater	1,2-D and 1,2-DCA	Aug. 2010	-	ND	ND	ND
FP1-MW46, 22-26 m	Ft. Pierce, FL, USA	Sediment	None ^c	Aug. 2010	-	ND	ND	ND
FP2-MW49, 26-27 m	Ft. Pierce, FL, USA	Sediment	None ^c	Aug. 2010	-	+	+	ND
FP3-MW49, 46-47 m	Ft. Pierce, FL, USA	Sediment	None ^c	Aug. 2010	-	+	+	ND
FP4-MW47, 47-48 m	Ft. Pierce, FL, USA	Sediment	None ^c	Aug. 2010	-	+	ND	ND
FP5-MW49, 95-98 m	Ft. Pierce, FL, USA	Sediment	None ^c	Aug. 2010	-	ND	ND	ND
FP-MW33, 13-14 m	Ft. Pierce, FL, USA	Groundwater	18,000 µg/L of 1,2-D	June, 2012	Propene	+	+	+
FP-MW26, 14-15 m	Ft. Pierce, FL, USA	Groundwater	17,000 µg/L of 1,2-D	June, 2012	Propene	+	+	+
FP-MW20, 20-21m	Ft. Pierce, FL, USA	Groundwater	810 µg/L of 1,2-D	June, 2012	Propene	+	+	+
DNA samples								
FP-MW-2S, 6-7 m	Ft. Pierce, FL, USA	DNA/Biobead	14,000 µg/L of 1,2-D	July, 2011	No Propene ^d	+	ND	ND
FP-MW-20, 20-21 m	Ft. Pierce, FL, USA	DNA/Biobead	810 µg/L of 1,2-D	Feb. 2011	Propene ^d	ND	ND	+
FP-MW-26, 14-15 m	Ft. Pierce, FL, USA	DNA/Biobead	17,000 µg/L of 1,2-D	Mar. 2011	Propene ^d	+	+	+
FP-MW-61, 20-21 m	Ft. Pierce, FL, USA	DNA/Biobead	140 µg/L of 1,2-D	May, 2011	Propene ^d	ND	+	+
FW-024	IFC site, Oakridge, TN, USA	DNA/GW	Multiple	Feb. 2004	NT	+	ND	ND
FW-103	IFC site, Oakridge, TN, USA	DNA/GW	Multiple	Feb. 2004	NT	+	+	+
FW-100-2	IFC site, Oakridge, TN, USA	DNA/GW	Multiple	Aug. 2005	NT	+	ND	+
FW-100-3	IFC site, Oakridge, TN, USA	DNA/GW	Multiple	Feb. 2004	NT	+	ND	+

GW; groundwater NT; not tested ND; not detected

- No dechlorination in microcosm after 90 days incubation.

^a Solid residues (trester) from wine making consisting mostly of grape skins.

^b Small amounts of 1-CP or 2-CP were detected in live microcosm but also in negative controls.

^c Ft. Pierce is contaminated with 1,2-D (up to 24,000 µg/L) but the sediments tested here were for wells outside plume area.

^d Dechlorination not tested on microcosm, data provided reflect field-site conditions; there was no propene detected in FP1-MW-2S.

DNA Isolation

The DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) was used to extract DNA from sediment-free cultures, with modifications to improve cell lysis (Ritalahti et al., 2004). DNA from solid and groundwater samples used the MO BIO Power Soil DNA kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) and the PowerWater DNA Isolation Kit (MO BIO Laboratories Inc.), respectively, following the manufactures recommendations.

RNA isolation and preparation of cDNA libraries

Biomass was collected from 10-20 mL of RC and KS culture suspensions, when 50-75% of the initial 1,2-D dose had been converted to propene. Cells were harvested by vacuum filtration onto a Durapore hydrophilic polyvinylidene fluoride membrane (25 mm diameter and 0.22 µm pore size) (Millipore, Billerica, MA). RNA extraction, DNase treatment, cDNA synthesis and purification were performed as described (Ritalahti et al., 2010a) cDNA libraries were established with degenerate primers B1R and RR2F targeting *Dhc* RDase genes (Krajmalnik-Brown et al., 2004). Primer walking procedures extended the partial *dcpA* and *dcpB* genes.

Assembly of the *dcpAB* gene cassette by primer walking

Because the genomes of *Dhc* strains KS and RC were not available, the primer *dcp_up120F* was designed to obtain the 5' end of the *Dhc dcpA* gene. The *dcp_up120F* primer design was based on the available genome information for *Dhgm lykanthroporepellens* strain BL-DC-9 and targeted a region 120 bp upstream of the *dcpA* gene (Dehly_1524) start position. Combined with the primer *dcpA*-1449R, PCR products of ~1,569 bp were predicted. The PCR reactions consisted of 1X PCR buffer, 2.5 mM MgCl₂, 250 µM of each deoxynucleoside triphosphate (ABI, Foster City, CA), primers (250 nM each), and 2.5 U of AmpliTaq polymerase (ABI, Foster City, CA). The following thermocycler temperature program was used for the amplification of the *dcpA* gene: 94°C for 2 min, 10 seconds (1 cycle); 94°C for 30 seconds, 56.0°C for 45 seconds, and 72°C for 2 min, 10 seconds (30 cycles); and 72°C for 6 min. Reactions with genomic DNA from *Dhgm* strain BL-DC-9 as template served as positive controls. Amplicons were separated on 1% (wt/vol) agarose gels, stained with ethidium bromide (1 µg/mL), purified with the Qiagen PCR Purification kit (Qiagen, Valencia, CA, USA) and sequenced. Alignments of sequences using SeqMan II software (DNASTAR, Lasergene version 7) and ClustalW (www.ebi.ac.uk/Tools/msa/clustalw2/) were used to assemble the missing 5' end and the *dcpA* start site coding sequence. In addition, the DNA Walking *SpeedUp*TM kit (Seegene, Seoul, South Korea) was used to extend the partial *dcpAB* sequence and amplify the entire *dcpB* gene. The procedure involved a series of consecutive PCR amplifications with primers targeting known sequence regions in combination with the kit's DNA walking-annealing control primers. The internal primers *dcpA*-360F and *dcpA*-1257F were used with *Dhc* culture RC and culture KS genomic DNA samples following the manufacturer's recommendations. The resulting chromosome walking PCR products were purified, sequenced and assembled with the SeqMan II software.

Quantitative real-time PCR (qPCR)

Dhc and *Dhgm* 16S rRNA gene-targeted PCR assays followed established protocols (see Table 7 for primer information and references). The IDT DNA Primer Quest software (<http://scitools.idtdna.com/Primerquest/>) was used to design qPCR primers *dcpA*-1257F and *dcpA*-1449R with TaqMan probe *dcpA*-1426 to enumerate *dcpA* gene copies (Table 7). First, the primers were used with SYBR Green chemistry to recognize non-specific amplification and/or primer-dimer formation (Hatt et al., 2012). Standard curves were generated with ten-fold serial dilutions

(10^8 to 10^0 copies) of the partial *Dhc* strain KS *dcpAB* gene fragment cloned into the TOPO TA (Invitrogen) pCRII plasmid. To confirm target specificity, melting curves were obtained with genomic template DNA from *Dhc* strains RC and KS and *Dhgm* strain BL-DC-9. Additionally, the qPCR amplicons were resolved in 1% agarose gels run at 120 volts for 30 min to assess amplicon size target-specific amplification. Reactions with sterile water (no-template DNA) and with genomic DNA from *Dhc* strain GT, which does not possess the *dcpA* gene, served as negative controls. After validation and optimization in SYBR Green qPCR, the primers were used with the TaqMan probe *dcpA*-1426 in assays as described (Ritalahti et al., 2006). All assays exhibited amplification efficiencies between $100 \pm 10\%$; (i.e., slope within -3.6 and -3.1), consistency across replicate reactions, and linear standard curves ($R^2 > 0.980$) (ABI, 2005; Bustin et al., 2009). A quantification limit of 30 copies per reaction was determined based on fluorescence signals above the cycle threshold value of 0.2 within the first 38 PCR cycles measured in 20 replicate assays (ABI, 2005; Bustin et al., 2009). The lowest value of the standard curve (3 copies per reaction) was the detection limit, and all non-template control assays fell below this value. The qPCR assay conditions for the reactions targeting the *Dhc* 16S rRNA gene have been published (Ritalahti et al., 2006). All known *Dhc* genomes possess one 16S rRNA gene copy and the enumeration of this gene is used to determine the *Dhc* cell numbers (Ritalahti et al., 2006). The number of *dcpA* genes per *Dhc* genome was calculated by dividing the number of *dcpA* genes copies detected/mL of culture by the total number of *Dhc* 16S rRNA genes detected in the same culture volume. For transcriptional analysis, cDNA generated from active 1,2-D-dechlorinating cultures served as template. All qPCR data were corrected for the loss (i.e., % recovery) of luciferase transcripts, which were used as an internal standard (Johnson et al., 2005a) and *rpoB* transcripts were quantified as a measure of general metabolic activity as described (Fung et al., 2007). The *rpoB* housekeeping gene is conserved in *Dhc* genomes and *rpoB* transcripts were quantified as a measure of general metabolic activity and for normalization (Chow et al., 2010; Fung et al., 2007; Rahm et al., 2006). When applicable, *dcpA* transcript abundances were normalized to *rpoB* or to *dcpA* gene copy numbers. Starved cultures (i.e., cultures that had consumed all 1,2-D for at least 1 month) served as baseline controls for the transcriptional studies. All samples were analyzed in triplicate at two dilutions (1:10 and 1:100) using the ABI 7500 fast quantitative real-time PCR (qPCR) instrument (Applied Biosystems) and the reported values represent the average of at least three biological replicate cultures (i.e., six pseudo-replicates per sample, or 18 datasets for the three biological replicates).

Cloning *dcpA* sequences from environmental samples and phylogenetic analysis.

Primers *dcpA*-360F and *dcpA*-1449R were designed for standard PCR reactions to detect, clone and sequence *dcpA* genes from samples of interest (Table 7). These primers were designed based on alignments of the *dcpA* sequences retrieved from the cDNA libraries of *Dhc* strains RC and KS and the *dcpA* gene (Dehly_1524) of *Dhgm* strain BL-DC-9. *dcpA* clone libraries were established using DNA isolated from the original sediment and groundwater samples listed in Table 6.

Table 7. Primers and probes used in the discovery of a biomarker for reductive dechlorination of 1,2-D.

Primer or probe	Sequence (5'→3')	Target	Purpose	Reference
RRF2 ¹	SHMGBMGWGATTTYATGAARR	RDaseA RRDFMK motif	Amplification of RDase-like genes	(Krajmalnik-Brown et al., 2004)
B1R ¹	CHADHAGCCAYTCRTACCA	RDaseB WYEW motif	Amplification of RDase-like genes	(Krajmalnik-Brown et al., 2004)
<i>dcpA</i> -1257F ²	CGATGTGCCAGCCATTGTGTCTTT	<i>dcpA</i> gene	<i>dcpA</i> gene quantification and primer walking	(Padilla-Crespo et al., 2014)
<i>dcpA</i> -1449R ²	TTTAAACAGCGGGCAGGTACTGGT	<i>dcpA</i> gene	<i>dcpA</i> gene quantification, direct & nested PCR with <i>dcpA</i> -360F	(Padilla-Crespo et al., 2014)
<i>dcpA</i> -1426 ²	FAM-ACGTCATCTCAGATGAAGGCAGAGCT-BHQ ³	<i>dcpA</i> gene	<i>dcpA</i> gene quantification, direct and nested PCR	(Padilla-Crespo et al., 2014)
<i>dcpA</i> -360F ²	TTGCGTGATCAAATTGGAGCCTGG	<i>dcpA</i> qPCR	<i>dcpA</i> gene quantification, direct and nested PCR with primer <i>dcpA</i> -1449R, primer walking	(Padilla-Crespo et al., 2014)
<i>Dhc</i> -1200F	CTGGAGCTAATCCCCAAAGCT	<i>Dhc</i> 16S rRNA gene	<i>Dhc</i> 16S rRNA gene quantification	(Ritalahti et al., 2006)
<i>Dhc</i> -1271R	CAACTTCATGCAGGCGGG	<i>Dhc</i> 16S rRNA gene	<i>Dhc</i> 16S rRNA gene quantification	(Ritalahti et al., 2006)
<i>Dhc</i> -1240Probe	FAM-TCCTCAGTTCGGATTGCAGGCTGAA-TAMRA ³	<i>Dhc</i> 16S rRNA gene	<i>Dhc</i> 16S rRNA gene quantification	(Ritalahti et al., 2006)
LuciF	TACAACACCCCAACATCTTCGA	Luciferase reference mRNA	Quantitation of internal standard	(Johnson et al., 2005a)
LuciR	GGAAGTTCACCGGCGTCAT	Luciferase reference mRNA	Quantitation of internal standard	(Johnson et al., 2005a)
<i>Luci</i> -probe	JOE-CGGGCGTGGCAGGTCTTCCC-BHQ ³	Luciferase reference mRNA	Quantitation of internal standard	(Johnson et al., 2005a)
<i>rpoB</i> -1648F	ATTATCGCTCAGGCCAATACCCGT	<i>Dhc rpoB</i> gene	<i>Dhc rpoB</i> gene quantification	(Fung et al., 2007)
<i>rpoB</i> -1800R	TGCTCAAGGAAGGGTATGAGCGAA	<i>Dhc rpoB</i> gene	<i>Dhc rpoB</i> gene quantification	(Fung et al., 2007)
<i>Dhc</i> -730F	GCGGTTTTCTAGGTTGTC	<i>Dhc</i> 16S rRNA gene	<i>Dhc</i> detection by PCR	(He et al., 2003a)
<i>Dhc</i> -1350R	CACCTTGCTGATATGCGG	<i>Dhc</i> 16S rRNA gene	<i>Dhc</i> detection by PCR	(He et al., 2003a)

Primer or probe	Sequence (5'→3')	Target	Purpose	Reference
BL-DC-631F	GGTCATCTGATACTGTTGGACTTGAGTATG	<i>Dhgm</i> 16S rRNA gene	<i>Dhgm</i> detection by PCR	(Yan et al., 2009)
BL-DC-796R	ACCCAGTGTTTAGGGCGTGGACTACCAGG	<i>Dhgm</i> 16S rRNA gene	<i>Dhgm</i> detection by PCR	(Yan et al., 2009)
dcp_up120F ²	GCTCCTGGCAGAGCCGTCAGT	120 bp upstream of <i>dcpA</i>	Amplification and assembly of <i>dcpA</i> gene start	(Padilla-Crespo et al., 2014)

¹ Abbreviations for degenerate nucleotides positions are as follows: R =A or G; K = G or T; M =A or C; S =C or G; W=A or T; Y =C or T; B =C, G, or T; D = A, G, or T; V =A, C, or G; H =A, C, or T

² Primer names are given based on their target position related to the *dcpA* start coordinates in *Dhgm lykanthroporepellens* strain BL-DC-9 sequenced genome. BHQ, Black Hole Quencher; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

Environmental DNA samples were subjected to nested PCR and using an initial PCR amplification reaction with 2 μ L of undiluted or 1:10 diluted template DNA with the degenerate primers B1R and RRF2 as described (Krajmalnik-Brown et al., 2004). Subsequently, a second (nested) round of PCR using the *dcpA*-specific primers *dcpA*-360F and *dcpA*-1449R was performed using 2 μ L of the DNA solution obtained from the first round of amplification (see Figure 6 for approximate binding sites for primers RRF2 and B1R as well as the *dcpA*-specific primers).

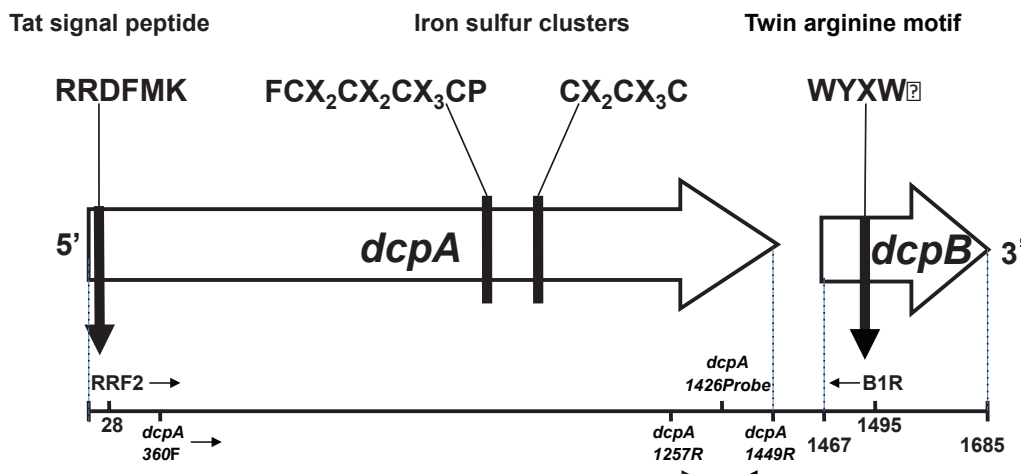


Figure 6. Arrangements of the *dcpA* gene and its corresponding *dcpB* genes in *Dhc* strains RC and KS. Approximate binding sites for the degenerate primers RRF2 and B1R as well as the *dcpA* specific primers designed in this study are indicated. Also shown are the characteristic dehalogenase features encoded by the *dcpA* gene which include the conserved Tat signal peptide RRXFFXK at the N-terminus and two iron sulfur clusters closer to the C-terminus in the form of FCXXCXXCXXXCP (or FCX2CX2CX3CP) and CXXCXXXC (or CX2CX3C). *dcpB* is located downstream of *dcpA* and encodes for a small highly hydrophobic protein with the conserved twin arginine motif in the form WYXW. The *dcpA* and *dcpB* genes (Dehly_1524 and Dehly_1523) in *Dhgm* strain BL-DC-9 also encode for a dehalogenase with these common features.

The expected amplicon size generated in nested PCR was 1,089 bp. The *dcpA*-specific PCR reactions consisted of (final concentrations) 1x PCR buffer, 2.5 mM of MgCl₂, 250 μ M of each deoxynucleoside triphosphate (ABI), 250 nM of each primer, and 2.5 U of AmpliTaq polymerase (ABI). The following temperature program was used to amplify the *dcpA* gene: 94°C for 2 min 10 s (1 cycle); 94°C for 30 s, 56.0°C for 45 s, and 72°C for 2 min 10 s (30 cycles); and 72°C for 6 min. The *dcpA* amplicons were cloned in the pCRII TOPO vector and transformed into *E. coli* TOP'10 competent cells (TOPO TA cloning kit, Invitrogen) following the manufacturer's recommendations. The QIAprep Spin Miniprep kit (Qiagen, Valencia, CA, USA) was used for plasmid isolation, the inserts were sequenced using primers M13F and M13R (http://tools.invitrogen.com/content/sfs/manuals/nupage_tech_man.pdf), and the DNA nucleotide sequences were translated (<http://web.expasy.org/translate/>) and aligned using ClustalW (Thompson et al., 1999) in MEGA version 5 (Tamura et al., 2011). Phylogenetic relationships were calculated from a total of 53 amino acid (aa) sequences using the neighbor joining tree method (Saitou et al., 1987) and evolutionary distances were computed using the number of differences method (Nei et al., 2000). Branch support values were estimated with a bootstrap test (1,000 replicates (Felsenstein, 1985)). The 53 translated nucleotide sequences used in the

phylogenetic tree comprised 48 partial *dcpA* sequences (~1 kb in length) obtained from environmental samples, the complete *dcpA* sequence of *Dhgm* strain BL-DC-9 RC, *Dhc* strain RC and strain KS, the translated nucleotide sequence of the pseudogene DET0162, and DET1538, encoding a putative RDase with unknown function (both identified on the genome of *Dhc* strain 195 (Seshadri et al., 2005). DET1538 served as the out-group for phylogenetic analyses.

Protein assays and blue native polyacrylamide gel electrophoresis (BN-PAGE)

Dhgm strain BL-DC-9 was used for in vitro enzyme assays, BN-PAGE and proteomics workflows because strain BL-DC-9 is a pure culture and its genome sequence is available. Strain BL-DC-9 harboring the *dcpA* gene was grown with 0.5 mM 1,2-D. Cells were harvested by centrifugation (10,000 x g for 20 min at 4°C) and lysed by bead beating (Tang et al., 2013b). The crude extracts were subjected to Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) to detect reductive dechlorination activity in gel slices following electrophoretic separation. Activity assays with individual gel slices were performed as described (Tang et al., 2013b). In the activity assays, the positive controls consisted of 1 mL of pelleted cell culture suspended in assay buffer while negative control vials received not protein.

2D-LC-MS/MS analysis

BN-PAGE enzyme assays were combined with proteomic workflows to identify RDase peptides present in the gel slices showing 1,2-D dechlorination activity. Coomassie-stained gel bands were excised from the SDS-PAGE gel and rinsed in HPLC-grade, degassed water (i.e., MilliQ water filtered through a 0.02-micron filter and bubbled with nitrogen gas for 30 min). In-gel digestion of proteins was performed as described (Shevchenko et al., 2006). Briefly, gel slices were cut into small pieces and destained for 30 min in 100 mM ammonium bicarbonate/acetonitrile (1:1, vol/vol) at room temperature along with intermittent vortexing. Gel-enmeshed proteins were further subjected to reduction, alkylation and overnight trypsin digestion at 37°C as described (Shevchenko et al., 2006), and peptides were obtained in 100 µL of extraction buffer (5% formic acid/acetonitrile, 1:2, vol/vol). The extracted peptide mix (50 µL) was pressure loaded onto an in-house packed biphasic MuDPIT (Multi Dimensional Protein Identification Technology) column packed with ~3 cm of strong cation exchange (SCX) resin (Phenomenex, Torrance, CA) and ~5 cm of reverse phase (RP) C18 resin (Phenomenex, Torrance, CA). The sample column was connected to a 15 cm RP packed front column (New Objective, Woburn, MA) and analyzed via 2D-LC-MS/MS using three salt pulses (30, 60 and 100% of 500 mM ammonium acetate) followed by a 120 min elution gradient of 100% Solvent A (95% H₂O, 5% acetonitrile, 0.1% formic acid to 60%) and Solvent B (30% H₂O, 70% acetonitrile, 0.1% formic acid). Peptide fragmentation data were collected using an LTQ or LTQ-Orbitrap (used in LTQ mode), operated in data-dependent mode and under the control of the Xcalibur software (Thermo Scientific). The LTQ-Orbitrap was set to 30K resolution while rest of precincts on either instrument was maintained as described (Brown et al., 2006; Chourey et al., 2010; Thompson et al., 2008). The MS/MS data obtained were searched against the *Dhgm* strain BL-DC-9 genome (NC_014314.1, downloaded from JGI, April 2012) using the MyriMatch algorithm (Tabb et al., 2007) along with the Bumbershoot software to translate spectra into peptide sequences (Holman et al., 2012). The MyriMatch searches used the following settings: fully tryptic peptides with a minimum length of 5 amino acids, mono precursor m/z tolerance set to 10 ppm, fragment m/z mass tolerance set to 50 ppm, number of missed cleavages 2 and a fixed modification of +57 Da on cysteine residues. The resultant datasets were sorted using IDPicker (v 3.0.463) set to following parameters: only fully tryptic peptides with 2 distinct peptides per protein with a false discovery rate (FDR) set to 0. Cellular localization

predictions for identified proteins were derived using subcellular localization tool PSORT v.3.0 (11). All chemicals for proteomic analysis were obtained from Sigma Chemical Co. (St. Louis, MO), trypsin was acquired from Promega (Madison, WI), formic acid (99%) was obtained from EM Science (Darmstadt, Germany), and HPLC-grade water and acetonitrile were purchased from Burdick and Jackson (Muskegon, MI).

Computational analyses

The DcpA sequences were analyzed for secretory signal peptides using the TatP (www.cbs.dtu.dk/services/TatP) and SignalP (<http://www.cbs.dtu.dk/services/SignalP/>) programs. The Compute pI/Mw program (http://us.expasy.org/tools/pi_tool.html) was used to predict the molecular weight and isoelectric point of DcpA. The DcpB sequences were analyzed with TMMOD (<http://www.liao.cis.udel.edu/website/servers/TMMOD/>) to predict protein topology based on transmembrane motifs. The presence of corrinoid and ribosome binding sites, and a putative Dehalobox (a stretch of nucleotides that resemble the FNR-box that bind to the promoter for transcription initiation) (Gábor et al., 2006; Smidt et al., 2000) were identified by visually inspecting the nucleotide sequences and by manually aligning the regions with known motifs.

Task 5: Effect of perturbations

In part, the outcomes of Task 6 guided the efforts under Task 5. Specifically, the observation that the presence of nitrate/nitrite is a key determinant for ethene formation at sites impacted with chlorinated ethenes triggered more detailed investigations. Nitrous oxide (N₂O) forms when nitrogenous compounds (e.g., NH₄⁺, NO₃⁻) are directly transformed by microorganisms or indirectly converted via coupled biotic-abiotic processes (Onley et al., 2018). Experimental efforts revealed that N₂O is an environmentally relevant compound inhibiting organohalide respiration. This finding has potential implications when biostimulation with reactive nitrogen (i.e., NH₄⁺) is considered at sites undergoing enhanced anaerobic bioremediation. Since organohalide-respiring Dehalococcoidia are corrinoid auxotrophs and strictly depend on the microbial community to produce cobamides, perturbation effects on the community can affect reductive dechlorination activity. Therefore, the experimental efforts under Task 5 explored the impact of cobamides on reductive dechlorination of cDCE and VC in *Dhc* strains expressing the VC RDases BvcA or VcrA. In addition, this research explored the effects of CFC-113 on *Dhc* reductive dechlorination activity because chlorofluorocarbons are common co-contaminants at sites impacted with chlorinated ethenes.

Inhibition of reductive dechlorination by N₂O

Bacterial strains and growth conditions

The impact of N₂O on reductive dechlorination was investigated using two organohalide-respiring isolates. *Geo* strain SZ, a corrinoid-prototroph capable of dechlorinating PCE and TCE to cDCE (He et al., 2003b; Sung et al., 2006a), and *Dhc* strain BAV1, a corrinoid-auxotroph capable of dechlorinating cDCE and VC to non-toxic ethene (He et al., 2003b; He et al., 2005; Löffler et al., 2013b). Both cultures were grown in 160 mL serum bottles containing a CO₂/N₂ (20/80, vol/vol) headspace and 100 mL of synthetic, bicarbonate-buffered (30 mM, pH 7.3), and reduced (0.2 mM Na₂S and 0.2 mM L-cysteine) mineral salt medium as described (Yan et al., 2018; Yan et al., 2016). Strain SZ cultures received 5 mM acetate as electron donor and 0.28 mM PCE (38 μmol/bottle), 10 mM fumarate, or both PCE and fumarate as electron acceptors, respectively, and a vitamin B₁₂-free Wolin vitamin solution (Wolin et al., 1963). *Dhc* strain BAV1 culture vessels

received 10 mL of hydrogen, 0.8 mM (90 $\mu\text{mol/bottle}$) *c*DCE or 0.25 mM (40 $\mu\text{mol/bottle}$) VC, and 5 mM acetate as electron donor, electron acceptor, and carbon source, respectively. *Dhc* strain BAV1 culture vessels received the Wolin vitamin stock solution containing vitamin B₁₂ to achieve a final concentration of 25 $\mu\text{g L}^{-1}$. For inoculation, culture vessels received 3% (vol/vol) inocula from actively dechlorinating cultures maintained under the same conditions. All experiments used triplicate culture vessels, and culture bottles without N₂O and without inoculum served as positive and negative controls, respectively. Culture vessels were incubated without agitation at 30°C in the dark with the stoppers facing up.

Inhibition experiments

For *Geo* strain SZ cultures, undiluted N₂O gas was directly added to incubation vessels using plastic syringes (BD, Franklin Lakes, NJ, USA) to achieve final aqueous N₂O concentrations of 9.5, 19.1 and 57.3 μM in PCE-amended cultures, of 191.4 μM and of 10 mM in fumarate-amended cultures, and of 191.4 μM in cultures grown with both PCE and fumarate. For *Dhc* strain BAV1 cultures, undiluted or 10-fold diluted (with N₂) N₂O gas was added with plastic syringes to achieve final aqueous phase N₂O concentrations of 9.5, 19.1 and 57.3 μM in *c*DCE-amended vessels, and of 2.9, 5.7 and 19.1 μM in VC-amended vessels. The aqueous phase concentrations (in μM) of N₂O in all medium bottles were calculated from the headspace concentrations using a dimensionless Henry's constant of 1.94 for N₂O at 30°C (Sander, 2015) according to $C_{\text{aq}} = \frac{C_{\text{g}}}{H_{\text{cc}}}$, where C_{aq} , C_{g} , and H_{cc} are the aqueous concentration (in μM), the headspace concentration (in $\mu\text{mol L}^{-1}$) and the dimensionless Henry's constant, respectively. Average dechlorination rates were calculated based on the continuous accumulation of dechlorination products (i.e., before stable product concentrations were observed). Since each reductive dechlorination step is associated with the release of one chloride ion (Cl⁻), the average dechlorination rates determined in growth experiments are reported as the total amount of Cl⁻ released per volume per unit time (i.e., $\mu\text{mol Cl}^{-} \text{L}^{-1} \text{d}^{-1}$).

Whole cell suspension dechlorination assays

Biomass for whole cell suspension assays was harvested by centrifugation at 10,000 x *g* at 4°C for 30 minutes from 1.6 L *Geo* strain SZ and *Dhc* strain BAV1 cultures that had dechlorinated three feedings of PCE and *c*DCE, respectively. Inside an anoxic chamber (Coy Laboratory Products Inc., MI, USA), the supernatants were decanted, and the pellets were suspended in 8 – 10 mL reduced mineral salts medium. For protein quantification, triplicate 0.2 mL (strain SZ) and 1.0 mL (strain BAV1) concentrated cell suspensions were transferred to 2-mL screw cap tubes containing 20 mg of 0.1 mm diameter glass beads, and cells were broken at room temperature using a Bead Ruptor (OMNI, GA, USA) at speed of 6.0 m s⁻¹ for three 10-min cycles with 2-min breaks. After centrifugation at 13,000 x *g* for 2 minutes to remove cell debris, protein content in the supernatants was estimated using the Bradford assay (Bradford, 1976) on a plate reader (BioTek Instruments, VT, USA). To ensure consistency between replicate experiments, cell suspensions of both isolates were freshly prepared following identical procedures.

Dechlorination assays for rate determinations were performed in 8-mL glass vials sealed with Teflon-lined butyl rubber septa held in place with aluminum crimps. Each vial contained 3.80 – 4.86 mL of reduced basal salts medium with 5 mM acetate and a N₂/CO₂ (80/20, vol/vol) headspace for *Geo* strain SZ and a H₂/CO₂ (80/20, vol/vol) headspace for *Dhc* strain BAV1. Electron donor was provided in at least 40-fold excess of the theoretical demand to ensure that reductive dechlorination was not electron donor limited. For *Geo* strain SZ cell suspensions, 36 – 1,100 μL of an aqueous 1 mM PCE stock solution were added directly to the assay vials to achieve

final aqueous PCE concentrations ranging from 5–150 μM . For *Dhc* strain BAV1 cell suspensions, 2 – 550 μL *c*DCE stock (from a 5.0 mM aqueous stock solution) or 12–630 μL VC stock (from a 2.0 mM aqueous stock solution) were added to achieve final aqueous *c*DCE and VC concentrations ranging from 1–500 μM and 3–150 μM , respectively. The total aqueous volume in all vials was 4.9 mL before introducing 0.1 mL of cell suspension. Replicate vials at each initial PCE and *c*DCE concentration received N_2O to achieve 0, 10, and 60 μM , and VC-amended vials received N_2O to achieve 0, 15, and 50 μM dissolved phase N_2O concentrations. N_2O concentrations were quantified in all assay vials prior to and at the end of the incubation period and determined constant N_2O concentrations. Following equilibration, each assay vial received 0.1 mL of the respective cell suspension (corresponding to 56.3 ± 2.2 μg protein per vial for *Geo* strain SZ and 19.7 ± 1.4 μg protein per vial for *Dhc* strain BAV1) using plastic syringes to start dechlorination activity. Vials that received 0.1 mL sterile mineral salt medium instead of cell suspension, and vials that received 0.1 mL heat-killed cell suspension served as negative controls. During the 6-hour assay incubation, 1.0 mL liquid samples were collected from sacrificial assay vials every 60 minutes and transferred to 20-mL glass auto-sampler vials containing 0.1 mL of 25 mM H_2SO_4 to terminate dechlorination activity. The cell titers and substrate concentrations were chosen such that the dechlorination rates could be determined within the 6-hour incubation period and no more than 80% of the initial chlorinated ethene concentration had been consumed at the end of the incubation period.

Analytical procedures

Chlorinated ethenes and ethene were analyzed with an Agilent G1888 headspace autosampler connected to an Agilent 7890 gas chromatograph (GC) equipped with a flame ionization detector (method detection limit ~ 0.2 μM) and a DB-624 capillary column (60 m length $\times$ 0.32 mm diameter, 1.8 μm film thickness) (Yan et al., 2016). Fumarate and succinate were quantified using an Agilent 1200 series high-performance liquid chromatography system equipped with an Aminex HPX-87H column and a dual-wavelength absorbance detector set to 210 nm (Onley et al., 2018). N_2O was analyzed by injecting 100 μL headspace samples into an Agilent 7890A GC equipped with an HP-PLOT Q column (30 m length $\times$ 0.320 mm diameter, 20 μm film thickness) and a micro-electron capture detector. The OD measurements were conducted with a PerkinElmer Lambda 35 UV-Vis spectrophotometer by transferring 1-mL cell suspension into a cuvette and recording readings at 600 nm.

Dechlorination kinetics and inhibition models

The shortest doubling times reported for *Geo* strain SZ and *Dhc* strain BAV1 are 6 hours (Sung et al., 2006a) and 2.2 days (He et al., 2003b; Löffler et al., 2013b; Sung et al., 2006a), respectively. Therefore, additional cell growth was considered negligible over the assay period ($< 6\text{h}$) and confirmed by constant OD_{600} values. For this reason, the Michaelis-Menten model, rather than the Monod model for systems involving cell growth, was used to analyze the cell suspension dechlorination data. Therefore, the half-velocity constant K_m , rather than the Monod half-saturation constant K_s , was applied in the analyses of cell suspension kinetic parameters. For each treatment at a different initial substrate concentration $[S]$, an initial dechlorination rate v , normalized to the amount of protein per vial, in units of $\text{nmoles Cl}^- \text{ released min}^{-1} \text{ mg protein}^{-1}$, was calculated from the sum of all dechlorination products measured with the GC. In brief, the amended PCE, *c*DCE or VC concentrations in the respective assay vials served as the initial substrate concentrations, and the corresponding dechlorination rates were determined from the slope of progression curves representing total Cl^- released. The linear regression analysis included five data points and at least three for the assays with a low initial chlorinated ethene concentration.

Thus, each datum point in the Michaelis-Menten plots represents a dechlorination rate extracted from one initial substrate concentration.

The maximum dechlorination rate $V_{\max}$ and the half-velocity constant K_m for each treatment were calculated using the Michaelis-Menten nonlinear regression method in the Enzyme Kinetics Module for SigmaPlot 13 (Systat Software Inc., Chicago, IL, USA). This software module evaluated competitive, noncompetitive, and uncompetitive inhibition models for best fit to the rate data based on the highest coefficient of determination (R^2), the lowest corrected Akaike's Information Criterion (AICc) values, and the lowest standard deviation of the residuals (Sy.x.). The best-fit model (i.e., noncompetitive inhibition) was used to determine the inhibitory constant, K_i , for N_2O on reductive dechlorination. For data visualization, Michaelis-Menten (V over $[S]$), Lineweaver-Burk ($1/V$ over $1/[S]$), and Dixon ($1/V$ over $[I]$) plots were generated using the SigmaPlot Enzyme Kinetics Module for each inhibition model and the different electron acceptors (i.e., PCE, cDCE and VC).

Impact of fixed nitrogen (NH_4^+) availability on *Dhc* reductive dechlorination activity

Cultures and growth conditions

Groundwater samples were collected in January 2014 from well PW4 located at a contaminated site in Australia and shipped on ice for immediate processing upon arrival (Baldwin et al., 2017). PW4 enrichment cultures were established in 160-mL serum bottles by combining 50 mL of site groundwater with 50 mL of defined mineral salts medium (He et al., 2005) with the following modifications: dithiothreitol (DTT) (0.5 mM) replaced L-cysteine as reducing agent to eliminate a possible N source. After complete dechlorination of cDCE to ethene, transfers (2%, v/v) were carried out in larger vessels (2-L glass bottles containing 1.7-L of medium) to accommodate larger sample requirements for proteomic analysis. Third Creek (TC) enrichment cultures were derived from TC sediment impacted with chlorinated solvent following established procedures (Şimşir et al., 2017). Neat cDCE (approximately 0.3 mM aqueous phase concentrations) as electron acceptor and lactate (5 mM), a fermentable substrate yielding hydrogen as electron donor for *Dhc*, were added to TC and PW4 incubation vessels. To assess the effect of NH_4^+ , cultures were established with NH_4^+ (5.6 mM or 0.08 g/L) amendment or without NH_4^+ (Table 8). The headspace consisted of N_2/CO_2 (80/20, v/v), except for negative controls of N_2 fixation activity, where argon replaced N_2 and NH_4^+ was omitted from the medium (Table 8).

Table 8. Overview of growth conditions to evaluate the effects of fixed nitrogen (i.e., NH_4^+) limitations.

Inoculum	NH ₄ ⁺ (5.6 mM)	Headspace	Reductant	e ⁻ Donor/ Carbon Source	e ⁻ Acceptor
PW4 culture	+	N ₂ /CO ₂	DTT	Lactate (500 μmol/bottle)	cDCE (0.3 mM)
	-				
	-	Argon/CO ₂			
<i>Dhc</i> strain BAV1	+	N ₂ /CO ₂	DTT	Hydrogen (200 μmol/bottle) / Acetate (500 μmol/bottle)	
	-				
<i>Dhc</i> strain GT	+				
	-				
Third Creek (TC) culture	+	N ₂ /CO ₂	DTT	Lactate (500 μmol/bottle)	
	-				
	-	Argon/CO ₂			

Dhc strains BAV1 and GT were grown in 160-mL serum bottles containing 100 mL of defined medium (Yan et al., 2013), with or without NH_4^+ (5.6 mM), neat cDCE (0.3 mM) as electron acceptor, hydrogen (207 μmol) as electron donor, and acetate (5 mM) as carbon source. All serum bottles were closed with butyl rubber stoppers (Geo-Microbial Technologies, Inc., Ochelata, OK) and incubated statically in the dark at 21°C. The calculation of reductive dechlorination rates assumed that one chloride ion (Cl^-) is released per reductive dechlorination step and are reported as total μmol of Cl^- released per liter per day (i.e., $\mu\text{M Cl}^- \text{d}^{-1}$).

DNA extraction

Biomass from PW4 and TC cultures was collected from 9 mL of culture suspension via vacuum filtration onto Durapore membranes (47 mm diameter, 0.22 μm pore size, Millipore, Billerica, MA). Biomass from 100 mL of well PW4 groundwater was collected with 0.22 μm Millipore Sterivex-GP cartridges as described (Ritalahti et al., 2010b). DNA was extracted from collected biomass or 0.5 g of TC sediment using the MoBio power soil DNA isolation kit (MO BIO, Carlsbad, CA) according to the manufacturer's recommendations. DNA was quantified using the Qubit dsDNA BR Assay (Life Technologies, Carlsbad, CA) and stored at -80°C.

RNA extraction, purification and reverse transcription

For RNA extraction, biomass from 15 mL of TC or PW4 cultures was collected via vacuum filtration onto 0.22 μm Durapore membrane filters and placed into 2 mL vials with 0.25 mL RNAlater (Qiagen, Germantown, MD). Total RNA was extracted from frozen filters using the RNeasy Mini Kit (Qiagen, Valencia, CA), quantified using the Qubit RNA BR Assay (Life Technologies), and processed as described (Yoon et al., 2015). DNA was removed using the Qiagen RNase-free DNase Set before cDNA was obtained with the Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) (Yoon et al., 2015). To estimate RNA loss, 2 μL (2×10^4 copies) of luciferase control mRNA (Promega, Madison, WI) was added as an internal standard after the cell lysis step (Yoon et al., 2015). The mRNA recovery ranged from 5 to 35% between samples.

qPCR

Established TaqMan qPCR assays were used to enumerate total bacterial 16S rRNA, *Dhc* 16S rRNA, *tceA*, *bvcA*, *vcrA* genes and transcripts (Ritalahti et al., 2006), as well as luciferase transcripts (Johnson et al., 2005a). New primer/probe combinations were designed to amplify Cornell-type *Dhc* 16S rRNA and *Dhc* nitrogenase genes and transcripts (Table 9). The Primer3 software (Rozen et al., 1999) (Applied Biosystems, Foster City, CA) was used to design primer/probe sets for the specific amplification of the *Dhc* nitrogenase genes *nifD*, *nifK*, and *nifH* (Table 9). Primer/probe sets *nifH-195C* and *nifH-PV* (Table 9) were designed to target the *nifH* gene of the N_2 -fixing Cornell-type *Dhc* and the non- N_2 -fixing Pinellas- and Victoria-type *Dhc*, respectively. A primer/probe set targeting the *Dhc* Cornell 16S rRNA gene was designed to distinguish N_2 -fixing *Dhc* (i.e., Cornell group) from the non- N_2 -fixing *Dhc* (Pinellas and Victoria groups). The specificity of the primers and probes was manually evaluated using BLAST analysis and confirmed experimentally.

qPCR assays were conducted using an ABI ViiA7 real-time PCR system equipped with ViiA7 Software (Life Technologies) (Ritalahti et al., 2006). Briefly, every 20 μL -reaction contained 10 μL of 2-fold concentrated TaqMan Universal PCR Master Mix (Applied Biosystems), 2 μL of DNA template, forward and reverse primers and the probe at final concentrations of 300 nM each, as well as UltraPure nuclease-free water (Invitrogen, Carlsbad, CA, USA). The qPCR cycle

conditions were as follows: 50 °C for 2 min and a hold at 95 °C for 10 min, followed by 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. The standard curves were generated using three independent, triplicate 10-fold serial dilution series of plasmid DNA carrying PCR amplicons of the respective target genes. Plasmids with target *Dhc* gene fragments including *nifD* (308 bp), *nifK* (467 bp), and *nifH-195C* (681 bp) from *Dhc* strain 195, and *nifH-PV* (469 bp) from *Dhc* strain GT were constructed by inserting PCR amplicons of the respective target genes into the PCR2.1 vector using the TOPO TA cloning kit following the manufacturer's protocol (Invitrogen, Carlsbad, CA). All qPCR assays met the MIQE requirements (Bustin et al., 2009) for assay design and evaluating qPCR experiments. Slope, y-intercept, R^2 and efficiency of qPCR assay standard curves were approximately -3.71, 40.1, 0.99, and 88-101%, respectively. The detection limit was 11.4 gene copies per reaction, and the linear range of the standard curves spanned 1.14E+02 to 1.14E+08 gene copies per reaction. The known *Dhc* strains harbor a single 16S rRNA gene and single copy *nif* operons and the gene copy numbers and the *Dhc* cell numbers are reported interchangeably. Transcript copy numbers are reported on a per gene basis as transcript-to-gene ratio (TGR) values.

Table 9. Primers and probes for qPCR and RT-qPCR analyses to assess N₂ fixation in *Dhc*.

Primer/probe sets *nifH*-195C and *nifH*-PV were designed to target the *nifH* gene of the N₂-fixing Cornell-type *Dhc* and the non-N₂-fixing Pinellas- and Victoria-type *Dhc*, respectively. Primer/probe sets targeting the *Dhc* Cornell 16S rRNA gene were designed to distinguish N₂-fixing *Dhc* (i.e., Cornell group) from the non-N₂-fixing *Dhc* of the Pinellas and Victoria groups.

Forward primer (5'→3')	Reverse Primer (5'→3')	TaqMan-MGB probe (5'→3')	Target gene (organism)
<i>Dhc</i> Cornell 16S-qF: AACTGAAGGTAATACCGCATGTGAT	<i>Dhc</i> Cornell 16S-qR: ATCCTCTCAGACCAGCTACCGA	<i>Dhc</i> Cornell 16S-qP: TAAGTCGGTTCATTAAAGC	16S rRNA (Cornell-type <i>Dhc</i>)
<i>nifD</i> -195-650qF: ATAACCCGTTTCGGTCTGTTC	<i>nifD</i> -195-769qR: TACGGCATTCCCTGGATTAAG	<i>nifD</i> -195-687P: CGAAATAACGGGCAATCTTGCGCA	<i>nifD</i> (Cornell-type <i>Dhc</i>)
<i>nifK</i> -195-37qF: ATCATTTCCAGCAAACGCATT	<i>nifK</i> -195-119qR: GGCTTCCCCATTCTTGACC	<i>nifK</i> -195-64qP: CGATAGCCCACAACC	<i>nifK</i> (Cornell-type <i>Dhc</i>)
<i>nifH</i> -195C-69qF: GCCCCGTATTTCGTCAGCC	<i>nifH</i> -195C-147qR: ATGTCCAGCGGGCTGAAAT	<i>nifH</i> -195C-96qP: TGCGGATTCCATTCTG	<i>nifH</i> (Cornell-type <i>Dhc</i>)
<i>nifH</i> -PV-362qF: AGCATGCCAGATTCATTACCG	<i>nifH</i> -PV-466qR: GGCTGCAACTGAACATAGGTCC	<i>nifH</i> -PV-400qP: TCCAAGACTATGGGTGTGAC	<i>nifH</i> (Pinellas- and Victoria-type <i>Dhc</i>)

Primer/probe design used the Primer3 software (Applied Biosystems, Foster City, CA) (Rozen et al., 1999). Abbreviations: q: qPCR, F: forward primer, R: reverse primer, P: probe

16S rRNA gene amplicon sequencing and data analysis

16S rRNA gene fragments were amplified from purified DNA with primer set 515F/806R targeting the V4 region of both bacterial and archaeal 16S rRNA genes (Caporaso et al., 2011). A DNA library was prepared according to established procedures and sequenced on an Illumina MiSeq platform (Caporaso et al., 2011). Raw sequencing reads were jointly paired, demultiplexed, and trimmed to a length of 250 bp using the QIIME v.1.9.1 software package (Caporaso et al., 2010). Chimeric sequences were removed using ChimeraSlayer with QIIME default parameters and the resulting sequences were aligned with the PyNAST algorithm. Grouping of sequences at the 97% sequence identity level and quality control processing was carried out using the UPARSE pipeline (Edgar, 2013). Taxonomy assignment was performed using the RDP classifier (Wang et al., 2007) trained against the updated Greengenes database (version May, 2013) (Werner et al., 2012) using QIIME (Caporaso et al., 2010). A total of 19,0930, 116,355, and 62,802 bacterial and archaeal sequences that met the quality standards were obtained from groundwater well PW4 samples, PW4 enrichment cultures amended with NH_4^+ (at the end of the incubation period, day 29), and PW4 enrichment cultures without NH_4^+ (day 143), respectively. The Third Creek (TC) samples yielded a total of 255,175, 199,249, and 152,250 16S rRNA gene amplicon sequences for TC sediment samples, TC enrichment cultures amended with NH_4^+ (day 88), and TC enrichment cultures without NH_4^+ (day 160), respectively.

Analytical procedures

Chlorinated ethenes, ethene and methane were analyzed by injecting 100- μL headspace samples into an Agilent 7890A gas chromatograph equipped with a flame ionization detector and a DB-624 capillary column (60 m length x 0.32 mm I.D., 18 μm film thickness). Standard curves were prepared as described (He et al., 2002).

Proteomics analysis and protein identification in PW4 groundwater and enrichment cultures

Biomass from 50 mL of PW4 enrichment cultures (+/- NH_4^+) and from 1,000 mL of well PW4 groundwater was collected on duplicate 0.22- μm sterile Sterivex-GP cartridges and 2 mL RNAlater was added as a preservative. Sterivex cartridges of field samples were shipped on ice via express carrier and immediately stored at -80°C . Total proteins were extracted from the biomass using a detergent-based extraction protocol and subjected to nanoLC-MS/MS analysis following established procedures (Chourey et al., 2013; Kleindienst et al., 2019). Briefly, membrane filters from Sterivex cartridges were removed from the housing, cut into small pieces, suspended in SDS-lysis buffer, and processed as described (Kleindienst et al., 2019). For nano liquid chromatography-tandem mass spectrometry (nano-LC-MS) (Ultimate 3000 HPLC system, Dionex, USA) analysis, the peptides were loaded onto an in-house prepared biphasic resin packed column (SCX and C18, Phenomenex, Torrance, CA) (Kleindienst et al., 2019) and subjected to an offline wash for 15 minutes. Peptides were eluted and subjected to chromatographic separation and measurements via a 24-hour Multi-Dimensional Protein Identification Technology approach (Brown et al., 2006). Measurements were carried out using a linear trap quadrupole mass spectrometer (Thermo Fisher Scientific, Germany) coupled to a nano-LC system and operated in data dependent mode via Thermo Xcalibur software V2.1.0 (Brown et al., 2006). For protein identification, the raw spectra were searched against an assembled database of genomes representing aquifer microbial communities (Chourey et al., 2013). Common contaminant peptide sequences from trypsin and keratin were concatenated and included in the database. Reverse

database sequences were also included as decoy sequences to calculate a false discovery rate. Database matching was done via Myrimatch v2.1 algorithm (Tabb et al., 2007) set to parameters as described (Xiong et al., 2015). For data analysis, spectral counts of identified peptides were normalized to obtain the normalized spectral abundance factor (NSAF) and used to compare protein expression across samples and time points (Paoletti et al., 2006). The NSAF values were multiplied by a random, constant number (100,000) for improved visualization.

Modulation of organohalide respiration by cobamides

Dehalococcoides mccartyi (Dhc) cultures

Dhc strain BAV1 (ATCC BAA-2100) and strain GT (ATCC BAA-2099) were grown in 160 mL serum bottles containing 100 mL defined, completely synthetic mineral salts medium amended with 5 mM acetate as carbon source, 10 mL hydrogen as electron donor, 5 μ L neat *c*DCE (0.60 mM aqueous phase concentration) or 2 mL VC (0.52 mM aqueous phase concentration) as electron acceptor as described (Yan et al., 2013). Following equilibration, triplicate bottles were amended with the Wolin vitamin mix (Wolin et al., 1963) not containing cyanocobalamin (vitamin B₁₂). The vessels received 36.9 nM of a purified cobamide. For inoculation, *Dhc* strain BAV1 or strain GT cell suspensions were centrifuged and the pellets suspended in cyanocobalamin-free mineral salts medium to avoid cyanocobalamin carryover (Yan et al., 2012). Cultures that failed to dechlorinate *c*DCE or VC received 10 μ M DMB from a filter-sterilized 3 mM aqueous stock solution to restore dechlorination activity. All culture vessels were incubated statically in dark at 30°C. The *c*DCE-to-ethene dechlorination rates were calculated based on VC and ethene quantification, each step associated with the release of one chloride ion and reported as μ moles of Cl⁻ released liter⁻¹ day⁻¹.

Cobamide biosynthesis, extraction and purification

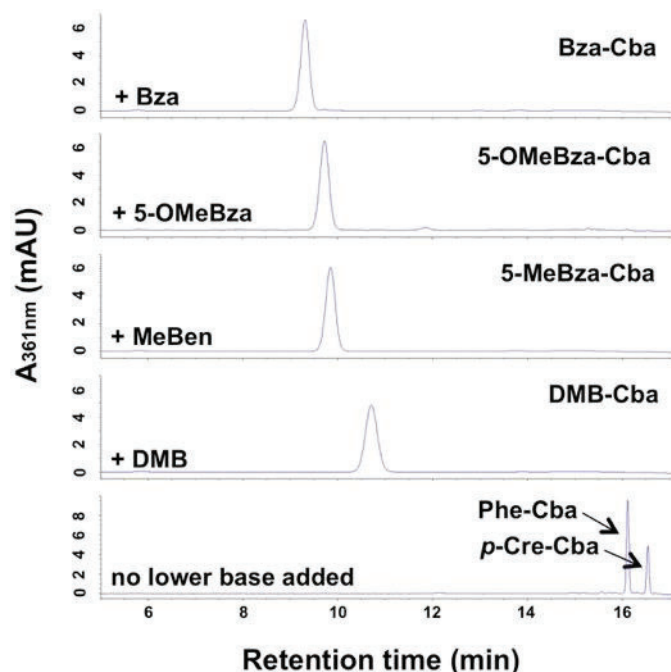
DMB-, 5-MeBza-, 5-OMeBza- and Bza-Cba were obtained via guided cobamide biosynthesis using *Sporomusa* sp. strain KB-1 (GenBank accession no. AY780559.1) cultures supplied with a single lower base compound (200 μ M). To achieve higher yields, 800 mL cultures of strain KB-1 were grown in 1.2 liter glass vessels using a modified medium (Yan et al., 2013) amended with yeast extract (2 g/L), casitone (2 g/L), betaine (50 mM) and a cyanocobalamin-free Wolin vitamin mix (Wolin et al., 1963). After 72-96 hours of static incubation at 30°C in the dark, cells were harvested from 1.6-2.4 L culture suspensions by centrifugation at 17,600 x g for 15 min at room temperature. Cell pellets were suspended in 15 mL deionized water, briefly incubated in a sonication water bath to achieve homogeneous suspensions, and 5-mL aliquots were transferred to sterile 50 mL plastic tubes. Total corrinoids were extracted in the cyano form (e.g., a cyanide group as the upper β -ligand) and purified using the potassium cyanide extraction method (Yan et al., 2013). Cobamide-containing fractions were separated by HPLC and manually collected from the detector outlet according to retention time and diode array detector response to remove any remaining traces of native phenolic cobamides (e.g., Phe-Cba and *p*-Cre-Cba). Cobamides were purified as described above. Cobamide absorbance was measured with a Lambda 35 UV/VIS spectrometer (PerkinElmer, Waltham, MA, USA) at 361 nm, and cobamide concentrations were estimated using a molar extinction coefficient of 28,060 mol⁻¹ cm⁻¹ (Pratt, 1972). The purified cyano form of DMB-Cba was indistinguishable from commercial cyanocobalamin. All experiments used purified DMB-Cba in control incubations to verify the absence of any inhibitory compounds introduced during the extraction process.

HPLC and LC-MS analysis

Cobamide purity and authenticity (e.g., distinct singular peaks and *m/z* values) were analyzed in a

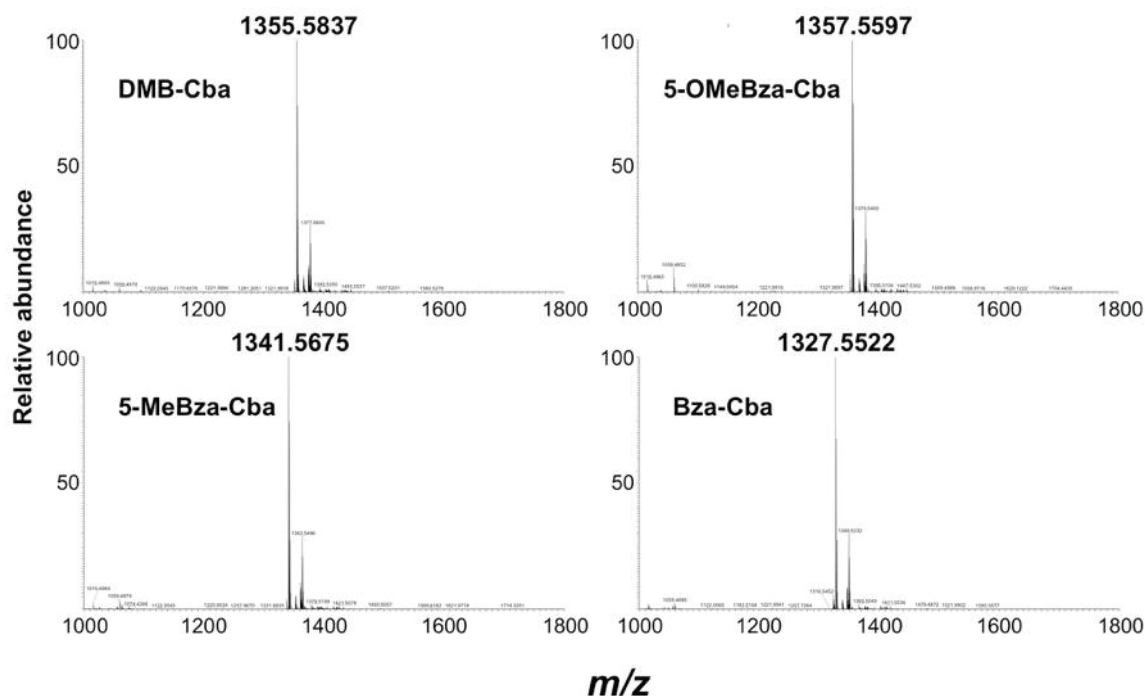
combined approach employing an Agilent 1200 HPLC system and a Thermo Fisher Orbitrap Exactive Plus LC/MS system (Figure 7 and Figure 8). For HPLC analysis, 20 μ L samples were injected onto an Eclipse XDB-C18 column (5 μ m, 4.6 x 150 mm) and separated with a flow rate of 1 mL per min at 30°C using 0.1% (v/v) formic acid ($\geq 88\%$, w/v) in water (eluent A) and 0.1% (v/v) formic acid in methanol (eluent B) as mobile phases. The column was equilibrated with 82% eluent A / 18% eluent B, and a linear change to 75% A / 25% B was applied following sample injection over a 12-min time period. Then, the eluent composition decreased immediately to 25% A / 75% B over 3 min followed by a 5-min hold, before the column was equilibrated to initial conditions. Cobamides were detected at 361 nm with an Agilent 1260 Infinity diode array detector and quantified by comparing integrated peak areas to 4-point calibration curves generated with purified cobamides. LC-MS analysis was performed using a Dionex Ultimate 3000 system with an inline diode array detector fitted to an Exactive Plus Orbitrap Mass Spectrometer with an electrospray ionization source. For LC-DAD-MS analysis, 10 μ L aliquots of each sample were injected onto a Kinetex XB-C18 column (2.6 μ m, 2.1 x 100 mm) (Phenomenex, Torrance, CA) and separated at a flow rate of 0.2 mL per min at 30°C using 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile (eluent B) as mobile phases. The gradient started with 100% A, changed linearly to 85% A after 2.8 min, 75% A after 5.2 min, 90% A after 5.44 min, and 100% A after 6.8 min with a 4.2 min hold to achieve column equilibration to starting conditions. The mass spectrometer was operated in full scan mode with a mass range of 750-1,800 m/z and a resolution of 140,000. All ion fragmentation was performed on each ion packet in a subsequent event after each full scan using a normalized collision energy of 20 eV with a stepped normalized collision energy of 50%. Electrospray ionization was performed in positive mode with the sheath gas set at 25 arbitrary units, the auxiliary gas at 10 arbitrary units, the spray voltage at 4,000 V, and a capillary temperature of 350°C. The DAD was set to detect at 361 nm as well as over a 3D field from 190-800 nm.

Figure 7. Guided biosynthesis of different naturally occurring benzimidazole cobamides using *Sporomusa* sp. strain KB-1.



HPLC chromatograms of cobamides (10 mg/L) extracted in the cyano form from *Sporomusa* sp. strain KB-1 cultures grown in the presence of DMB, 5-MeBza, 5-OMeBza, or Bza (200 μ M each). Cultures not amended with a lower base produced the strain KB-1 native cobamides carrying phenol and 4-methylphenol lower bases (bottom panel). mAU, milliabsorbance units.

Figure 8. Confirmation of cobamide authenticity by measuring m/z values using LC-MS analysis. The calculated molecular weights for the cyano form of DMB-Cba, 5-MeBza-Cba, 5-OMeBza-Cba and Bza-Cba are 1355.37, 1341.34, 1357.34, and 1327.32, respectively.



Corrinoid extraction from *Dhc* cultures

Dhc cells and culture supernatant were separated by filtration using a 47 mm diameter 0.22 μ m pore size membrane (Pall Life Sciences, Port Washington, NY). Intracellular corrinoids were extracted and purified following the KCN extraction protocol (Yan et al., 2013). Supernatant-associated corrinoids were reduced into the upper ligand-free, cobalt(II) form by reductants (e.g., HS⁻, L-cysteine, dithiothreitol) present in the medium (Assaf-Anid et al., 1994; Chiu et al., 1996; Lesage et al., 1998). To convert cob(II)amides back to the oxidized cyano-cob(III)amide forms, which can be separated and quantified with the established HPLC method, culture supernatants were reacted with ambient air in the presence of KCN for 24 hours (Schneider et al., 1987). This method was validated by extracting a mixture of cobamides with different lower bases from 100 mL of medium, and the average recovery efficiencies from triplicate vessels were 93.4%, 90.6%, and 91.1% for DMB-Cba, 5-OMeBza-Cba and 5-Bza-Cba, respectively. Following the 24-hour incubation, culture supernatants were loaded onto a C₁₈ Sep-Pak cartridge and washed with 40 mL distilled water. Absorbed corrinoids were eluted with 3 mL methanol, vacuum dried, and suspended in 0.5 mL distilled water.

Cobamide uptake in *Dhc*

Triplicate 100 mL cultures of *Dhc* strain BAV1 and strain GT were grown with 5 μ L cDCE and a cobamide mixture containing equimolar concentrations of DMB-Cba, 5-OMeBza-Cba and 5-Bza-

Cba (36.9 nM each). As soon as *c*DCE was completely dechlorinated to ethene, cobamides remaining in the culture supernatants were recovered, purified and quantified as described above.

Analytical methods

Dhc cells were harvested onto 0.22 μ m membrane filters (Merck Millipore Ltd., Darmstadt, Germany) and the genomic DNA was extracted from the filters using the MO BIO Soil DNA Isolation kit (MO BIO, Carlsbad, CA) as described (Yan et al., 2012). *Dhc* 16S rRNA gene copies were enumerated by quantitative PCR (qPCR) with a ViiA 7 real-time PCR system (Life Technologies, Grand Island, NY) using primer pair Dhc1200F/Dhc1271R and probe Dhc1240probe following established protocols (Ritalahti et al., 2006). Chlorinated ethenes and ethene were analyzed with an Agilent 7890 gas chromatograph equipped with a flame ionization detector and a DB-624 capillary column (60 m x 0.32 mm x 1.8 μ m) (Sung et al., 2006a; Sung et al., 2006b).

Effects of CFC-113 on *Dhc* reductive dechlorination activity

Environmental samples

Sediment samples were collected in May 2011 from the Third Creek in Knoxville, TN (35°56'52.3"N 83°56'17.7"W), the Shady Valley unincorporated community in Johnson County, TN (36°31'10.4"N 81°55'60"W), and the Axton Cross brown field site located alongside the Housatonic River in CT, USA (41°19'1.7"N 73°5'25"W). The Third Creek and the Axton Cross sampling sites have a history of contamination with chlorinated solvents including PCE, TCE, 1,1,1-TCA, and carbon tetrachloride, whereas the Shady Valley sample was collected within a pristine, ecologically sensitive area. Sediment was transferred to sterile glass jars, which were completely filled, sealed, and stored at 4 °C until use.

Effects of CFC-113 and transformation products on reductive dechlorination

Effects of CFC-113 on reductive dechlorination of chlorinated ethenes were tested using the commercial, *Dhc*-containing bioaugmentation consortium SDC-9. Cultivation occurred in 160-mL glass serum bottles with 100 mL reduced ($\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ and L-cysteine, 0.2 mM each), bicarbonate-buffered (30 mM), defined mineral salt medium (Yan et al., 2013) amended with 10 μ L of TCE, 5 mM lactate, and a N_2 - CO_2 (80/20, v/v) headspace. Volumes of 1, 5, and 10 μ L of neat CFC-113 were added by syringe to achieve aqueous phase concentrations of 7.6, 38, and 76 μ M, respectively, which reflect concentrations that have been observed in contaminated groundwater (Höhener et al., 2003; Jackson et al., 1999; Lesage et al., 1990; Parker et al., 2005; Weidhaas et al., 2013). The effects of CFC-113 transformation products on reductive dechlorination of TCE were evaluated by individually adding 76 μ M of CTFE, TFE, and *c*DFE (i.e., 1.3, 2.1, and 1.2 mL, respectively) to SDC-9 cultures. For reversibility experiments, the headspace of SDC-9 cultures, which had been incubated with 10 μ L CFC-113 for 17 days, was evacuated (30 inHg for 15 min) and purged with a N_2 - CO_2 (80/20, v/v) (25 psig) for three cycles. The residual aqueous phase CFC-113 concentration was below the method detection limit of 0.1 μ M. The detection limit of CFC-113 was determined by the signal-to-noise ratio approach (Vial et al., 1999). Following the purge, 10 μ L of neat TCE was added with a microliter syringe. *Dhgm lykanthroporepellens* strain BL-DC-9, a bacterium that dechlorinates 1,2-D to propene (Moe et al., 2009), was used to evaluate the inhibitory effect of CFC-113 on reductive dechlorination of 1,2-D. Strain BL-DC-9 was incubated in 160-mL vessels containing the same mineral salt medium amended with 10 μ L of 1,2-D, 10 μ L of CFC-113, 5 mM acetate and 10 mL of hydrogen. The pseudo-first-order rate constants for TCE

reductive dechlorination were obtained by plotting the natural log of TCE concentrations as a function of time using four early time points. The standard error of the slope was determined using the LINEST function in Excel 2016 (Microsoft Corp., Redmond, WA). Culture bottles without CFC-113, CTFE, TFE, or cDFE served as positive controls. Negative control bottles received TCE or 1,2-D in addition to CFC-113, CTFE, TFE, or cDFE but were not inoculated. Triplicate incubation vessels were established for each condition and sealed with butyl rubber stoppers (Geo-Microbial Technologies, Ochelata, OK), and incubated without agitation at room temperature (21-24 °C) in the dark.

Microbial reductive dehalogenation of CFC-113

Microcosms were established in 160-mL glass serum bottles containing 100 mL of mineral salt medium amended 5 mM lactate. Inside an anoxic chamber, each bottle received 5 grams (wet weight) of solid material, was closed with a butyl rubber stopper, and the headspace was flushed with N₂-CO₂ (80/20, v/v) before 10 µL of CFC-113 was added. Active microcosms with transformation products were transferred (3%, v/v) to fresh medium amended with 10 µL of CFC-113. Autoclaved control microcosms amended with CFC-113 accompanied each experiment.

Abiotic degradation by reactive mineral phases

To explore abiotic transformation of CFC-113, 10 g (dry weight) of the reactive mineral phases, including mackinawite, green rust, magnetite, and manganese dioxide were separately added to 100 mL of deoxygenated 100 mM Tris buffer (pH 7.4) in 160-mL glass serum bottles. Positive control incubations received TCE to confirm reactivity of the respective mineral phases. CFC-113 and TCE were added aseptically to achieve final aqueous concentrations of 100 µM, and all bottles were incubated at room temperature in the dark for 4 weeks on a rotary shaker (120 rpm) in horizontal position. X-ray diffraction (XRD) measurements were conducted using X'pert PRO (PANalytical Inc., Natick, MA) as described (Im et al., 2014). These analyses confirmed the identity of mackinawite and green rust.

Abiotic reductive dehalogenation by vitamin B₁₂

Abiotic reductive dehalogenation of CFC-113 with vitamin B₁₂ (cyanocobalamin) was examined in 160-mL glass serum bottles containing 100 mL Tris buffer (100 mM, pH 7.4) with 60 mL N₂ headspace (Im et al., 2014). Vitamin B₁₂ stock solutions (1 mM) were prepared fresh for each experiment, filter-sterilized (0.22 µm membrane filter) and added to a final concentration of 10 µM. Neat CFC-113 (6 µL, 50 µmol) was added with a microliter syringe, and gaseous CTFE (1.2 mL, 50 µmol) was added using 3-mL plastic syringes. A 100 mM titanium(III)-citrate stock solution was prepared as described (Im et al., 2014) and 5 mL were added to the bottles to generate the Co(I) supernucleophile. All stock solutions were prepared using deionized, degassed water (Milli-Q Corporation, Bedford, MA), and the Hungate technique was applied to avoid oxygen contamination.⁴² Control bottles that lacked either vitamin B₁₂ and/or titanium(III)-citrate accompanied each experiment.

Co-metabolic reductive dehalogenation by corrinoid-producing bacteria

In order to evaluate the feasibility of co-metabolic reductive dehalogenation by corrinoid-producing bacteria, experiments with *Sporomusa ovata* strain H1 (DSMZ 2662) were conducted. *S. ovata* can *de novo* synthesize the corrin ring system and produces phenolyl- and p-creosolyl cobamides (Stupperich et al., 1989; Yan et al., 2013). *S. ovata* was grown in 160-mL glass serum bottles containing 100 mL of corrinoid-free mineral salts medium amended with 15 mM betaine and with a N₂-CO₂ (80/20, v/v) headspace (Im et al., 2014). CFC-113 (1.5 µL) and CTFE (0.3 mL)

were aseptically added to replicate cultures to achieve aqueous concentrations of 11 and 18 μM , respectively. Negative control bottles received CFC-113 or CTFE but were not inoculated.

Analytical methods

Fluoride ions (F^-) were monitored by ion chromatography using a Dionex ICS 2100 system equipped with a 4 mm $\times$ 250 mm IonPac AS18 hydroxide-selective anion-exchange column (Sunnyvale, CA). TCE, cDCE, VC, ethene, CFC-113, and 1,2-D were analyzed using an Agilent 7890 gas chromatograph equipped with a G1888 headspace autosampler, a DB-624 capillary column (60 m $\times$ 0.32 mm $\times$ 1.8 μm), and a flame ionization detector. One-mL aqueous phase samples were collected periodically for quantification of each compound. Quantification was achieved based on external standard curves following established procedures (Löffler et al., 1997a). Briefly, standards were prepared by adding a known amount of each compound to bottles with the same liquid-to-headspace ratio as that for the culture being analyzed. Liquid compounds were added directly using dedicated microliter syringes (Hamilton, Reno, NV), and gaseous compounds were added using 3-mL plastic syringes. Due to the coelution of CTFE and TFE on the DB-624 column, these compounds were quantified using an HP-PLOT/Q column (30 m $\times$ 0.53 mm $\times$ 40 μm). The formation of transformation products including cDFE was measured by direct injection of headspace samples (100 μL) into an Agilent 7890 gas chromatograph with a DB-624 capillary column (60 m $\times$ 0.32 mm $\times$ 1.8 μm) connected to an Agilent 5975C mass selective detector. The formation of cDFE was confirmed by comparing retention times and by GC-MS spectra to those of an authentic cDFE standard. The retention times observed for each analyte are listed in Table 10. To calculate the total amounts of halogenated compound in the incubation vessels, dimensionless Henry's constants were used (Table 10).

Table 10. Retention times of the analytes and dimensionless Henry's constants used for calculations of aqueous phase concentrations.

Compound	Retention time (min) [†]	Dimensionless Henry's constant	Ref. [‡]
Trichloroethene (TCE)	6.37	0.294	(Washington, 1996)
<i>cis</i> -1,2-Dichloroethene (cDCE)	5.60	0.154	(Yang et al., 2000)
Vinyl chloride (VC)	3.43	1.077	(Yang et al., 2000)
Ethene	2.85	8.5	(Yang et al., 2000)
1,1,2-Trichloro-1,2,2-trifluoroethane (CFC-113)	4.34	16.5	TOXNET (Toxicology Data Network) *
Chlorotrifluoroethene (CTFE)	3.04	9.7	TOXNET *
Trifluoroethene (TFE)	2.91	17.7	(USEPA, 2012)
<i>cis</i> -1,2-Difluoroethene (cDFE)	2.88	9.2	(USEPA, 2012)
1,2-Dichloropropane (1,2-D)	6.65	0.298	(Lucius et al., 1990)

[†] Retention times were determined using the GC-FID equipped with G1888 headspace autosampler and a DB-624 capillary column (60 m $\times$ 0.32 mm $\times$ 1.8 μm).

[‡] References report dimensionless Henry's constants.

* <https://infocus.nlm.nih.gov/2015/11/04/toxnet-the-nlm-toxicology-databases/>

Task 6: Database mining

Data collection and preprocessing

Groundwater monitoring datasets were collected from 1,056 wells in 10 sites in the U.S. contaminated with PCE. Each dataset contained *Dhc* 16S rRNA gene copy numbers and geochemical parameters, including pH, ORP, concentrations of DO, NO₃⁻, NO₂⁻, Fe²⁺, SO₄²⁻, Cl⁻, TOC, CH₄, chlorinated solvent, and ethene (Table 11).

Table 11. Summary of chlorinated ethenes and ethene concentrations and geochemical parameters in the data sets after preprocessing.

(A) Chlorinated ethenes and ethene (N = 222 measurement sets ^a)

Variable	Unit	Mean	Max	Min	Std. dev.
PCE	mg/L	1,943.5	149,300.0	0.0	14,392.4
TCE	mg/L	243.4	10,000.0	0.2	1,017.3
cDCE	μg/L	1,030.9	51,000.0	0.2	3,887.3
VC	μg/L	987.7	38,000.0	0.2	3,596.4
Ethene	μg/L	398.5	19,200.0	0.0	1,798.8

(B) Geochemical parameters (N = 222 measurement sets ^a)

Variable	% Missing ^b	Unit	Mean	Max	Min	Std. dev.
Methane	0.5	μg/L	3,524.0	34,600.0	0.0	5,901.7
pH	0.5	-	6.8	9.2	5.6	0.6
DO	0.5	mg/L	1.7	47.1	0.0	3.7
Redox	1.8	mV	-66.9	227.6	-445.0	98.7
Nitrate	3.6	mg/L	2.9	63.9	0.0	6.9
Nitrite	23.4	mg/L	0.5	12.0	0.1	0.9
Ferrous	14.4	mg/L	5.6	190.0	0.0	20.4
Sulfate	0.5	mg/L	44.5	666.0	0.0	82.6
TOC	0.0	mg/L	374.5	8,300.0	0.8	1,231.8
Chloride	3.6	mg/L	63.5	470.0	2.4	83.8

^a A set contains biogeochemical and contaminant data from a single time point measurement in a well.

^b Indicates the percentage of missing measurements for each parameter.

All datasets were screened for (i) wells with at least three sampling time points, (ii) consistent information for at least six of the geochemical parameters mentioned above, (iii) quantitative microbial information (i.e., *Dhc* biomarker gene qPCR data), and (iv) concentrations of chlorinated ethenes exceeding the maximum contaminant levels (MCLs). Datasets not meeting these criteria were excluded from the analysis. Following this prescreening, 222 measurement sets from 35 wells at five sites collected between October 2008 and January 2014 were analyzed further. Each measurement set contained biogeochemical information of a single time point. For the 35 wells included in the analysis, the sampling interval was 3.4 months (95% confidence interval 3.0-3.8), which was chosen as prediction interval in this study.

The chlorinated ethene detoxification potential (DP) was the dependent variable and was categorized into three classes based on the concentrations of toxic chlorinated ethenes and environmentally benign ethene in each observation. The three DP classes were categorized as follows: Class 1 (i.e., High DP) included wells with ethene. In Class 2 (i.e., Medium DP) wells, dechlorination stalled at VC, and in Class 3 (i.e., Low DP) wells, PCE/TCE were stable or dechlorination stalled at cDCE. The class identification numbers 1, 2, and 3 (Table 12) were assigned for categorization only, thus the dependent variable is ordinal, not numerical. Class categorization was sequential and after assignment of observations to Class 1 and Class 3, the remaining observations were categorized as Class 2.

Table 12. Thresholds for categorization of chlorinated ethene detoxification potential (DP) using molar fractions of contaminants and the non-toxic product ethene.

Category	Threshold (%molar fraction) ^a	Reductive dechlorination potential
Class 1	Ethene% > 10	Ethene formation
Class 3	If (PCE+TCE) % ≥ 60, or if (PCE+TCE) % ≤ 60, Ethene% < 0.5, VC% < 20	PCE, TCE stable or cDCE most abundant
Class 2	All other datasets (high VC%)	VC most abundant

^a (PCE+TCE)% indicates the molar fraction of PCE and TCE, (VC)% indicates the molar fraction of VC, and (Ethene)% indicates the fraction of ethene among the total amount (i.e., 100%) of chlorinated ethenes plus ethene.

The thresholds for categorization into these three classes were established based on recommendations made in prior work (Stroo et al., 2013) and the actual distribution of each parameter in the datasets used in this study. (Stroo et al., 2013) suggested that bioaugmentation is not necessary (i.e., Class 1) when the molar fraction of ethene exceeds 10%. Since there were no suggested threshold values for Class 3 (i.e., Low DP), different threshold values were evaluated to determine appropriate values for the current dataset. The combined PCE/TCE molar fraction ranging between 40 to 80%, and the VC molar fraction ranging between 5 to 20% were tested, but the mean and median of contaminant and ethene fractions using different thresholds were not significantly different (data not shown). The current values were selected considering the central tendency of fractions of contaminants and ethene and the evenness of sample distribution to optimize the model performance. Geochemical and *Dhc* 16S rRNA gene abundance information determined at time point *n* was used to predict a DP class at time point *n+1* for a total of 187 individual well sampling events. Based on the available datasets, a 3-month window was chosen to develop a data mining model and to investigate correlations between biogeochemical measurements and the 3-month-ahead dechlorination potential. In 60% of the wells (21 out of 35), changes in DP class were observed within the monitoring period, suggesting dynamic changes occurred during the 3-month window.

Correlation analysis and preselection of independent variables

Since the dependent variable (i.e., the DP class) is ordinal and the sample size is relatively small, Spearman's rank correlation coefficients (ρ) were calculated as a non-parametric method to

assess the relationship between variables using SPSS Statistics 22 (SPSS Inc., Chicago, IL). The Spearman's correlation coefficient is computed from Equation 1

$$\rho = 1 - \frac{6 \sum d_i^2}{n(n^2-1)} \quad \text{Equation 1}$$

where n is a sample size and d_i is a difference between ranks of raw variables. If the correlation between DP class and a parameter was statistically significant at the 99% confidence level ($p < 0.01$), the parameter was selected as an independent variable for the data mining procedure.

Classification and regression tree (CART) model

CART, a widely used decision tree model, was applied to the preprocessed groundwater monitoring dataset representing 35 wells from five sites. The CART model was originally developed to solve classification and regression problems for both numerical and categorical dependent variables (Breiman et al., 1984; De'ath et al., 2000). The CART algorithm produces a classification tree for categorical dependent variables and a regression tree for continuous dependent variables, respectively. Similar to other decision tree models, the CART model has advantages compared to other machine learning models, such as (i) suitability for nonlinear structure, (ii) ranking of the relative importance of independent variables, (iii) clear classification rules (i.e., a white box model), (iv) easy output interpretation, (v) dealing with missing values, and (vi) simultaneous handling of continuous and categorical variables (Kim et al., 2009; Pal et al., 2003). The CART algorithm repeatedly split the data into two mutually exclusive subgroups by setting a splitting threshold on a single explanatory variable to make the best separation of the dependent variable (De'ath et al., 2000). Since the CART algorithm partitions the data into homogeneous groups, a measure to define homogeneity of each group is required. The Gini impurity index was adopted as a splitting criterion for the CART model. An index value of zero would indicate a completely homogeneous group. The index was computed according to Equation 2.

$$I_{Gini} = 1 - \sum_{i=1}^m f_i^2 \quad \text{Equation 2}$$

where i is an element of a set $\{1, 2, \dots, m\}$ and f_i is the fraction of i^{th} element in the set (Breiman et al., 1984; De'ath et al., 2000). To apply the CART algorithm to the preprocessed groundwater monitoring datasets, seven geochemical parameters (CH_4 , ORP, NO_3^- , NO_2^- , Fe_2^+ , SO_4^{2-} , TOC) and the *Dhc* 16S rRNA gene copy number were used as independent variables and the three defined DP classes were used as the dependent variable. The dataset was partitioned into a training set for model construction and a test set for prediction performance evaluation. The commonly used partitioning ratio between the training and the test set of 70:30 (i.e., 129 training cases) was chosen. In addition, a partitioning ratio of 80:20 (i.e., 146 training cases) was simulated to investigate the sensitivity of model training to the length of training data. For coherence test, a 10-fold cross-validation was conducted for 100 randomly shuffled datasets using the SAS Enterprise Miner Workstation 12.3 (SAS Inc, CA, USA).

The CART algorithm also calculates relative importance scores for each variable based on the usefulness of the variables over all possible splits (Raju et al., 2006). If a variable, which has been chosen as a splitting (i.e., primary) parameter in a split, can be a candidate for a surrogate parameter in other (multiple) splits, the variable is considered to be relatively more important than other variables. The CART algorithm sets the score of the most important parameter as 1, and derives the relative importance scores for other parameters that were used to construct the CART model.

Performance evaluation and selection of representative models

The performance of the CART model was evaluated using the receiver operating characteristic (ROC) analysis (Fawcett, 2006). The true positive rate is an intuitive metric for performance evaluation, which describes the case when the positive instance is correctly classified as positive (Duman et al., 2012; Park et al., 2011; Sokolova et al., 2009). The false positive rate describes a case when the negative instance is incorrectly classified as positive. A ROC graph is defined by false positive rate and true positive rate as x- and y-axes, respectively, and a discrete classification model for a 2-class problem produces a single point in the ROC graph (Figure 9a) (Fawcett, 2006). The area under the ROC curve (AUC), a single scalar value calculated from the ROC graph, is often used to compare the performance of classification models (Figure 9b) (Bradley, 1997).

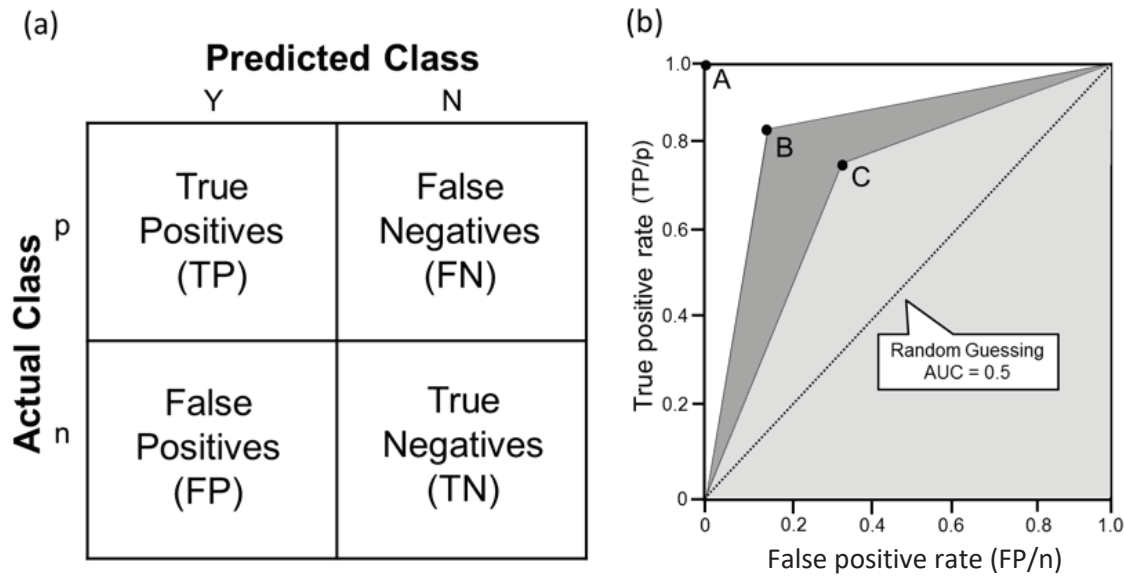


Figure 9. Performance evaluation and selection of representative models. An example of (a) a confusion matrix for a 2-class problem and (b) a ROC space to evaluate performance of a classifier (adopted from (Fawcett, 2006)). Each point in (b) represents a pair of TPR and FPR of a classification model. The shaded areas indicate the area under the ROC curve (AUC) of classifier B and C. AUC is a single scalar value calculated from the ROC curve and used to compare the performances of classification models. The diagonal line from (0,0) to (1,1) describes a classification model, in which AUC is 0.5 (random guessing). Y and N in (a) indicate the numbers of cases classified as positives and negatives by a model, while p and n are the numbers of true (observed) positive and negative cases, respectively.

In general, AUC greater than 0.7 is considered acceptable (Harvey et al., 2015). Following a previous suggestion (Fawcett, 2006; Hand et al., 2001), three different points representing each class were produced in the ROC graph, and the overall performances (AUC_{total}) were estimated by averaging AUC values of the three classes. Based on the AUC_{total} values of generated models, a model with the highest AUC value for both the test and the training sets was selected as the representative model.

Task 7: Apply new tools to DoD sites impacted with chlorinated solvents

Task 7 efforts aimed at the demonstration and validation of the high-throughput qPCR approach (Methods section, Task 1) and the proteomics pipeline (Methods section, Task 2) for analysis of groundwater samples collected from sites impacted with chlorinated ethenes. The successful completion of Task 7 would demonstrate the utility and the value of the new approaches for contaminated site assessment, bioremediation monitoring, and providing additional lines of evidence that contaminant attenuation occurs in impacted groundwater aquifers. Crucial for the successful completion of Task 7 was access to groundwater samples from sites impacted with chlorinated ethenes. Since the focus was on the demonstration of the new tools, we sought to obtain sample from well-characterized military sites with the following characteristics and information available:

- Chlorinated ethenes are the major contaminants
- Types of contaminants and concentrations
- Geochemical data (e.g., nitrate, sulfate, dissolved oxygen, methane, ferrous iron, total organic carbon, ethene)
- Past treatment history (e.g., biostimulation, bioaugmentation, physical-chemical treatment)
- Presence of *Dhc*

The presence of *Dhc* was crucial for validation because the targeted proteomics approach was designed to detect *Dhc* biomarker proteins. We sought samples with high, intermediate, and low *Dhc* abundances to (i) demonstrate the utility of the tool at sites where *Dhc* exceed 10^6 cells/L, and to assess the minimum *Dhc* titer that would allow detection and quantification of *Dhc* protein biomarkers using the targeted proteomics approach. The intent was to utilize the same samples for the demonstration and validation of the proteomics pipeline and the high-throughput qPCR approach. Since the proteomics pipeline was ready for field-testing in 2016 before the RD-qChip v2 was available, our plan was to process the samples for immediate application of the proteomics pipeline and freeze nucleic acid samples for later analysis. Table 14 list the sites from which samples could be obtained for the demonstration and validation efforts in 2016. Unfortunately, the samples did not meet the criteria listed above and were processed using regular qPCR to measure *Dhc* biomarker genes and using the proteomics pipeline. Following the completion of the RD-qChip v2 design in 2018, we attempted to obtain additional samples that would meet the criteria listed above but we encountered difficulties leveraging efforts with sampling events at (military) sites impacted with chlorinated ethenes. Access to samples was a major hurdle to complete Task 7 and we finally were successful coordinating sampling events in 2020 at Naval Air Station North Island, Coronado, CA, Installation Restoration Site 9 (sample collection in February/March 2020) and Site 5 (April/May 2020). In addition, samples have been received from Naval Air Station Point Mugu (March 2020), and additional samples will be collected at Lordstown Ordnance Plant site in Ohio in March 2020. Two University of Tennessee students have been *on site* at Naval Air Station North Island to support the sampling efforts. We anticipate that the qPCR data will be available in early summer 2020 and we will then complete a peer-reviewed manuscript detailing the design and application of the high-throughput qPCR approach for site assessment and bioremediation monitoring.

qPCR Analysis

Field samples were collected from each identified DoD site and shipped on wet ice as either collected groundwater in 1-liter closed containers or Sterivex-GP cartridges (Millipore, Billerica, MA, Cat.#: SVGPL10RC) used to process groundwater. The groundwater containers were immediately stored at 4°C, then processed (Ritalahti et al., 2010b) thru Sterivex-GP cartridges to collect the biomass and then stored at -80°C to preserve the collected biomass. The Sterivex-GP cartridges that were already processed upon arrival were immediately stored at -80°C to preserve the collected biomass.

The Sterivex-GP cartridges were processed independently for either DNA or protein analysis. DNA was isolated from the Sterivex-GP cartridges using the MoBio PowerLyzer PowerSoil Kit (MoBio, Carlsbad, CA) according to the manufacturer's recommendations. DNA concentrations were quantified using the Qubit dsDNA BR Assay (Life Technologies, Grand Island, NY) according to the manufacturer's manual. DNA solutions were stored at -80°C until analysis.

For qPCR analysis, each sample was diluted to three different dilutions using nuclease-free water (i.e. 1:10, 1:100 and 1:1000) to determine if any contaminants were present that would interfere with the qPCR analysis. The *Dhc* 16S rRNA gene assay was chosen to demonstrate any contaminant interference for each sample. Upon analysis of the qPCR results, if the results determined no interfering contaminants present, the most dilute sample that gave the best fit within the template DNA standard curve was chosen for further qPCR analysis (i.e. 1:10 dilution). Table 13 outlines the gene assays which were chosen for complete field site sample analysis and qPCR was performed on each sample.

Table 13. qPCR gene assays chosen to identify key dechlorinators present in groundwater collected at contaminated DoD sites.

Name	Gene	Gene Function
General Bacteria	16S rRNA	Phylogenetic
<i>Dehalococcoides mccartyi</i>	16S rRNA	Phylogenetic
<i>Dehalobacter mccartyi</i>	16S rRNA	Phylogenetic
<i>Dehalogenimonas</i> spp.	16S rRNA	Phylogenetic
<i>Dehalococcoides mccartyi</i> strain GT	<i>vcrA</i>	cDCE → Ethene
<i>Dehalococcoides mccartyi</i> strain BAV1	<i>bvcA</i>	cDCE → Ethene
<i>Dehalococcoides mccartyi</i> strain 195	<i>tceA</i>	TCE → VC
<i>Dehalobacter</i> sp. strain CF	<i>cfrA</i>	CF/1,1,1-TCA RDase
<i>Dhgm lykanthroporepellens</i> strain BL-DC-9	<i>dcpA</i>	1,2-D → Propene
' <i>Ca. Dhgm etheniformans</i> ' strain GP	<i>cerA</i>	VC → Ethene

Demonstration and validation of the proteomics pipeline

Groundwater samples for biomass collection

For validation of the proteomics approach, groundwater samples were obtained from DoD sites impacted with chlorinated ethenes as well as a contaminated site outside the United States. The intent was to leverage efforts with sample collection at DoD sites. Groundwater samples from injection and monitoring wells were collected using low-flow sampling methods at three distinct sites impacted with chlorinated ethenes. Sample 33NA-4 was extracted from an undisclosed contaminated site outside the U.S. and biomass was received on Sterivex 0.22 μm filter units. Samples M17 and M18 were extracted in August 2016 at the Naval Support Activity Mechanicsburg site in the U.S. undergoing chemical oxidation (hydrogen peroxide/chelated iron catalyst) and mineral injections, as well as supplementation of organic compounds (*i.e.*, lactate) to stimulate indigenous dechlorinators. Biomass was collected *on site* and the Sterivex 0.22 μm filter units were shipped on ice. Samples 97, 116, and 129 were collected in September 2016 from wells at the Offutt Airforce Base in the United States, in which injections of emulsified vegetable oil, zero-valent iron and a *Dhc*-containing bioaugmentation consortium had occurred. The samples were collected in July 2016. Groundwater samples were received in sterile 1 L bottles and filtered through Sterivex units to concentrate the biomass immediately after arrival. All Sterivex cartridges were stored at -80°C prior to protein extraction and digestion.

Sample preparation for global and targeted proteomics analyses

Included in the analysis were the groundwater samples (M17, M18, 97, 116 and 129) listed in Table 14 and consortium BDI. Filtered cells from ax3enic cultures of *Dhc* strains 195, FL2, BAV1 ($n=2$ biological replicates), the BDI Consortium, as well as the M17, M18, 97, 116 and 129 groundwater samples ($n=1$) were processed by adding two mL of a detergent based lysis buffer (4% sodium dodecyl sulfate [SDS] in 100 mM Tris-HCl, pH 8.0) to the Sterivex cartridges followed by incubation in a water bath at 97°C for 15 minutes and incubation at room temperature for 1 hour. The SDS lysis buffer was recovered and the filters rinsed once more with fresh lysis buffer. As previously described, proteins were extracted from cell lysates by trichloroacetic acid (TCA) precipitation and proteolytically digested with trypsin following denaturation and disulfide bonds being reduced and blocked (Yang et al., 2012).

Frozen filter membranes with biomass from the 33NA4 groundwater sample ($n=1$) were removed from the cartridges and cut into ~ 1 cm pieces using a sterilized razor blade and then suspended in 5 mL of SDS lysis buffer (5% SDS in 50 mM Tris-HCl, pH 8.5; 0.15 M NaCl, 0.1 mM EDTA; 1mM MgCl_2 ; 50 mM DTT). Cells were heat-lysed as described earlier (Chourey et al., 2010) and the supernatant containing the whole cell lysate transferred to new tubes. Proteins were then precipitated by adjusting the samples to 20% TCA (v/v). Lysate mixes were centrifuged at 21,000 g x 20 min to obtain a protein pellet which was washed with chilled acetone, air dried and solubilized in 6 M guanidine buffer (Giannone et al., 2011). Following protein solubilization, proteolysis was initiated using trypsin (Yang et al., 2012). All peptide solutions were desalted on 200 μL C18 stage tips (Thermo Scientific, Waltham, MA) and stored at -80°C prior to global proteomics analysis. For targeted proteomics runs, volumes of processed samples were loaded directly onto capillary back columns and desalted off-line.

Table 14. Summary of contaminated sites used for both qPCR analysis and proteomic analysis.

Site	Well ID	Sample ID	Comments
Battelle-NSA-Mechanicsburg	SO3M48-35-082516	M48	Samples were filtered <i>on site</i> and Sterivex cartridges were received. Filter M48 was grey and filters M17 and M18 were red/brown in color prior to extraction. No <i>Dhc</i> biomarker genes detected with qPCR for M48.
	SO3M17-35-082416	M17	
	SO3M18-36-082416	M18	
Dover Airforce Base	MW605S	MW605S	Received groundwater, not Sterivex cartridges. <i>Dhc</i> 16S rRNA gene copies/mL were below 3.2E+04 and no proteomics analysis was performed. Only samples with <i>Dhc</i> greater than 1E+05 copies/mL were chosen for proteomics analyses.
	MW605D	MW605D	
	MW608S	MW608S	
	MW102S	MW102S	
	MW102D	MW102D	
Offutt Airforce Base	IMW09-85-091716	85	Received groundwater with either milky or black color. Samples #85 and #94 contained some type of suspended oily substance(s) and it was very challenging to collect biomass. Multiple cartridges had to be combined for biomarker extraction.
	SMW010-129-091716	129	
	IMW99-29-091716	29	
	IMW010-116-091816-L	116	
	IMW09-94-091816-L	94	
	IMW09-97-091716	97	
	IMW010-132-091716	1332	
Charleston Airforce Base	MW29-21	MW29-21	Sterivex cartridges were received. <i>Dhc</i> 16S rRNA gene copies/mL were generally below the E+5 threshold. No proteomics analysis was performed.
	MW29-8	MW29-8	
	MW025	MW025	
	MW88-6	MW88-6	
	MW29-6	MW29-6	
	MW29-30	MW29-30	
	MW89-7	MW89-7	
Outside the United States	33NA-4	33NA-4	Preliminary global proteomics detected <i>Dhc</i> proteins including reductive dechlorination biomarkers.

Identification of targeted peptides and proteins in groundwater by LC-MRM-MS

To validate the identification of peptide signals, the following criteria were required: (A) Co-elution of all five selected transitions; (B) similar transition ratios between the signals detected in groundwater to those observed in the pure cultures; and (C) the peak group had to be observed in all three technical replicate runs. (D) For a subset of the target proteins ($n=6$), strong agreement between transition ratios (coefficients of variation $< 20\%$ in at least three or more of the final monitored transitions) and retention times (≤ 3 mins difference) of the endogenous peptides with the spiked standards were required. For the six target proteins, a collective set of 11 synthetic unlabeled peptide standards were obtained (Thermo Fisher) as purified lyophilized solids ($>95\%$ purity), reconstituted to $5 \text{ pmol}/\mu\text{L}$ solutions in solvent A (0.1% formic acid, 2% acetonitrile) and $1 \mu\text{L}$ of each standard solution spiked to the same peptide sample amounts of BDI and the groundwater samples that were loaded for technical reproducibility. For additional support, high mass accuracy and resolution global proteomics data filtered at a peptide-FDR level $< 1\%$ were used to verify the presence of the target peptides and proteins in the groundwater samples.

Global proteomics data analysis

Protein intensity values from each global proteomics dataset were calculated by summing together the MS1-level intensities of peptide precursors that were derived from IDPicker using IDPQuantify (Chen et al., 2013). Protein abundance values were normalized by dividing the protein intensity values by their length (*i.e.*, number of amino acids), performing a $\log_2$ transformation, and mean central tendency adjusted with the software platform Inferno RDN (<https://omics.pnl.gov/software/infernordn>).

Using the Perseus software (Tyanova et al., 2016), proteins in pure cultures of *Dhc* strains 195, FL2 and BAV1 that were stochastically sampled by requiring quantified proteins to be observed in both biological replicates per strain. For the BDI consortium and groundwater sample sets ($n=2$ and 3 technical replicates, respectively) proteins observed in at least one run were considered for comparison to targeted results. Additional information regarding the treatment of global proteomics data can be found in the primary literature (Solis et al., 2019). All proteins identified by LC-MS/MS were clustered at $> 85\%$ amino acid sequence identity with the UClust algorithm of the analysis tool USearch v10.0.9 (Edgar, 2010).

Results and Discussion

The overarching objective was to advance MBTs and their application to more effectively assess, monitor, optimize, predict, and manage reductive dechlorination processes at contaminated DoD sites. Specifically, innovative high-throughput qPCR technology and environmental proteomics workflows were explored to characterize dechlorinating microbial communities and activities. The meaningful implementation of advanced MBTs requires an in-depth understanding of the microorganisms contributing to contaminant degradation and their ecology. Therefore, this project also aimed at discovering new microbes and novel biomarkers involved in the degradation of chlorinated solvents, and at elucidating geochemical controls over the reductive dechlorination process.

Information and data, including figures and tables, contained in this section are largely documented in the following peer-reviewed publications:

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Summary of key findings

1. The enrichment and isolation efforts demonstrated that reductive dechlorination of chlorinated ethenes to environmentally benign ethene is not limited to *Dehalococcoides mccartyi* (*Dhc*) strains. At least some members of the genus *Dehalogenimonas* (*Dhgm*) have the ability to use TCE, cDCE, and VC as growth-supporting electron acceptors to produce ethene. *Dhgm* 16S rRNA genes were frequently detected and outnumbered *Dhc* in more than half of 1,173 groundwater samples collected from aquifers impacted with chlorinated ethenes, suggesting *Dhgm* contribute to reductive dechlorination and detoxification.
2. RDases require a corrinoid cofactor for activity; however, *Dhc* are corrinoid-auxotroph and rely on the microbial community to supply this essential nutrient. Detailed physiological studies demonstrated that the type and quantity of corrinoid provided to *Dhc* determines reductive dechlorination rates and extents. This finding has implications for bioremediation because corrinoid limitations, both in terms of quantity and quality, can limit *Dhc* reductive dechlorination activity.
3. *dcpA* was identified as a novel biomarker gene for 1,2-dichloropropane (1,2-D) reductive dechlorination to environmentally benign propene.
4. *cerA* was identified as a novel reductive dechlorination biomarker for VC-to-ethene reductive dechlorination.
5. BvcA encoded by the *bvcA* gene was confirmed as a DCE and VC RDase.
6. The design and testing of RD-qChip v1 OpenArray® plate accommodating 54 target gene assays led to the improved RD-qChip v2 OpenArray® plate design with 112 target genes for monitoring the degradation of chlorinated solvents. This OpenArray® offers several advantages over the conventional qPCR technology including reduced cost per qPCR assay, improved accuracy, and richer informational content to allow knowledge-based decision-making.
7. Global proteomics identified *Dhc* biomarker proteins in axenic cultures of *Dhc* strains 195, FL2, and BAV1. Targeted proteomics validated the selection of 37 peptides with adequate MRM-MS characteristics for monitoring applications via LC-MRM-MS. *Dhc* strain-level resolution can be provided by targeted proteomics where RDase peptides indicative of specific reductive dechlorination steps were detected.

8. The application of a targeted proteomics approach for the identification of *Dhc* biomarker proteins in contaminated groundwater is feasible; however, further optimization is needed to determine absolute quantitative amounts of the target proteins.
9. The application of machine learning-based data mining to biogeochemical and microbial (i.e., qPCR) data sets collected from sites impacted with chlorinated ethenes demonstrated the value of this approach for generating predictive models and identifying the most promising remediation strategy at a specific site.
10. The data mining approach identified oxidized nitrogen species as relevant geochemical parameters affecting *in situ* reductive dechlorination potential, and physiological studies demonstrated that nitrous oxide (N₂O) is strong inhibitor of reductive dechlorination.
11. Biostimulation with NH₄⁺ can enhance reductive dechlorination activity when fixed nitrogen is a limiting nutrient; however, a “do nothing” approach that relies on indigenous diazotrophs (i.e., N₂-fixing microorganisms) can achieve similar dechlorination end points and avoids the potential for stalled dechlorination due to inhibitory levels of NH₄⁺ or transformation products (i.e., N₂O).
12. 1,1,2-trichloro-1,2,2-trifluoroethane (CFC-113), a commonly observed co-contaminant, inhibits reductive dechlorination by *Dhc* in a concentration-dependent manner, causing cDCE stalls. Reductive dehalogenation of CFC-113 can alleviate the inhibition.
13. The efforts to extract information from existing databases identified key shortcomings, including a lack of standardized sampling protocols, inconsistent monitoring schedules and parameter sets, and poor data curation and availability. Building a systematic, comprehensive and open-access database that combines microbiological, geochemical, physical and hydrogeological aquifer/plume data, as well as information about treatment, should be a high priority.
14. The database mining efforts highlighted the value of *Dhc* biomarker gene enumeration. The analysis demonstrated that ratios of *Dhc* to total bacterial 16S rRNA genes and of *bvcA* + *vcrA* to total bacterial 16S rRNA genes exceeding 0.1%, and a ratio of *vcrA* + *bvcA* to *Dhc* 16S rRNA genes near unity are useful normalized, measurable parameters for predicting detoxification (i.e., ethene formation) at sites impacted with chlorinated ethenes where *Dhc* are key dechlorinators.

Task 1: RD-qChip – Design qPCR assays and validate with defined samples

The aim of Task 1 was the design of the RD-qChip QOpenArray® plate for biomarker genes relevant for the reductive dechlorination process of chlorinated contaminants, in particular chlorinated ethenes. A number of organism-specific (i.e., phylogenetic) and process-specific (i.e., functional) biomarkers have been identified and selected for inclusion in the design of the RD-qChip. Organism-specific biomarkers provide useful information, especially when a firm link between the target organism and the process of interest has been established. Phylogenetic biomarkers for RD-qChip design included 16S rRNA genes of relevant dechlorinators and other microorganisms known to have direct/indirect roles in the reductive dechlorination process. Table 15 summarizes biomarker genes for anaerobic dechlorination of chlorinated solvents.

Table 15. Summary of the current knowledge of organism-specific (i.e., 16S rRNA genes) and pathway-specific (i.e., functional) biomarker genes involved in the transformation of chlorinated solvents.

Dechlorination step	RDase gene	Organism	Reference
PCE → TCE	-	<i>Desulfitobacterium</i> sp.	(Bouchard et al., 1996), (Gerritse et al., 1996)
PCE/TCE → cDCE	<i>pceA</i>	<i>Sulfurospirillum</i> spp.	(Löffler et al., 1997b) (Neumann et al., 1996)
PCE → TCE	<i>pceA_{TCE}</i>	<i>Sulfurospirillum</i> SL2-PCE	(John et al., 2009) (Buttet et al., 2013)
PCE/TCE → cDCE	<i>pceA_{DCE}</i>	<i>Sulfurospirillum</i> SL2-PCE	(Buttet et al., 2013)
PCE → TCE	<i>pceA</i>	<i>Sulfurospirillum</i> -ACS _{TCE}	(Yang et al., 2017a)
PCE/TCE → cDCE	<i>pceA</i>	<i>Sulfurospirillum</i> -ACS _{cDCE}	(Yang et al., 2017a)
PCE/TCE → cDCE	<i>pceA</i>	<i>Desulfitobacterium</i> spp.	(Reinhold et al., 2012)
PCE/TCE → cDCE	<i>pceA</i>	<i>Geobacter lovleyi</i>	(Amos et al., 2007c) (Wagner et al., 2012)
PCE/TCE → cDCE	<i>pceA</i>	<i>Desulfuromonas</i> sp.	(Lee et al., 2011b; Löffler et al., 2000)
PCE/TCE → cDCE	<i>pceA</i>	<i>Dehalobacter</i> sp.	(Maillard et al., 2003)
PCE → TCE	<i>pceA</i>	<i>Dhc</i>	(Fung et al., 2007)
PCE → TCE	<i>pteA</i>	<i>Dhc</i>	(Zhao et al., 2017a)
PCE/TCE → cisDCE/tDCE	<i>mbrA</i>	<i>Dhc</i>	(Cheng et al., 2009)
TCE → VC	<i>tceA</i>	<i>Dhc</i>	(Johnson et al., 2005b; Ritalahti et al., 2006) (Holmes et al., 2006)
cDCE → ethene	<i>vcrA</i>	<i>Dhc</i>	(Holmes et al., 2006) (Ritalahti et al., 2006) (Lee et al., 2013; Molenda et al., 2016)
cDCE → ethene	<i>bvcA</i>	<i>Dhc</i>	(Ritalahti et al., 2006) (Sung et al., 2006b) (Holmes et al., 2006; Molenda et al., 2016)
TCE → ethene	<i>vcrA</i>	<i>Dhc</i>	(Zhao et al., 2019a)
TCE → ethene	<i>cerA</i>	<i>Dhgm</i>	(Yang et al., 2017c)
VC → ethene	<i>cerA</i>	<i>Dhgm</i>	(Yang et al., 2017c)
tDCE → VC	<i>tdrA</i>	<i>Dhgm</i>	(Molenda et al., 2016)
1,2-D → propene	<i>dcpA</i>	<i>Dhc</i>	(Padilla-Crespo et al., 2014)
1,2-D → propene	<i>dcpA</i>	<i>Dhgm</i>	(Padilla-Crespo et al., 2014)
1,1,1-TCA → 1,1-DCA	<i>cfrA</i>	<i>Dehalobacter</i> sp. CF	(Tang et al., 2013b; Wang et al., 2017)

Dechlorination step	RDase gene	Organism	Reference
1,1,1-TCA → CA	<i>ctrA</i>	<i>Desulfitobacterium</i> sp. PR	(Ding et al., 2014)
1,1,2-TCA → 1,2 DCA	<i>tmrA</i>	<i>Dehalobacter</i> sp. UNSWDHB	(Wong et al., 2016)
1,1,1-TCA→1,1-DCA	<i>thmA</i>	<i>Dehalobacter</i> sp. THM1	(Zhao et al., 2017b)
1,1-DCA → CA	<i>dcrA</i>	<i>Dehalobacter</i> sp. DCA	(Tang et al., 2013b)
1,2-DCA → Ethene	<i>dcaA</i>	<i>Desulfitobacterium dichloroeliminans</i> DCA1	(Kunze et al., 2017; Marzorati et al., 2007)
1,2-DCA → Ethene	<i>dcaA</i>	<i>Dehalobacter</i> sp. WL	(Grostern et al., 2009)
CF → DCM	<i>tmrA</i>	<i>Dehalobacter</i> sp. UNSWDHB	(Wong et al., 2016)
CF → DCM	<i>cfrA</i>	<i>Dehalobacter</i> sp. CF	(Tang et al., 2013b; Wang et al., 2017)
CF → DCM	<i>ctrA</i>	<i>Desulfitobacterium</i> sp. PR	(Ding et al., 2014)
CF → DCM	<i>thmA</i>	<i>Dehalobacter</i> sp. THM1	(Zhao et al., 2017b)
1,2,3,4-TeCB → 1,2,4-TCB	<i>cbrA</i>	<i>Dhc</i> CBDB1 and DCMB5	(Adrian et al., 2007b; Pöritz et al., 2015)
1,2,3-TCB → 1,3-DCB			(Pöritz et al., 2015; Wagner et al., 2009)
2,4,6-TCP → 2,4 DCP	<i>crdA</i>	<i>Desulfitobacterium hafniense</i> PCP-1	(Boyer et al., 2003; Gauthier et al., 2006)
Polychlorinated phenols → DCPs	<i>cprA</i>	<i>Desulfitobacterium</i> sp.	(Gauthier et al., 2006; Thibodeau et al., 2004; Villemur et al., 2006) (Bisaillon et al., 2010)
1,2,4,5-TeCB→ 1,3-DCB/1,4-DCB	<i>tcBA</i>	<i>Dehalobacter</i> sp. TeCB1	(Alfán-Guzmán et al., 2017)
1,2,4-TCB→ 1,3-DCB/1,4-DCB			
DCM → Acetate	-	<i>Dehalobacter</i> sp.	(Justicia-Leon et al., 2014; Justicia-Leon et al., 2012)
DCM → Acetate, formate	-	<i>Dehalobacterium formicoaceticum</i>	(Chen et al., 2020; Chen et al., 2017; Mägli et al., 1998; Mägli et al., 1996)
DCM → Acetate, hydrogen	-	<i>Ca. Dichloromethanomonas elyunquensis</i>	(Chen et al., 2020; Kleindienst et al., 2017)

Target gene selection

Members of phylogenetically diverse genera, including *Sulfurospirillum*, *Dehalobacter*, *Desulfitobacterium*, *Desulfuromonas*, and *Geobacter*, have been isolated and demonstrated to reductively dechlorinate PCE and TCE to cDCE (Gerritse et al., 1996; Holliger et al., 1998a; Miller et al., 1997; Scholz-Muramatsu et al., 1995; Sung et al., 2006a; Sung et al., 2003). Some of these organisms are prolific PCE/TCE dechlorinators and can contribute to the enhanced dissolution of DNAPL in source zones (Amos et al., 2007a). qPCR assays targeting the 16S rRNA genes of these known PCE/TCE dechlorinators were included in the design of the RD-qChip. Of particular interest are members of the species *Dehalococcoides mccartyi* (*Dhc*), which form a distinct organismal group known for complete reductive dechlorination to non-toxic ethene (Flynn et al., 2000). Since the first report of *Dhc* appeared in 1997 (Maymó-Gatell et al., 1997), several additional *Dhc* strains with the ability to dechlorinate DCEs and VC to ethene have been isolated and characterized (Löffler et al., 2013b). Based on 16S rRNA gene differences, the *Dhc* genus has been divided into the Pinellas, Victoria and Cornell subgroups (Hendrickson et al., 2002). qPCR assays targeting the entire *Dhc* genus (i.e., strains belonging to the Pinellas, Victoria and Cornell

subgroups) and the Cornell subgroup only were designed and included in the RD-qChip design (Cornell group-specific assays only included in RD-qChip v2). The presence of *Dhc* biomarkers at sites impacted with chlorinated ethenes has been considered a key determinant for detoxification potential (i.e., ethene formation). Phylogenetically related to *Dhc* is the genus *Dhgm*, and its members were described as obligate organohalide-respiring bacteria utilizing chlorinated alkanes as electron acceptors (Key et al., 2017; Moe et al., 2009). Recent findings suggest that members of the genus *Dhgm* can also use chlorinated ethenes as electron acceptors, and *Dhgm* sp. strain WBC-2 has been shown to catalyze the reductive dechlorination of tDCE to VC (Molenda et al., 2016). Efforts described in more detail below identified ‘*Candidatus* Dehalogenimonas etheniformans’ strain GP as the first member of the *Dhgm* genus capable of utilizing TCE, 1,1-DCE, cDCE and VC as respiratory electron acceptors (Yang et al., 2017c). Thus, the RD-qChip design included a qPCR assay targeting biomarker genes of *Dhgm*, including the *Dhgm* 16S rRNA gene. Also included in the design were assays that target all bacteria and methanogenic archaea using general primers for these respective domains. The term “all” indicates that the design of these primers was based on the sequences available in public databases and does not imply that comprehensive amplification of all members of the respective domains is achieved.

Dhc are strictly organohalide-respiring bacteria and growth requires a suitable chlorinated compound (i.e., the contaminant) as electron acceptor. Based on this observation, a high abundance of *Dhc* 16S rRNA genes suggests that the major chlorinated contaminants serve as electron acceptors for growth. In such a scenario, the measurement of an organism-specific biomarker does provide information about a specific process of interest. Still, the mere presence of *Dhc* determined by measuring *Dhc* 16S rRNA genes does not conclusively demonstrate that the target contaminants, such as chlorinated ethenes, are effectively degraded. All of the known *Dhc* strains possess RDase genes to perform organohalide respiration but not all *Dhc* strains have RDases to dechlorinate chlorinated ethenes (Löffler et al., 2013b).

Here, the power of process-specific biomarker genes come into play, and the qPCR assays targeting functional genes can generate information about the potential for specific reductive dechlorination reactions to occur. Obvious targets are genes encoding RDases that catalyze carbon-chlorine bond cleavage during reductive dechlorination. The *Dhc* *bvcA* and *vcrA* genes encoding RDases linked to cDCE and VC reductive dechlorination are included among those process-specific biomarkers. Genome and metagenome sequencing efforts identified hundreds of (putative) *Dhc* RDase gene sequences, but functional assignments have been made in only a few instances. The focus was on the design of qPCR assays targeting RDase genes with functional assignment; however, the design of RD-qChip v2 targeted several putative *Dhc* RDase genes, including RDase genes identified in the SDC-9 metagenome with unknown function. The inclusion of these putative RDase genes was justified because the SDC-9 consortium is frequently used for bioaugmentation treatment at DoD sites.

Dhc and *Dhgm* possess “*fdhA*” genes, which were initially annotated as genes encoding formate dehydrogenase alpha subunit (FdhA). While *Dhgm* isolates can oxidize formate, *Dhc* cannot and strictly depend on hydrogen as electron donor. A recent study implicated the *Dhc* “*fdhA*” gene in a completely different function and reported that it encodes a complex iron-sulfur molybdoenzyme (CISM) that is part of a multi-protein reductive dehalogenase complex (Kublik et al., 2016; Zinder, 2016). In *Dhc*, CISM appears to play a crucial role in respiratory electron transfer to the chlorinated electron acceptor, and thus in reductive dechlorination. Specifically, CISM was proposed to serve an integral role as an electron-channeling module between the Hup hydrogenase and the RDase in the respiratory chain of *Dhc* (Kublik et al., 2016). The *Dhc* genes encoding CISM are highly

conserved and suitable targets for qPCR assays to measure the presence and abundance of these bacterial groups, and these targets were included in the design of RD-qChip v2. Of note, CISM proteins are highly expressed in growing *Dhc* cells suggesting that the expression of this gene and the production of the CISM protein may serve as a general biomarker of *Dhc* dechlorination activity (discussed below, Validation of RD-qChip v2). Recently, the presence of CISM gene cluster was reported in ‘*Candidatus* Dehalogenimonas etheniformans’ strain GP (Yang, et al., 2017).

Dhc strictly depend upon hydrogen as electron donor and one or more hydrogenases have roles in electron transfer to the chlorinated electron acceptor. *Dhc* possess five different gene clusters that encode for membrane-bound *Hup*, *Ech*, *Hyc*, *Hym* hydrogenases and a cytoplasmic *Vhu* hydrogenase (Ahsanul Islam et al., 2010). The *Hup* hydrogenase forms a complex with CISM and is involved in electron transfer during reductive dechlorination (Kublik et al., 2016). Therefore, the RD-qChip design included qPCR assays targeting the *hup*, *ech*, *hyc*, *hym*, and *vhu* genes.

The known *Dhc* strains are corrinoid auxotrophs and strictly require external corrinoid for reductive dechlorination activity (Men et al., 2012; Yan et al., 2018; Yan et al., 2013; Yan et al., 2012; Yan et al., 2016; Yi et al., 2012). The bioinformatic analysis of *Dhc* genomes revealed that *Dhc* strains cannot *de novo* synthesize corrinoid and instead have to import cobamides or precursors from the environment. *Dhc* strains possess genes encoding functions for corrinoid transport (*btuF*, *btuC* and *btuD*), cobinamide salvage and activation (*cbiZ*, *cbiB*, *cobU* and *cobD*) and modification (*cobA* encodes a corrinoid adenosyltransferase), and lower base activation (*cobT*, *cobS*, *cobC*) (Men et al., 2017; Yan et al., 2018; Yan et al., 2016). Based on these observations, genes encoding functions for corrinoid salvage and modification were included in the RD-qChip design. More details about the gene target included in the designs of RD-qChip v1 and v2 are provided in the Methods section of this report. A complete list of phylogenetic biomarkers included in RD-qChip design v1 and v2 are shown in Table 3 and Table 4.

Primer and probe design

Several qPCR assays targeting the genes of interest were available in the peer-reviewed literature, but upon careful evaluation, the majority of primers (and probes) had to be redesigned to meet the more stringent criteria of the QuantArray®. Specifically, the QuantArray® approach requires all primers and probes hybridize to their target genes at a uniform temperature.

Prior to incorporation on the array plate, the specificity and amplification efficiency of the primer pairs were validated using SYBR Green chemistry. Unproductive primer pairs were discarded, and new primers were designed and tested. Only primer pairs that met the requirements as outlined in the Methods section under Task 1 (qPCR assay validation) were evaluated in regular qPCR assays using linear amplification probes (i.e., TaqMan chemistry).

Standard curves

Standard curves were prepared as described in the Methods section under Task 1 (Standard Curve Preparation). The RD-qChip v1 comprised a total of 56 qPCR assays targeting 42 biomarker genes. Note that a few genes were targeted with more than one primer/probe combination. RD-qChip v2 comprised a total of 112 qPCR assays targeting 102 different biomarker genes. Multiple assays targeting the same gene was done for internal control purposes (i.e., to assess consistency across the array plate). The rationale was that different assays targeting the same gene should result in similar gene abundance values.

Validation of the RD-qChip approach with defined samples

Validation of RD-qChip v1

The validation of RD-qChip v1 custom OpenArray® plates used DNA standards containing mixed target pDNAs. Mixture 1 included phylogentic gene targets (*Dhc*, *Dhb*, *Dhgm*, *Geo*, etc.) and Mixture 2 included other process-specific target genes such as *vcrA*, *bvcA*, *tceA*, *cerA*, *cbrA*, etc. A 10-fold serial dilution series of the plasmid DNAs-mixture were prepared ranging from 2.02E+08 to 2.02E+01 target gene copies/μL corresponding to 1.58E+06 to 1.58E-02 gene copies per 33-nL well as given in Table 16.

More details on how the mixtures were prepared are outlined in the Methods section above.

Table 16. The dilution series of a mixture of pDNAs used as DNA standards for validation of TaqMan assays on RD-qChip v1.

Concentration (ng/μL)	1.0E+00	1.0E-01	1.0E-02	1.0E-03	1.0E-04	1.0E-05	1.0E-06	1.0E-07	1.0E-08
Copies/μL	2.02E+08	2.02E+07	2.02E+06	2.02E+05	2.02E+04	2.02E+03	2.02E+02	2.02E+01	2.02E+00
Copies/33 nL	1.58E+06	1.58E+05	1.58E+04	1.58E+03	1.58E+02	1.58E+01	1.58E+00	1.58E-01	1.58E-02

RD-qChip v1 amplification efficiencies and standard curves for each target gene were analyzed against the same qPCR criteria outlined in the Methods section (qPCR assay validation) for single qPCR assays. Assays that yielded sub-optimal results or did not result in reproducible amplification were redesigned using the same method described in the Methods section (Primer and probe design for qPCR).

Assay-specific standard curves were constructed by preparing duplicate custom OpenArray® plates (two plates for pDNA mixture 1 and two plates for pDNA mixture 2) (Figure 10).

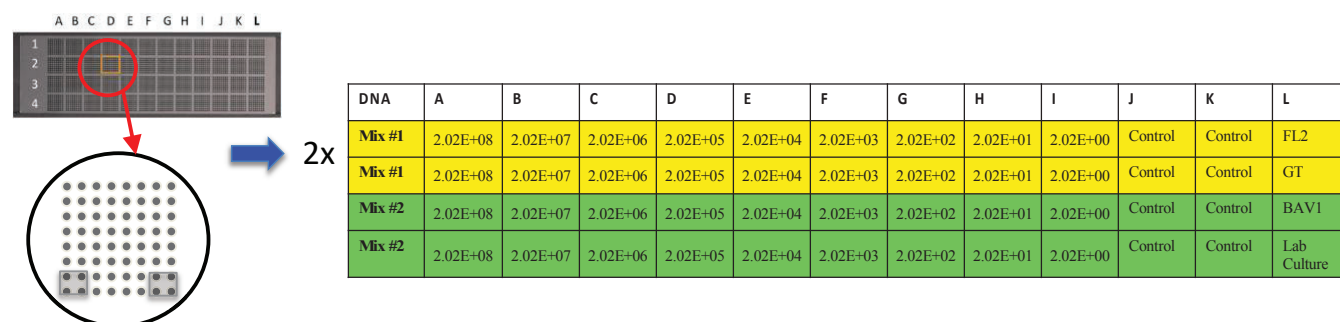


Figure 10. Experimental design for determining limits of detection and reproducibility of RD-qChip v1 qPCR assays.

The results from the duplicated plate runs were combined and used to construct assay-specific standard curves. The threshold cycle number (C_{rt}) is used to determine the cell gene copy number for each dilution and is a default parameter of QuantStudio 12K Flex OpenArray[®] gene expression analysis software (Expression Suite V1.0). A linear regression best-fit line (Equation 3) was constructed based on the C_t values and log₁₀-gene copy numbers to determine assay specific parameters including slope, y-intercept, and efficiency (Equation 3 and Equation 4). (Table 17) includes assay specific parameters obtained following the validation runs of two RD-qChip v1 array plates.

$$C_t = \text{slope} \times \log_{10}(\text{gene copy number}) + \text{yintercept} \quad \text{Equation 3}$$

$$\text{Efficiency (\%)} = (10^{\frac{1}{\text{slope}}} - 1) \times 100 \quad \text{Equation 4}$$

Table 17. RD-qChip v1 experimental testing and outcomes (i.e., rejected or accepted).

A reject decision (rows highlighted in red) meant that the assay did not meet the performance criteria and new primers and probes were designed or the assay was eliminated.

Assay #	Target	Assay Name	Genus	Slope	Y-intercept	R ²	Efficiency (%)	Rejected or accepted
1	16S	ARCH1_16S	<i>Archaea-General</i>	-2.47	36.69	0.895	154.15	Rejected
2	16S	BAC1_16S	<i>Bacteria-General</i>	-2.41	31.84	0.955	159.71	Rejected
3	16S	GEOGC_16S	<i>Geobacter daltonii</i> FRC-32	-3.48	39.92	0.999	93.82	Accepted
4	16S	ADEH_16S	<i>Anaeromyxobacter</i>	no amplification				Rejected
5	16S	DHB_16S	<i>Dehalobacter restrictus</i> PER-K23	-3.51	39.41	1.000	92.71	Accepted
6	16S	DFORM_16S	<i>Dehalobacterium formicoaceticum</i>	-3.59	40.78	0.991	90.01	Accepted
7	16S	DHB1_16S	<i>Dehalobacter</i> CF50	-3.34	38.85	1.000	99.40	Accepted
8	16S	DHC1_16S	<i>Dehalococcoides</i>	-3.34	38.60	0.999	99.40	Accepted
9	16S	DHC2_16S	<i>Dehalococcoides</i>	-3.39	40.53	1.000	97.12	Accepted
10	16S	DHC3_16S	<i>Dehalococcoides</i>	-3.32	38.96	1.000	99.97	Accepted
11	16S	DHGM_16S	<i>Dehalogenimonas</i>	-3.48	39.37	1.000	93.79	Accepted
12	16S	DSM1_16S	<i>Desulfuromonas</i>	No amplification				Not tested
13	16S	DSM2_16S	<i>Desulfuromonas michiganensis</i>	-4.36	33.86	0.952	87.07	Rejected
14	16S	DF1_16S	<i>Dehalobium</i>	-3.37	45.66	0.998	97.84	Accepted
15	16S	DHB2_16S	<i>Ca</i> DIEL	-3.48	40.42	0.999	93.99	Accepted
16	16S	DHB3_16S	<i>Ca</i> DIEL	-3.37	38.99	0.999	98.13	Accepted
17	16S	DHB4_16S	<i>Dehalobacterium</i>	-3.54	39.21	0.999	91.80	Rejected
18	16S	ACMB_16S	<i>Acidaminobacter</i>	-3.55	41.28	0.999	91.43	Accepted
19	16S	NTSP_16S	<i>Nitrospira</i>	-3.42	40.72	1.000	95.91	Accepted
20	16S	SPIRO1_16S	<i>Spirochaetes</i>	-3.33	40.02	0.997	99.52	Accepted
21	16S	SPIRO2_16S	<i>Spirochaetes</i>	-3.18	38.07	0.964	106.21	Accepted
22	16S	DSV_16S	<i>Desulfovibrio</i>	-3.35	39.57	0.998	98.68	Accepted
23	16S	SPG_16S	<i>Sphingobacteria</i>	-3.35	39.56	0.999	98.69	Accepted
24	16S	METSP1_16S	<i>Methanospirillum</i>	-3.27	38.67	0.999	102.12	Accepted

Assay #	Target	Assay Name	Genus	Slope	Y-intercept	R ²	Efficiency (%)	Rejected or accepted
25	16S	METSP2_16S	<i>Methanospirillum</i>	-3.49	41.65	0.999	93.56	Accepted
26	16S	VAD27_16S	<i>Rikenellaceae</i>	-3.41	40.34	1.000	96.28	Accepted
27	RDase	GEO_PCEA	<i>Geobacter</i>	-3.61	41.61	0.998	89.37	Accepted
28	RDase	DHC195_PCEA	<i>Dehalococcoides</i>	-3.42	39.36	1.000	96.25	Accepted
29	RDase	DHCCBDB_PCEA	<i>Dehalococcoides</i>	-3.44	42.68	0.998	95.39	Accepted
30	RDase	SFP_PCEA	<i>Sulfurospirillum</i>	-3.48	40.10	1.000	93.78	Accepted
31	Hydrogenase	DHCP_HYCE	<i>Dehalococcoides</i>	-3.56	41.09	0.999	90.82	Accepted
32	RDase	DF_PCEA	<i>Desulfitobacterium</i>	-3.40	39.48	1.000	96.92	Accepted
33	RDase	DHCFL2_TCEA	<i>Dehalococcoides</i>	-3.26	38.00	1.000	102.48	Accepted
34	RDase	DHC_CBRA	<i>Dehalococcoides</i>	-3.40	40.62	1.000	96.98	Accepted
35	RDase	DHC_MBRA1	<i>Dehalococcoides</i>	-3.42	40.24	1.000	96.07	Accepted
36	RDase	DHC_MBRA2	<i>Dehalococcoides</i>	-3.53	40.15	0.996	91.89	Accepted
37	RDase	DHC_DCPA	<i>Dehalococcoides</i>	No amplification				Rejected
38	RDase	DHC_VCRA1	<i>Dehalococcoides</i>	-3.53	40.15	0.996	91.89	Accepted
39	RDase	DHC_BVCA1	<i>Dehalococcoides</i>	No amplification-plasmid has an issue				Rejected
40	RDase	DHC_VCRA2	<i>Dehalococcoides</i>	No amplification				Rejected
41	RDase	DHC_BVCA2	<i>Dehalococcoides</i>	No amplification-plasmid had an issue				Rejected
42	B12	DHCP_CBIZ1	<i>Dehalococcoides</i>	-3.24	39.00	0.991	103.51	Accepted
43	B12	DHCP_CBIZ2	<i>Dehalococcoides</i>	-3.54	41.19	0.999	91.55	Accepted
44	B12	DHCP_COBU	<i>Dehalococcoides</i>	-3.53	40.84	0.996	91.99	Accepted
45	VC Oxidase	ETNE	<i>Mycobacterium</i>	-3.24	37.45	1.000	103.38	Accepted
46	VC Oxidase	ETNC1	<i>Mycobacterium</i>	-3.38	40.83	1.000	97.51	Accepted
47	VC Oxidase	ETNC2	<i>Uncultured bacterium</i>	-3.46	41.03	1.000	94.52	Accepted
48	CISM	DHC_FDHA	<i>Dehalococcoides</i>	-3.41	39.87	1.000	96.32	Accepted
49	Hydrogenase	DHCP_VHUA	<i>Dehalococcoides</i>	-3.25	37.27	1.000	103.31	Accepted
50	Hydrogenase	DHCP_FEHYMC1	<i>Dehalococcoides</i>	-3.26	39.43	1.00	102.48	Accepted
51	Hydrogenase	DHCP_FEHYMC2	<i>Dehalococcoides</i>	-3.63	42.44	0.995	88.73	Accepted
52	Hydrogenase	DHC_HUPL	<i>Dehalococcoides</i>	-3.24	38.20	0.981	103.40	Accepted
53	Sulfate reductase	SUL_APS	<i>Desulfovibrio</i>	No amplification				Rejected
54	Cytochrome c	ADEH_CYTC1	<i>Anaeromyxobacter</i>	No amplification				Not tested
55	Control	MIC_LUCI	<i>Photinus</i>	-3.42	39.16	0.999	95.92	Accepted

The criteria for selecting productive qPCR assays included: Amplification efficiency between 85 and 110%, standard curve slope between -3.1 to -3.6, and coefficient of determination $R^2 > 0.995$. As summarized in Table 17, most assays met these qPCR criteria. Some assays did not yield amplification products or resulted in non-specific or poor amplification. The Total Archaea and Total Bacteria 16S rRNA gene-targeted assays showed amplification efficiencies of $>120\%$, which was attributed to the degeneracy required in the primer sequences to amplify across a broad phylogenetic range of organisms, or to the use of a plasmid DNA mixture as a standard, which may impact annealing of degenerate primers. Assays, which did not meet the criteria (Table 17) were redesigned and validated before a new order of RD-qChips was placed. The exceptions were the assays for Total Archaea and Total Bacteria because optimization was not possible

and the information obtained would still be useful for comparative analysis and data interpretation. To verify the consistency between the RD-qChips, two chips were run in parallel with the same DNA mixtures. Figure 11 shows technical replicates across two separate v1 array plates at six dilutions (1.58 E+06, 1.58E+05, 1.58E+04, 1.58E+03, 1.58E+02, 1.58E+01 gene copies per 33 nL reaction volume). Most of the Crt (relative threshold cycle) values obtained from duplicate chips fell into the 95% confidence ellipse (green ellipse) (Figure 11). The slope of the blue fitted linear line (i.e., 0.9888, ~45-degree) reflects the replicability of assays across duplicate chips. The outlier Crt values belong to unproductive assays. Overall, the results demonstrate high reproducibility of assays within an array plate and between RD-qChip v1 array plates.

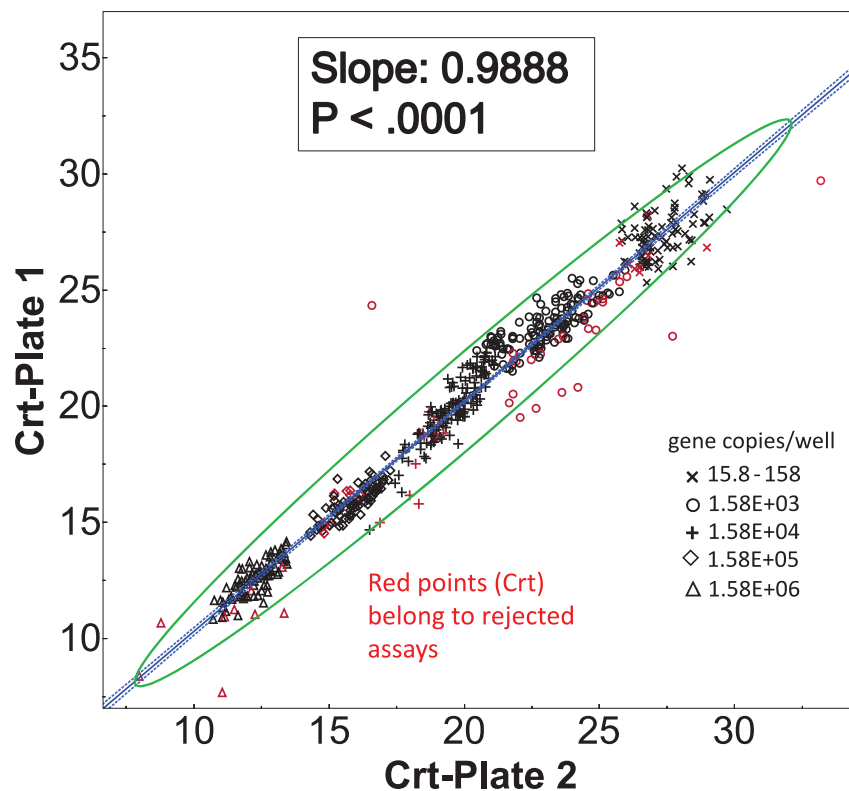


Figure 11. Assay replication results across duplicate RD-qChip v1. The green ellipse shows a 95% confidence interval. The blue line represents a linear fit with a slope of 0.9888 and a p-value < 0.0001.

The sensitivity of the QuantArray was tested by Crt responses against 10-fold serial dilutions of DNA standards. K-means statistical clustering (Hartigan et al., 1979) was applied to six dilutions (1.58 E+06, 1.58E+05, 1.58E+04, 1.58E+03, 1.58E+02, 1.58E+01 gene copies per 33 nL reaction volume) (Figure 12) using JMP statistical software. As seen in Figure 12, the Crt values clustered into five distant groups and amplification variability was observed in higher dilutions (i.e., 158 to 15.8 gene copies/33nL). Higher variability at low target copy numbers was expected due to the small reaction volume of 33 nL and has been observed previously (Mayer-Blackwell et al., 2014; Morrison et al., 2006). At lower dilutions with higher template DNA concentrations, linear correlation within accepted ranges was obtained.

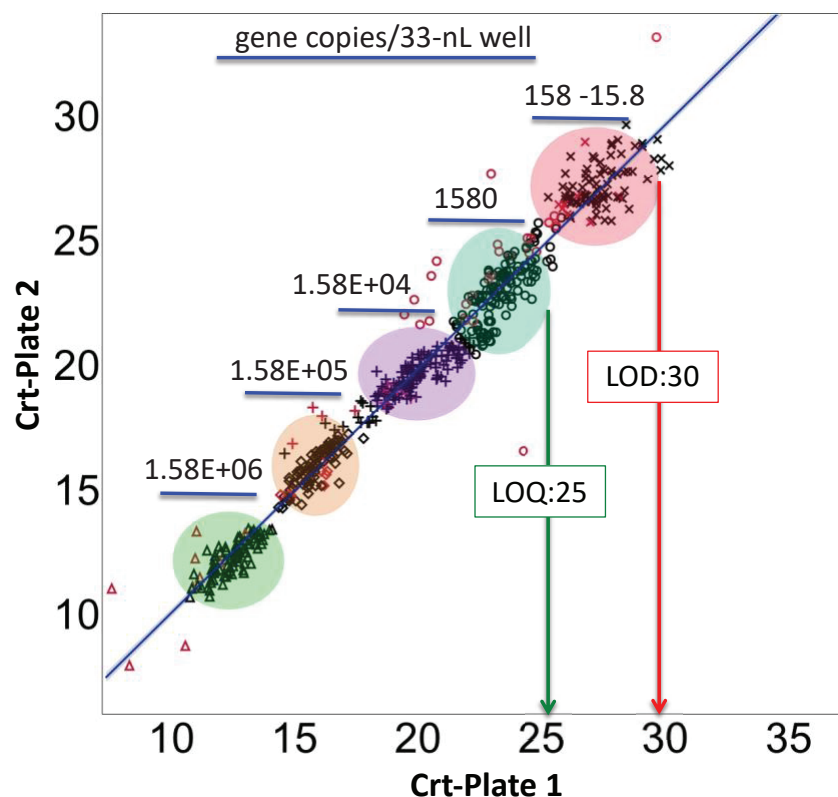


Figure 12. K-means statistical clustering applied to six DNA dilutions.

The most common method applied for data analysis for traditional qPCR is called as the Ct method (baseline threshold method). Applied Biosystems has developed an alternative method known as the Crt method (relative threshold method), which has proven to be more robust for analyzing data generated by the Applied Biosystems QuantStudio 12K Flex RealTime PCR System for the OpenArray plates (https://assets.thermofisher.com/TFS-Assets/LSG/brochures/CO28730-Crt-Tech-note_FLR.pdf).

It has been reported that the relative threshold method produces more reproducible Crt values for replicate assays on the same plate and lower standard deviations across replicate samples. The Crt method also enables faster analysis of the data sets, and was used for the analysis of data sets generated with the RD-qChip array plates.

To determine the limit of detection (LOD), frequencies of detection of each serial dilution were calculated (Table 18). The LOD is typically assumed to be the highest Crt value observed for a truly positive sample and used to determine what acceptable Crt values are for the specific assay. The LOD might be different for each assay, and all assays were considered carefully while defining the LOD for RD-qChip v1. The lowest DNA dilution with at least 80% detection frequency was selected as LOD. Accordingly, the LOD was $Crt \approx 30$, in other words, the LOD was determined to be 15.8 gene copies per 33 nL reaction volume with 95.9% detection frequency, which equals approximately $1.0E+03$ gene copies/ μ L of sample or 0.01 pg of DNA/ μ L of sample (Table 18, Figure 12). With an initial target gene abundance of 1.58 copies per 33-nL well, a detection frequency of >50% was achieved, indicating a high detection sensitivity of the approach.

Table 18. Frequencies of detection of DNA standards using RD-qChip v1.

Gene Copies/33 nL	1.58E+06	1.58E+05	1.58E+04	1.58E+03	1.58E+02	15.8	1.58	0.158
Detection Frequency (%)	95.9	97.4	97.4	97.9	95.8	95.9	57.2	7.6

The limit of quantification (LOQ) is the highest DNA dilution of each assay that results in an amplification signal within the linear range of the standard curve for the particular assay. For the RD-qChip v1, the LOQ was the dilution with 1,580 initial gene copies per 33-nL reaction volume (i.e, Crt ≈25). This initial target concentration equals approximately 1.0E+05 gene copies/μL of sample or 1 pg of DNA/μL of sample. A discernable signal was obtained at lower template concentrations and Crt values smaller than the LOQ; however, if this signal does not fit the linear standard curve, it was considered detectable but non-quantifiable (DNQ).

To achieve lower detection and quantification limits, DNA extracts can be concentrated, or larger volumes of groundwater can be filtered and used for DNA extraction. For example, assuming 100% DNA extraction efficiency, the practical detection limit (PDL) will be ~ 1.0E+04 copies/mL of sample if a 10-mL volume of original sample is filtered and the DNA is collected in a total volume of 50 μL (based on minimum detection limit value of 7.92 determined for qChip v1). If 1 L of groundwater is filtered and the DNA is eluted in a total volume of 50 μL, the PDL becomes ~ 1.0E+02 copies/mL of groundwater or ~ 1.0E+05 copies per L of groundwater.

The PDL for RD-qChip v1 can be calculated according to the following formula (Mayer-Blackwell et al., 2014):

$$PDL = MDL * \left(\frac{VDNA\ sample}{Vsample} \right) * \left(\frac{Vmastermix}{VDNA\ added} \right) * \left(\frac{1}{Vwell} \right) * \left(\frac{1000\ \mu L}{1\ mL} \right) \quad \text{Equation 5.}$$

$$PDL = 15.8 * (50/1 \times 10^6) * (5/1.2) * (1/0.033) * (1000\ \mu L/1\ mL) = 99.7\ \text{gene copies/mL} \sim 1.0E+05\ \text{gene copies/L}$$

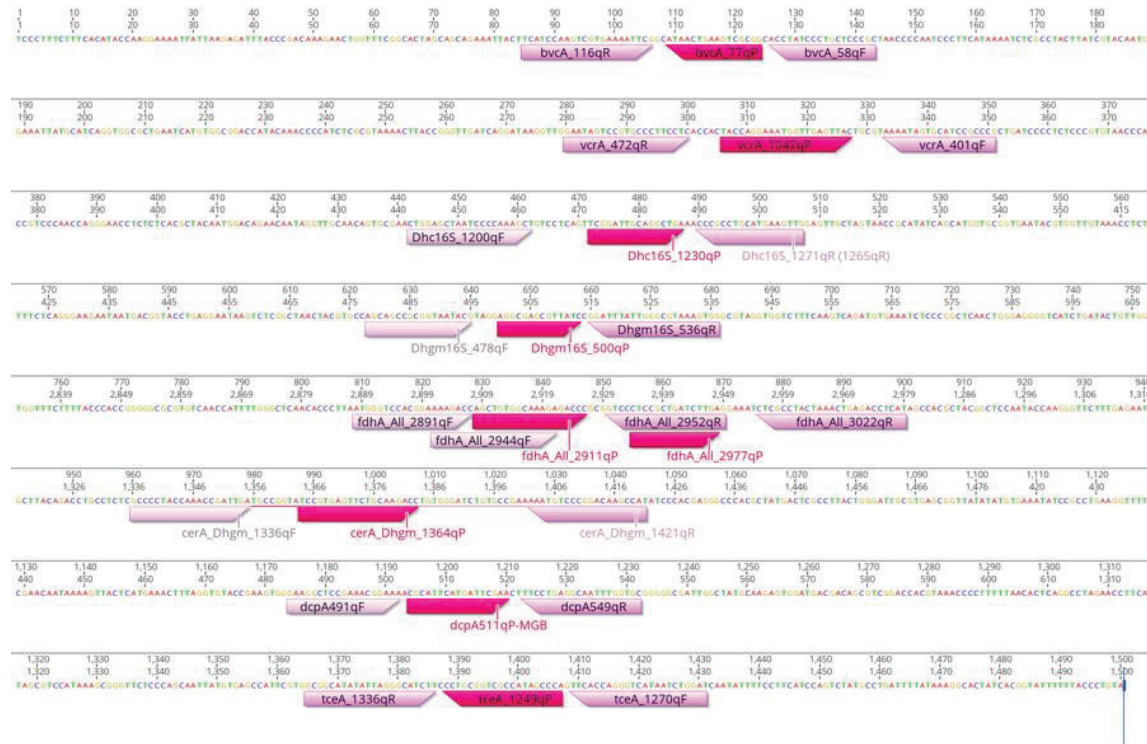
Validation of RD-qChip v2

For validation of RD-qChip v1 array plates, two pDNA mixtures (DNA Mixture 1 for 16S rRNA genes and DNA Mixture 2 for functional genes located on RD-qChip v1) of individual plasmids were used for standard curve preparation. The results indicated that the use of pDNA mixtures affected the amplification efficiencies of some assays and produced higher than expected LOD and LOQ values. Possible reasons for these observations included dilution/pipetting errors during the preparation pDNA mixtures, or unintended interactions between pDNAs (e.g., hairpin secondary structure formation). Further, the preparation of standard pDNA mixtures is a cumbersome and time-consuming effort. In order to overcome these issues for RD-qChip v2 and avoid isolation and mixing of 102 pDNAs, 11 linear DNA fragments (super oligos) were designed with each having 6-12 target gene fragments ranging between 100-300 bp in length. The target gene fragments were chosen using Geneious version 11.0.2 software to generate a final linear DNA fragment with a size of about 2,000 bp. The Mfold web server (<http://unafold.rna.albany.edu/>) (Zuker, 2003) was used to check folding, hybridization prediction, and determine the formation of unintended secondary structures. The designed linear DNA fragment super oligos that passed this test were uploaded to the IDT gBlocks® Gene Fragments Design Tool to check complexity and codon optimization of the sequence. One of the designed super oligos is depicted in Figure 13. The linear dsDNA fragments (LDF) were custom synthesized by IDT and validated by performing qPCR on 384-well plates.

The experimental efforts demonstrated the utility of the LDFs for more efficient construction of standard curves, and the decision was made to transition to the LDF standard curve approach. Unexpectedly, IDT was not able to synthesize all of the requested LDFs because the company had changed their synthesis pipeline. Response from IDT: *“We had made a change to our synthesis process in April 2018 which needed some time for optimizations just like any new process would. During this time, we observed an uptick in delayed orders and also the failure rates. We are in process of wrapping up that optimization and hope to meet your expectations better in future. Despite this situation we had at our end, I completely understand the frustration you are going through and I deeply apologize for the inconvenience you had to experience.”* IDT Team.

In order to overcome this problem, linear DNA fragments carrying 6-10 target gene fragments between 100-300 bp in length were redesigned using Geneious version 11.0.2 software to generate larger LDFs with a size of 1,500 bp. The Mfold web server was used to check as previously described for folding, hybridization prediction, and determine the formation of unintended secondary structures. The designed LDFs were loaded into Life Technologies GenArt Strings DNA Fragments Assistant Software to check secondary structure, GC content, presence of repetitive sequences and long base runs in the LDFs. The LDFs that passed the test were ordered from Invitrogen (Invitrogen, Carlsbad, CA). An example for a designed synthetic LDF is depicted in Figure 13.

Figure 13. Example for a custom-designed linear DNA fragment (LDF) for preparation of qPCR standard curves for the RD-qChip v2.



Using synthetic LDFs, operator-introduced errors and the formation of unintended secondary structures that affect amplification can be effectively removed. Further, this approach is much less laborious and time-consuming than the preparation of a standard mixture of 102 pDNAs (102 biomarker targets included in the RD-qChip v2 design). This approach eliminates the need to clone 102 genes or gene fragments into plasmids, transform them into *E. coli*, confirm insertion by sequencing, and isolate plasmids. This refined approach addresses the aforementioned problems with reduced amplification efficiencies and lower LOD and LOQ values.

For validation of RDase qChip v2, synthetic linear DNA fragments served as templates for standard curve preparation for validation of TaqMan® qPCR assays on RD-qChip v2. To prepare standard curves, 14 synthetic LDFs were mixed together to achieve 1.22E+08 target gene copies/μL. Then a 10-fold serial dilution series of the LDF- mixture was prepared ranging from 1.22 E+08 to 1.22E+01 target gene copies/μL corresponding to 1.61E+06 to 1.61E-01 gene copies per 33-nL well (Table 19).

Table 19. Dilution series of standard LDF mixture assayed on replicate plates of RD-qChip v2.

Concentration (ng/μL)	0.2	0.02	0.002	2.0E-04	2.0E-05	2.0E-06	2.0E-07	2.0E-08
Target gene copies/μL	1.22E+08	1.22E+07	1.22E+06	1.22E+05	1.22E+04	1.22E+03	1.22E+02	1.22E+01
Target gene copies/33-nL well	1.61E+06	1.61E+05	1.61E+04	1.61E+03	1.61E+02	1.61E+01	1.61E+00	1.61E-01

The sensitivity and specificity of the assays included in RD-qChip v2 were validated by calculation of amplification efficiencies and linear regression coefficients based on standard curve analysis using Equation 3 and Equation 4. For this purpose, a standard curve for each target assay was constructed by plotting the C_{rt} values against log₁₀ gene copies of the dilution series of the LDF standard mixture. An example of a standard curve generated based on the enumeration of a standard LDF mixture on RDqChip v2 for the *Dhc* 16S rRNA assay is illustrated in Figure 14.

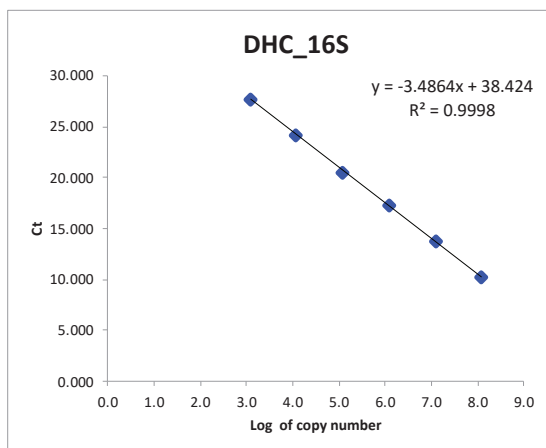


Figure 14. Standard curve generated for the *Dhc* 16S rRNA assay by plotting C_{rt} values against the log gene copy numbers of the dilution series of the LDF standard mixture.

The slope, linear regression standard coefficient (R²) and amplification efficiency (%) values of the standard curve generated for each TaqMan® assay based on data obtained from the run of the standard LDF mixture on the RD-qChip v2 plates were determined. The slope, linear regression standard coefficient (R²) and amplification efficiency (%) values from the run of the LDF standard mixture on a RD-qChip v2 plate are shown in Table 20.

Table 20. Experimental validation of RD-qChip v2 performance. Shown are the amplification efficiencies, linear regression coefficients, Y-intercepts, and slopes obtained from standard curves generated from the run of the LDF DNA standard mixture on the RD-qChip v2.

#	Target Gene Category	Assay Name	Target (genus, species, strain, or gene)	Slope	Y-intercept	R ²	Efficiency (%)
1	16S	AMYXO_16S	<i>Anaeromyxobacter</i>	-3.55	37.06	0.999	91.43
2	16S	BAC_16S	Total Bacteria	-3.52	36.48	0.998	92.34
3	16S	DCM_ACMB_16S	<i>Acidaminobacter</i> ^a	-3.45	37.51	1.000	95.02
4	16S	DCM_DSV_16S	<i>Desulfovibrio</i> ^a	-3.40	37.09	1.000	96.89
5	16S	DCM_METSP_16S	<i>Methanospirillum</i> ^a	-3.46	36.00	1.000	94.66
6	16S	DCM_NTSP_16S	<i>Nitrospira</i> ^a	-3.39	36.50	1.000	97.10
7	16S	DCM_SPG_16S	<i>Sphingobacteria</i> ^a	-3.40	36.25	1.000	96.85
8	16S	DCM_SPIRO_16S	<i>Spirochaetes</i> ^a	-3.53	37.51	0.999	92.04
9	16S	DCM_VAD27_16S	<i>Rikenellaceae</i> ^a	-3.49	37.00	1.000	93.48
10	16S	DIEL_16S	' <i>Ca. Diel</i> ' ^a	-3.49	37.59	0.999	93.48
11	16S	DF1_16S	<i>Dehalobium chlorocoercia</i> DF1	-3.33	35.97	0.999	99.73
12	16S	DEFO_16S	<i>Dehalobacterium formicoaceticum</i>	-3.41	36.30	0.999	96.57
13	16S	DHB_16S	<i>Dhb</i>	-3.38	35.44	1.000	97.47
14	16S	DHC_16S	<i>Dhc</i>	-3.48	38.30	1.000	93.92
15	16S	DHC_16S	<i>Dhc</i>	-3.49	38.42	1.000	93.57

#	Target Gene Category	Assay Name	Target (genus, species, strain, or gene)	Slope	Y-intercept	R ²	Efficiency (%)
16	16S	DHCCOR_16S	<i>Dhc</i> Cornell subgroup	-3.49	37.62	1.000	93.50
17	16S	DHGM_16S	<i>Dehalogenimonas</i>	-3.54	37.44	0.998	91.72
18	16S	DSF_16S	<i>Desulfitobacterium</i>	-3.44	35.80	1.000	95.15
19	16S	FLIPS_16S	<i>Sphaerochaeta</i>	-3.50	37.42	1.000	93.22
20	16S	GEO_1_16S	<i>Geobacter</i>	-3.47	37.00	1.000	94.23
21	16S	GEO_2_16S	<i>Geobacter</i>	-3.59	37.75	1.000	90.06
22	16S	METH_16S	Methanogenic Archaea	-3.50	36.74	1.000	93.06
23	16S	SFP_16S	<i>Sulfurospirillum</i>	-3.42	36.13	1.000	96.21
24	Control	LUC	Firefly luciferase	-3.31	37.42	0.999	100.62
25	Control	RPOB_DHC_ALL	<i>Dhc</i> RNA polymerase β -subunit	-3.31	36.25	1.000	100.64
26	B12	BTUC_DHC_C	<i>Dehalococcoides</i> Cornell subgroup	-3.45	38.39	0.999	95.04
27	B12	BTUC_DHC_P_V	<i>Dhc</i> Pinellas & Victoria subgroups	-3.44	36.32	0.999	95.39
28	B12	BTUD_DHC_P	<i>Dhc</i> Cornell subgroup	-3.54	37.47	1.000	91.54
29	B12	BTUD_DHC_V	<i>Dhc</i> Pinellas subgroup	-3.37	37.89	0.999	98.04
30	B12	BTUD_DHC_C	<i>Dhc</i> Victoria subgroup	-3.45	36.53	1.000	95.03
31	B12	BTUF_DHC_ALL	<i>Dhc</i>	-3.43	36.34	0.999	95.66
32	B12	CBIZ_P	<i>Dhc</i> Pinellas subgroup	-3.43	37.17	1.000	95.78
33	B12	CBIZ_V_C	<i>Dhc</i> Cornell & Victoria subgroups	-3.51	37.60	0.999	92.63
34	B12	COBA_DHC_ALL	<i>Dhc</i>	-3.50	36.37	1.000	93.20
35	B12	COBC_DHC_P	<i>Dhc</i> Pinellas subgroup	-3.48	37.80	1.000	93.74
36	B12	COBC_DHC_C_V	<i>Dhc</i> Cornell & Victoria subgroups	-3.35	36.99	0.999	98.98
37	B12	COBS_DHC_P1	<i>Dhc</i> Pinellas subgroup	-3.43	36.22	1.000	95.57
38	B12	COBS_DHC_P2	<i>Dhc</i> Pinellas subgroup	-3.51	35.96	0.998	92.63
39	B12	COBS_DHC_C_V	<i>Dhc</i> Cornell & Victoria subgroups	-3.44	37.47	1.000	95.33
40	B12	COBT_DHB	<i>Dhb</i>	-3.42	35.93	1.000	96.17
41	B12	COBT_DHC_ALL	<i>Dhc</i>	-3.38	36.58	1.000	97.54
42	B12	COBT_DHC_ALL	<i>Dhc</i>	-3.42	36.22	0.999	96.12
43	B12	COBT_DSF	<i>Desulfitobacterium</i>	-3.45	36.61	1.000	95.03
44	B12	COBU_DHC_C	<i>Dhc</i> Cornell subgroup	-3.43	36.68	1.000	95.83
45	B12	COBU_DHC_P	<i>Dhc</i> Pinellas subgroup	-3.43	36.55	1.000	95.55
46	B12	COBU_DHC_V	<i>Dhc</i> Victoria subgroup	-3.26	36.87	0.999	102.79
47	ETC	CISM_DHC_ALL_1	<i>Dhc</i>	-3.39	36.81	1.000	97.26
48	ETC	CISM_DHC_ALL_2	<i>Dhc</i>	-3.42	36.87	1.000	96.04
49	ETC	CISM_DHC_ALL_2	<i>Dhc</i>	-3.44	36.93	1.000	95.22
50	Hydrogenase	ECHE_DHC_ALL_1	<i>Dhc</i>	-3.54	37.24	0.999	91.56
51	Hydrogenase	ECHE_DHCALL_2	<i>Dhc</i>	-3.59	38.05	1.000	89.98
52	Dehydrogenase	FDHA1_DHGM_BLDC9	<i>Dhgm lykanthroporepellens</i> BLDC-9	-3.57	37.07	0.999	90.59
53	Dehydrogenase	FDHA2_DHGM_BLDC9	<i>Dhgm lykanthroporepellens</i> BLDC-9	-3.46	37.12	1.000	94.60
54	Dehydrogenase	FDHA3_DHGM_BLDC9	<i>Dhgm lykanthroporepellens</i> BLDC-9	-3.46	37.50	1.000	94.61
55	Hydrogenase	HUPL_DHC_ALL_1	<i>Dhc</i>	-3.41	36.22	1.000	96.44
56	Hydrogenase	HUPL_DHC_ALL_2	<i>Dhc</i>	-3.55	36.85	1.000	91.45
57	Hydrogenase	HYCE_DHC_ALL_1	<i>Dhc</i>	-3.44	36.58	1.000	95.31
58	Hydrogenase	HYCE_DHC_ALL_2	<i>Dhc</i>	-3.46	37.04	1.000	94.55

#	Target Gene Category	Assay Name	Target (genus, species, strain, or gene)	Slope	Y-intercept	R ²	Efficiency (%)
59	Hydrogenase	HYMB_DHC_ALL	<i>Dhc</i>	-3.43	36.55	1.000	95.75
60	Hydrogenase	HYMC_DHC_ALL_1	<i>Dhc</i>	-3.39	36.76	0.999	97.24
61	Hydrogenase	HYMC_DHC_ALL_2	<i>Dhc</i>	-3.49	37.18	1.000	93.29
62	Hydrogenase	VHUA_DHC_ALL	<i>Dhc</i>	-3.38	35.80	0.999	97.44
63	Nitrogenase	NIFD_DHC_C	<i>Dhc</i> Cornell subgroup	-3.49	36.81	0.999	93.33
64	Nitrogenase	NIFD_DHV	<i>Desulfovibrio vulgaris</i>	-3.44	36.72	1.000	95.14
65	Nitrogenase	NIFH_DHC_C	<i>Dhc</i> Cornell subgroup	-3.46	38.02	1.000	94.41
66	Nitrogenase	NIFH_DHC_P	<i>Dhc</i> Pinellas subgroup	-3.38	37.46	1.000	97.50
67	Nitrogenase	NIFK_DHC_C	<i>Dhc</i> Cornell subgroup	-3.36	35.92	1.000	98.40
68	RDase	BVCA	<i>Dhc</i>	-3.39	36.25	1.000	97.20
69	RDase	CBRA	<i>Dhc</i>	-3.40	37.21	1.000	96.79
70	RDase	CERA	<i>Dhgm etheniformans</i> strain GP	-3.46	36.92	1.000	94.68
71	RDase	CF_ALL	<i>Dehalobacter/Desulfitobacterium</i>	-3.47	35.92	1.000	94.17
72	RDase	CFRA	<i>Dhb</i>	-3.37	37.68	1.000	97.92
73	RDase	CTRA_THMA	<i>Desulfitobacterium/Dehalobacter</i>	-3.56	37.40	1.000	90.90
74	RDase	DCAA_DHB_WL	<i>Dhb</i>	-3.48	36.49	1.000	93.93
75	RDase	DCAA_DSF	<i>Desulfitobacterium</i>	-3.49	36.77	1.000	93.61
76	RDase	RDHA-AWM53_01188-RM	' <i>Ca. Diel</i> ' ^a	-3.47	36.48	1.000	94.12
77	RDase	RDHA-AWM53_01801-RM	' <i>Ca. Diel</i> ' ^a	-3.47	37.90	1.000	94.00
78	RDase	RDHA-AWM53_00864-RM	' <i>Ca. Diel</i> ' ^a	-3.49	37.11	1.000	93.31
79	RDase	DCMB_1041_RDHA	<i>Dhc</i>	-3.45	36.33	1.000	94.90
80	RDase	DCMB_1339_RDHA	<i>Dhc</i>	-3.41	36.30	1.000	96.65
81	RDase	DCMB_1434_RDHA	<i>Dhc</i>	-3.35	35.81	1.000	98.96
82	RDase	DCPA	<i>Dhc /Dhgm</i>	-3.42	37.40	1.000	96.23
83	RDase	DCRA_DHB	<i>Dhb</i>	-3.49	37.04	1.000	93.26
84	RDase	DHGM_GP_00862 RDHA	<i>Dhgm etheniformans</i> strain GP	-3.40	36.30	1.000	96.88
85	RDase	DHGM_GP_01297 RDHA	<i>Dhgm etheniformans</i> strain GP	-3.53	37.34	0.999	92.04
86	RDase	DHGM_GP_01300 RDHA	<i>Dhgm etheniformans</i> strain GP	-3.46	37.37	1.000	94.65
87	VC Oxidase	ETNC	<i>Mycobacterium</i>	-3.35	36.36	0.999	98.64
88	VC Oxidase	ETNE	<i>Mycobacterium</i>	-3.34	35.72	1.000	99.12
89	VC Oxidase	ETNE_MB	<i>Mycobacterium</i> & uncult. bact.	-3.32	34.81	1.000	100.11
90	VC Oxidase	ETNE_MPO	<i>Mycobacterium, Ochrobactrum, Pseudomonas</i>	-3.39	35.32	0.999	97.13
91	VC Oxidase	ETNE_NCD	<i>Nocardioide</i> s uncult. bacterium	-3.44	34.93	0.999	95.44
92	VC Oxidase	ETNE2	Uncultured bacterium	-3.43	37.36	1.000	95.73
93	RDase	HPR1A_RDHA_P	<i>Dhc</i> Pinellas subgroup	-3.50	37.03	1.000	92.93
94	RDase	HPR2A_RDHA_C	<i>Dhc</i> Cornell subgroup	-3.49	37.03	1.000	93.42
95	RDase	MBRA	<i>Dhc</i>	-3.47	36.95	1.000	94.32
96	RDase	PCB1_CG4_CG5	<i>Dhc</i>	-3.44	36.77	1.000	95.12
97	RDase	PCB2_CG1	<i>Dhc</i>	-3.40	36.55	1.000	96.67
98	RDase	PCEA_195	<i>Dhc</i>	-3.42	36.81	0.998	96.14
99	RDase	CBDBA1588_DHC_RDHA	<i>Dhc</i>	-3.37	36.72	1.000	98.11
100	RDase	PCEA_DHB_DSF	<i>Dhb/Desulfitobacterium</i>	-3.50	37.75	1.000	93.00
101	RDase	PCEA_GEO	<i>Geobacter</i>	-3.45	36.89	1.000	94.92
102	RDase	PCEA_SFP	<i>Sulfurospirillum</i>	-3.42	36.64	1.000	96.20
103	RDase	SDC9_23241-RDHA	<i>Dhc</i>	-3.48	37.34	1.000	93.82
104	RDase	SDC9_45582-RDHA	<i>Desulfitobacterium</i>	-3.49	38.01	1.000	93.46

#	Target Gene Category	Assay Name	Target (genus, species, strain, or gene)	Slope	Y-intercept	R ²	Efficiency (%)
105	RDase	SDC9_07142-RDHA	<i>Dhc</i>	-3.42	36.77	1.000	96.19
106	RDase	SDC9_07143-RDHA	<i>Dhc</i>	-3.34	35.41	0.999	99.43
107	RDase	SDC9_48350-RDHA	<i>Dhb/Desulfitobacterium</i>	-3.38	35.55	1.000	97.61
108	RDase	SDC9_23243-RDHA	<i>Dhc</i>	-3.45	36.98	1.000	94.92
109	RDase	SDC9_09280-RDHA	<i>Dhb/Desulfitobacterium</i>	-3.40	35.38	0.999	96.68
110	RDase	TCEA	<i>Dhc</i>	-3.43	36.20	1.000	95.77
111	RDase	VCRA	<i>Dhc</i>	-3.42	37.36	1.000	96.04
112	RDase	VCRA	<i>Dhc</i>	-3.39	37.32	0.998	97.07

^a Populations detected in a DCM-degrading enrichment culture RM harboring ‘*Candidatus* Dichloromethanomonas elyunquensis’ (‘*Ca.* Diel’) (Kleindienst et al., 2017).

The data shown in Table 20 demonstrate that the amplification efficiency values varied between 90 and 103%, the R² values ranged between 0.998 and 1.000, and the slope values ranged from -3.26 to -3.59. The assays with amplification efficiencies of 90-110%, slope of 3.1-3.6 and R²≥0.995 were accepted as positive assays in the highlight of MIQE guidelines. Based on these results, all TaqMan assays included in the RD-qChip v2 design met the MIQE criteria and were accepted as validated assays.

In order to check consistency and reliability of the RD qChip v2, two RD-qChip plates were run at different times with freshly prepared standard mixtures of LDFs. The comparison of Crt values across replicate RD-qChip v2 plates is illustrated in Figure 15. Linear regression, confidence interval, prediction interval and confidence ellipses were calculated and plotted using R v3.6.1 (Wickham, 2016) and ggplot2 v3.2.1 (Team, 2018) for seven dilutions of LDF-standard mixture spanning a concentrations range from 1.61E+00 to 1.61E+06 gene copies/well.

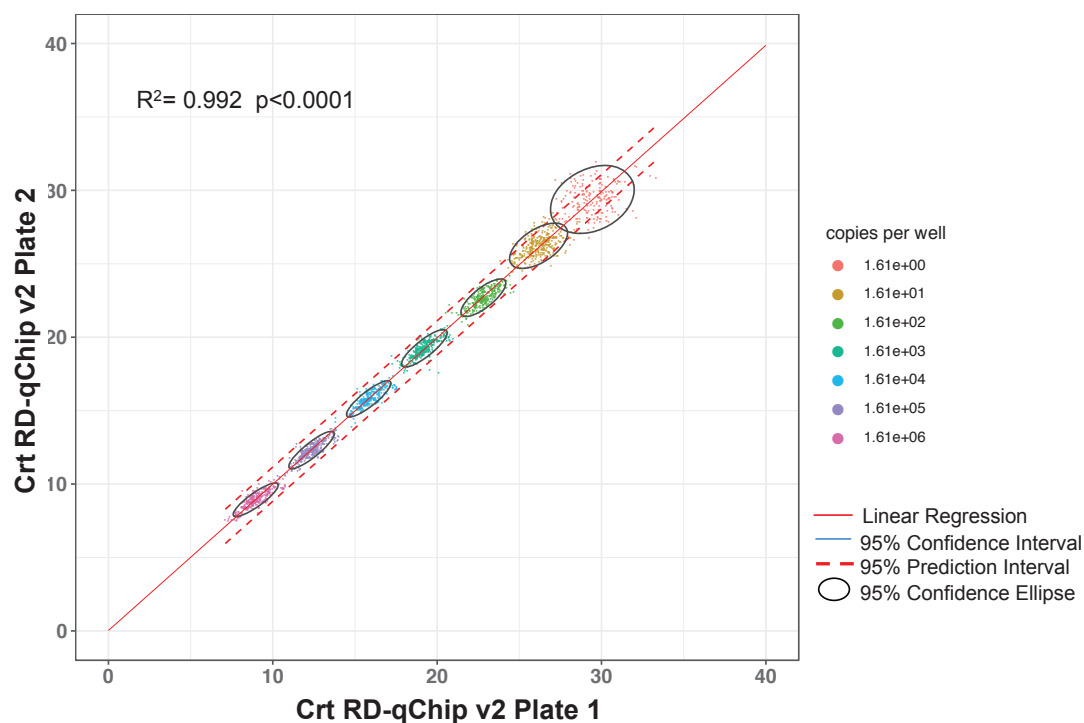


Figure 15. The comparison of Crt values across replicate RD-qChip v2 array plates.

Linear regression analysis with R v3.6.1 showed linear correlation between Crt values of two replicate RD-qChip v2 plates obtained from run of the standard mix of LDFs. Crt values obtained from replicate RD-qChip v2 plates demonstrated a significant positive correlation (Pearson's $r = 0.992$ $p < 0.0001$), indicating high reproducibility, reliability and consistency of different RD-qChip v2 array plates (Figure 15). The increase in dilution ratio led data variability increased at highest dilution ratios, presumably due to the propagation of pipetting inconsistencies. Similar observations have been reported in other studies using nL high-throughput qPCR platforms (Grembi et al., 2019; Zhao et al., 2019b).

The limit of detection (LOD) and limit of quantification (LOQ) are considered important parameters for the validation of qPCR assays. The LOD is defined as the lowest concentration (highest Crt value), at which 95% of the positive samples could be detected but may not be quantified. The LOQ is the lowest concentration of target DNA that can be quantified with acceptable precision and accuracy. In the context of qPCR, the LOQ is the lowest concentration that is in a linear range of the standard curve. As seen in Figure 16, the LOD was determined to be $\sim 1.6 \times 10^1$ gene copies/well (~ 120 -760 gene copies/ μL). The Crt for the LOQ was in the range of 23.9-28.5 for the assays and ~ 10 -16 gene copies were required per well (760-1,200 copies/ μL) to allow enumeration of the target gene.

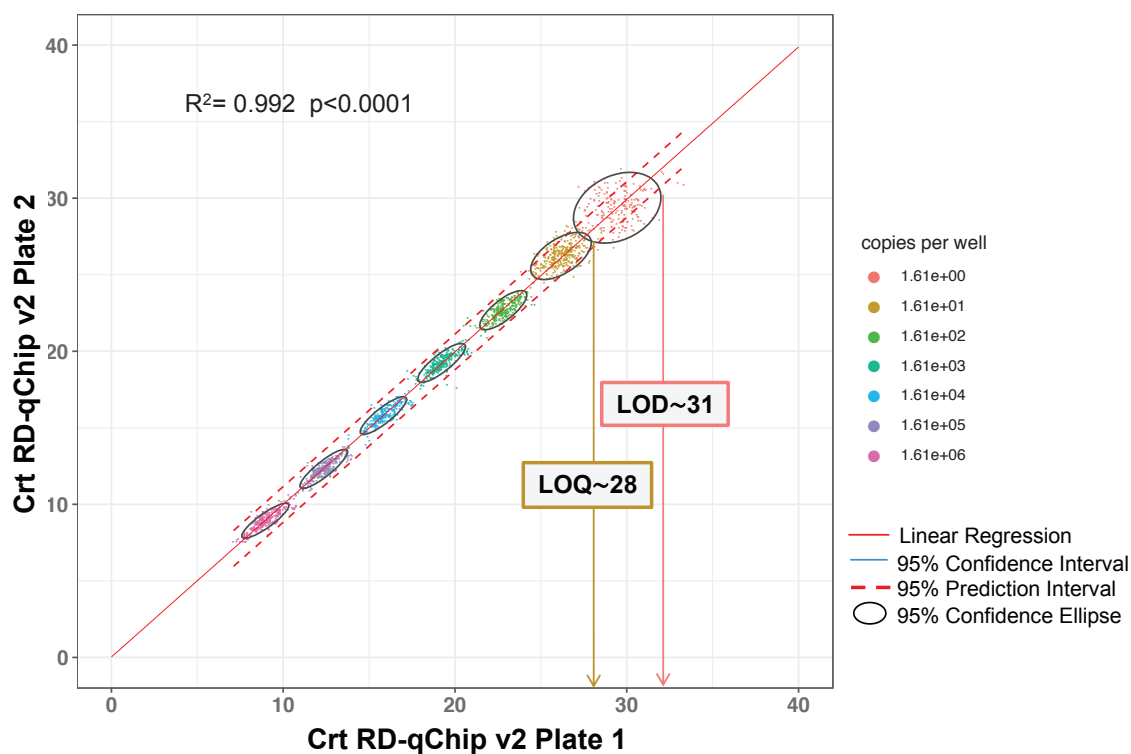


Figure 16. Ten-fold dilutions of mixed standard of linear DNA fragments to evaluate the sensitivity and specificity of RD qChip v2.

Dilutions ranged from 1.6×10^0 to 1.6×10^6 gene copies/well and LOD and LOQ values are indicated.

Of value for real world applications is the practical detection limit (PDL), which can be calculated according to Equation 5 (Mayer-Blackwell et al., 2014).

For example, if 1 L of groundwater is filtered, the DNA is eluted in a total volume of 50 μL

(assuming 100% DNA extraction efficiency) and the assumed minimum detection limit (MDL) is 5 gene copies/well, the practical detection limit for the RD-qChip v2 for a 1-L groundwater sample can be calculated as:

MDL : 5 gene copies/well (~1.6-10 gene copies/well, 5 was taken as an average)

V_{sample} : 1 L

$V_{\text{DNA sample}}$: 50 μL

V_{well} : 0.033 μL

$V_{\text{mastermix}}$: 5 μL

$V_{\text{DNA added}}$: 2 μL

$\text{PDL} = 5 * (50/1.0\text{E}+06) * (5/2) * (1/0.033) * (1000 \mu\text{L}/1 \text{ mL}) = \sim 19 \text{ gene copies/mL} = \sim 1.9\text{E}+04 \text{ gene copies/L}$

Assuming an LOQ value of 16 gene copies/well, we can use Equation 5 to calculate the practical quantification limit (PQL) according to:

$\text{PQL} = 16 * (50/1.0\text{E}+06) * (5/2) * (1/0.033) * (1000 \mu\text{L}/1 \text{ mL}) = 60.6 \text{ gene copies/mL} = 6.06\text{E}+4 \text{ gene copies/L}$

These calculations indicate that the RD-qChip v2 is able to detect 19 target gene copies/mL when 1 L of groundwater is used for biomass collection and DNA isolation. When the PQL is determined using Equation 5 (MDL is replaced with the LOQ value), the data reveal that the RD-qChip v2 can quantify ~ 61 target gene copies/mL when 1 L of groundwater is used for biomass collection and DNA isolation.

In order to lower the detection limits for target genes, larger volume of groundwater could be used for DNA extraction or the isolated DNA can be concentrated using commercially available kits (e.g., Zymo DNA Clean&Concentrator kit).

Analysis of DNA samples from pure and mixed cultures on the RD qChip v2

In order to validate RD-qChip v2, DNA samples were obtained from *Dhc* strain 195, *Dhc* strain GT, and the *Dhc*-containing bioaugmentation consortium SDC-9.

Quantitative analysis of 16S rRNA genes

The 16S rRNA gene assays placed on the RD-qChip v2 are listed in Table 21, and the results of 16S rRNA gene-targeted assays for defined laboratory samples are presented in Figure 17. *Dhc* strain 195 template DNA resulted in positive amplification of the following assays: BAC_16S (Total Bacteria 16S rRNA gene, $8.81\text{E}+06$ gene copies/mL), DHC_16S (all *Dhc* 16S rRNA genes, $8.93\text{E}+06$ gene copies/mL) and DHCCOR_16S (16S rRNA genes of Cornell subgroup *Dhc*, $8.60\text{E}+06$ gene copies/mL). The results for the different assays generated consistent quantitative data, what was expected because the target genes exist as single copy genes on the known *Dhc* genomes. No amplification was observed for other 16S rRNA gene-targeted assays. Experiments with *Dhc* strain GT genomic DNA as template also generated the expected results and the BAC_16S and DHC_16S assays determined $8.35\text{E}+06$ and $1.15\text{E}+07$ gene copies/mL, respectively whereas the DHCCOR_16S assay, which specifically targets 16S rRNA genes of the Cornell group, did not generate amplicons consistent with the affiliation of strain GT with the Pinellas subgroup.

Table 21. 16S rRNA gene-targeted assays included on the RD-qChip v2.

Assay Name	Target group, genus, or species
AMYXO_16S	<i>Anaeromyxobacter</i>
BAC_16S	Total Bacteria
DCM_ACMB_16S	<i>Acidaminobacter</i> ^a
DCM_DSV_16S	<i>Desulfovibrio</i> ^a
DCM_METSP_16S	<i>Methanospirillum</i> ^a
DCM_NTSP_16S	<i>Nitrospira</i> ^a
DCM_SPG_16S	<i>Sphingobacteria</i> ^a
DCM_SPIRO_16S	<i>Spirochaetes</i> ^a
DCM_VAD27_16S	<i>Rikenellaceae</i> ^a
DIEL_16S	‘ <i>Ca. Diel</i> ’
DF1_16S	<i>Dehalobium chlorocoercia</i> DF1
DEFO_16S	<i>Dehalobacterium formicoaceticum</i>
DHB_16S	<i>Dhb</i>
DHC_16S	<i>Dhc</i>
DHCCOR_16S	<i>Dhc</i> Cornell subgroup
DHGM_16S	<i>Dhgm</i>
DSF_16S	<i>Desulfitobacterium</i>
FLIPS_16S	<i>Sphaerochaeta</i>
GEO_1_16S	<i>Geobacter</i>
GEO_2_16S	<i>Geobacter</i>
METH_16S	Methanogenic Archaea
SFP_16S	<i>Sulfurospirillum</i>

^a Populations detected in a DCM-degrading enrichment culture RM harboring ‘*Candidatus* Dichloromethanomonas elyunquensis’ (‘*Ca. Diel*’) (Kleindienst et al., 2017).

The application of the RD qChip v2 to genomic DNA collected from consortium SDC-9 demonstrated the presence of methanogens, *Dhc*, *Desulfitobacterium*, *Sulfurospirillum*, *Sphaerochaeta* and *Desulfovibrio*. *Dhc* had the highest abundance at 4.91E+07 cells/mL. *Desulfitobacterium* and *Sphaerochaeta* were present in cell abundances of 1.80E+06 cells/mL and 1.95E+06 cells/mL, respectively. The abundances of methanogenic Archaea and *Desulfovibrio* were determined to be 5.30E+04 cells/mL and 6.0E+06 cells/mL. *Sphaerochaeta* are unique spirochetes with fermentative metabolisms that have been found in various anaerobic enrichment cultures (Ritalahti et al., 2012). Although the role of *Sphaerochaeta* in dechlorinating consortia is unclear, it was hypothesized that these bacteria fulfill relevant supporting functions (e.g., maintaining redox homeostasis, capturing inhibitors, providing essential nutrients) (Caro-Quintero et al., 2012; Zhuang et al., 2014).

Shotgun sequencing of the SDC-9 consortium has been performed and several genera including *Dhc*, *Desulfitobacterium*, *Sulfurospirillum*, *Desulfovibrio*, *Bacteroides*, *Clostridium* and *Methanocorpusculum* were identified (Dang et al., 2018). Some *Sulfurospirillum* species are known PCE/TCE dechlorinators and possess a specific *pceA* gene; however, this particular *pceA* RDase gene was not represented in the metagenome dataset. The application of the RD-qChip v2 detected and quantified *Sulfurospirillum* 16S rRNA genes in consortium SDC-9; however, the PCEA_SFP assay targeting the *pceA* of *Sulfurospirillum* spp. was negative. This finding is

consistent with the metagenome analysis and suggested that at least one non-dechlorinating *Sulfurospirillum* species exists in consortium SDC-9. A recent study reported shotgun metagenome data for consortium SDC-9 grown with PCE (Kucharzyk et al., 2020). This study found *Dhc* and *Desulfitobacterium* to be most abundant in consortium SDC-9 and also detected *Desulfovibrio*, *Methanocorpusculum*, *Pelosinus*, *Clostridium*, *Bacteroides* and *Sporomusa*; however, *Sulfurospirillum* was not reported. These findings suggest that *Desulfitobacterium* is involved in PCE and TCE reductive dechlorination to cDCE, and one or more *Dhc* strains dechlorinate cDCE to ethene. The differences in relative abundances of microbial populations and the presence/absence of certain species in the SDC-9 consortium that various studies have reported can be attributed to the specific growth conditions, the sampling time for biomass collection, sample handling, and the analytical (bioinformatic) pipelines used for data analysis.

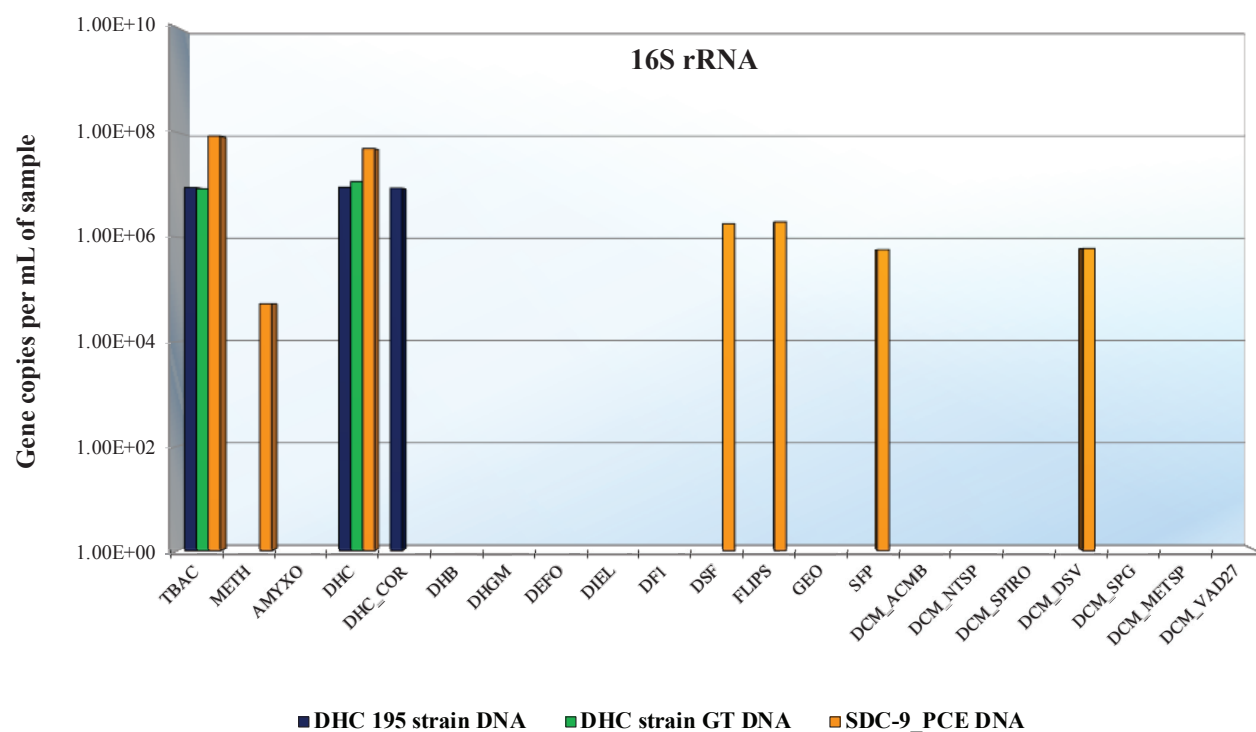


Figure 17. Abundance of 16S rRNA genes determined with the RD qChip v2 using DNA samples from *Dhc* strain 195 and strain GT and consortium SDC-9 grown with and PCE. For assay information, please see Table 21.

Quantitative analysis of RDase genes

The abundance results obtained from RD-qChip v2 for RDase genes in axenic *Dhc* cultures (strains 195 and GT) and consortium SDC-9 are depicted in Figure 18 and the assay list for RDases is presented in Table 22. For *Dhc* strain 195, the assays targeting *pceA* and *tceA* gave positive amplification and the abundance of *pceA* and *tceA* genes were determined to be 1.07E+07 and 8.57E+06 gene copies/mL, respectively. The DET1545 gene encodes an RDase specific to *Dhc* strain 195 and was detected with the HPR2A_RDHA_C assay (specific to the *Dhc* Cornell subgroup) at an abundance of 8.55E+06 gene copies/mL. Although the substrate specificity of DET1545 is not known, the expression of this gene has been commonly reported in studies of *Dhc* strain 195 grown with PCE or TCE (Fung et al., 2007; Rahm et al., 2008a).

Table 22. Assays targeting RDase and VC oxidase genes included in the RD-qChip v2 design.

Assay	Target (genus, species, strain, or gene)	Gene locus tag		
		<i>Dhc</i> 195 ^b	<i>Dhc</i> GT ^b	SDC-9 ^c
BVCA	<i>Dhc</i> strain BAV1, KBDCA1, KBDCA2, and KBDCA3	-	-	-
CBRA	<i>Dhc</i> strains DCMB5 and CBDB1	-	-	-
CERA	<i>Dhgm etheniformans</i> strain GP	-	-	-
CF_ALL	<i>Dhb</i> sp. CF, <i>Dsf</i> sp. PR, <i>Dhb</i> sp. THM1, <i>Dhb</i> sp. strain UNSWDHB	-	-	-
CFRA	<i>Dhb</i> sp. CF	-	-	-
CTRA_THMA	<i>Dsf</i> sp. PR, <i>Dhb</i> sp. THM1	-	-	-
DCAA_DHB_WL	<i>Dhb</i> sp. WL	-	-	-
DCAA_DSF	<i>Dsf dichloroeliminans</i> LMG P-21439 and strain DCA1	-	-	-
RDHA-AWM53_01188-RM	<i>Ca. Diel</i> ^a	-	-	-
RDHA-AWM53_01801-RM	<i>Ca. Diel</i> ^a	-	-	-
RDHA-AWM53_00864-RM	<i>Ca. Diel</i> ^a	-	-	-
DCMB_1041_RDHA	Most <i>Dhc</i>	-	-	-
DCMB_1339_RDHA	Most <i>Dhc</i>	-	DehalGT_1389	-
DCMB_1434_RDHA	Most <i>Dhc</i>	-	-	-
DCPA	<i>Dhc</i> strains KS and RC / <i>Dhgm lykanthroporepellens</i> BL-DC-9 and NSZ-14	-	-	-
DCRA_DHB	<i>Dhb</i> sp. DCA	-	-	-
DHGM_GP_00862 RDHA	<i>Dhgm etheniformans</i> strain GP	-	-	-
DHGM_GP_01297 RDHA	<i>Dhgm etheniformans</i> strain GP	-	-	-
DHGM_GP_01300 RDHA	<i>Dhgm etheniformans</i> strain GP	-	-	-
ETNC	<i>Mycobacterium</i>	-	-	-
ETNE	<i>Mycobacterium</i>	-	-	-
ETNE_MB	<i>Mycobacterium</i> and uncultured bacterium	-	-	-
ETNE_MPO	<i>Mycobacterium</i> , <i>Ochrobactrum</i> , <i>Pseudomonas</i>	-	-	-
ETNE_NCD	<i>Nocardioides</i> and uncultured bacterium	-	-	-
ETNE2	Uncultured bacterium	-	-	-
HPR1A_RDHA_P	<i>Dhc</i> Pinellas subgroup	-	DehalGT_0241	-
HPR2A_RDHA_C	<i>Dhc</i> Cornell subgroup	DET1545	-	-
MBRA	<i>Dhc</i> strains DCMB5, MB and CBDB1	-	-	-
PCB1_CG4_CG5 (pcbA4)	<i>Dhc</i> strains CG5, CG4 and 11a5	-	-	-
PCB2_CG1(pcbA1)	<i>Dhc</i> strains CG1, CG3 and GY50	-	-	-
PCEA_195	<i>Dhc</i> strain 195	DET0318	-	-
CBDBA1588_DHC_RDHA	<i>Dhc</i> strains CBDB1, KBVC1, 11a5, IBARAKI, UCH007, BTF08, GT and KB1	-	DehalGT_1312	-
PCEA_DHB_DSF	<i>Dhb restrictus</i> DSM 9455 and strain PER-K23; <i>Dsf hafniense</i> strains PCS-S, Y51 and TCE1; <i>Dsf</i> sp. strains PCE-S and CR1	-	-	SDC9_48357

Assay	Target (genus, species, strain, or gene)	Gene locus tag		
		<i>Dhc</i> 195 ^b	<i>Dhc</i> GT ^b	SDC-9 ^c
PCEA_GEO	<i>Geobacter lovleyi</i> strains SZ and KB1	-	-	-
PCEA_SFP	<i>Sfp</i> sp. SL2-2 and SL2-1; <i>Sfp halorespirans</i> DSM 13726 and strain PCE-M2; <i>Sfp multivorans</i> DSM 12446 <i>Sfp</i> sp. mixed culture SL2 PceA (DCE) and SL2 PceA (TCE)	-	-	-
SDC9_23241-RDHA	Specific to <i>Dhc</i> in SDC-9 consortium (NCBI nr database: <i>Dhc</i> strain UCH-ATV1 and uncultured bacterium)	-	-	SDC9_23241
SDC9_45582-RDHA	Specific to SDC-9 consortium (NCBI nr database: <i>Desulfitobacterium hafniense</i> strain PCE-S and DCB-2)	-	-	SDC9_45582
SDC9_07142-RDHA	Specific to <i>Dhc</i> in SDC-9 consortium (NCBI nr database: <i>Dhc</i> strains UCH-ATV1 and uncultured bacterium)	-	-	SDC9_07142
SDC9_07143-RDHA	Specific to <i>Dhc</i> in SDC-9 consortium (NCBI nr database: <i>Dhc</i> strain UCH-ATV1 and uncultured bacterium)	-	-	SDC9_07143
SDC9_48350-RDHA	Specific to SDC-9 consortium (NCBI nr database: <i>Dsf hafniense</i> strains PCE-S, Y51 and TCE1; <i>Dsf</i> sp. CR1; <i>Dhb restrictus</i> DSM 9455 and PER-K23; <i>Dhb</i> sp. CF and DCA)	-	-	SDC9_48350
SDC9_23243-RDHA	Specific to <i>Dhc</i> in SDC-9 consortium, (NCBI nr database: <i>Dhc</i> strains VS, KBTCE3, 195, KBTCE2, CG3, UCH007, CG4, CG1, GY50 and UCH-ATV1)	DET0180	-	SDC9_23243
SDC9_09280-RDHA	Specific to SDC-9 consortium (NCBI nr database 39.6% aa similarity to RDase in <i>Dhb</i>)	-	-	SDC9_09280
TCEA	<i>Dhc</i> strains FL2, 195, ANAS, KBTCE3, KBTCE2, 11a5, UCH007, AD14-1 and BTF-08, <i>Dhc</i> strain(s) in SDC-9 consortium	DET0079	-	SDC9_10429
VCRA	<i>Dhc</i> strains VS, KBTCE1, KBVC1, KBVC2, WBC2, IBARAKI, UCH007, BTF08, GT, ANAS2, <i>Dhc</i> strain(s) in SDC-9 consortium	-	DehalGT_1237	SDC9_07164

Dhc: *Dehalococcoides mccartyi*, *Dsf*: *Desulfitobacterium*, *Dhb*: *Dehalobacter*, *Sfp*: *Sulfurospirillum*

^a Populations detected in a DCM-degrading enrichment culture RM harboring ‘*Candidatus* Dichloromethanomonas elyunquensis’ (*Ca.* Diel) (Kleindienst et al., 2017).

^b The minus sign (-) indicates that the *Dhc* strain(s) does not have the target gene for the given qPCR assay and no amplification was observed.

^c The locus tags for RDase genes of SDC-9 cultures grown with PCE are shown (Kucharzyk et al., 2020). The minus (-) sign indicates no amplification for the given qPCR assay.

The SDC-9_23243 assay encoding a putative RDase was detected in DNA extracted from PCE-grown SDC-9 culture and axenic *Dhc* 195 cultures. With this assay, the DET0180 gene for *Dhc* 195 and the SDC-9 gene 23243 were amplified and gene copies/mL were determined as 8.16E+06 and 5.74E+07, respectively. DET1545 and DET0180 have been commonly reported as putative

RDases in *Dhc* strain 195 grown with PCE/TCE (Fung et al., 2007; Rahm et al., 2008a). The presence of these putative RDases has also been reported for ANAS1 and ANAS2 *Dhc* strains (Lee et al., 2011a).

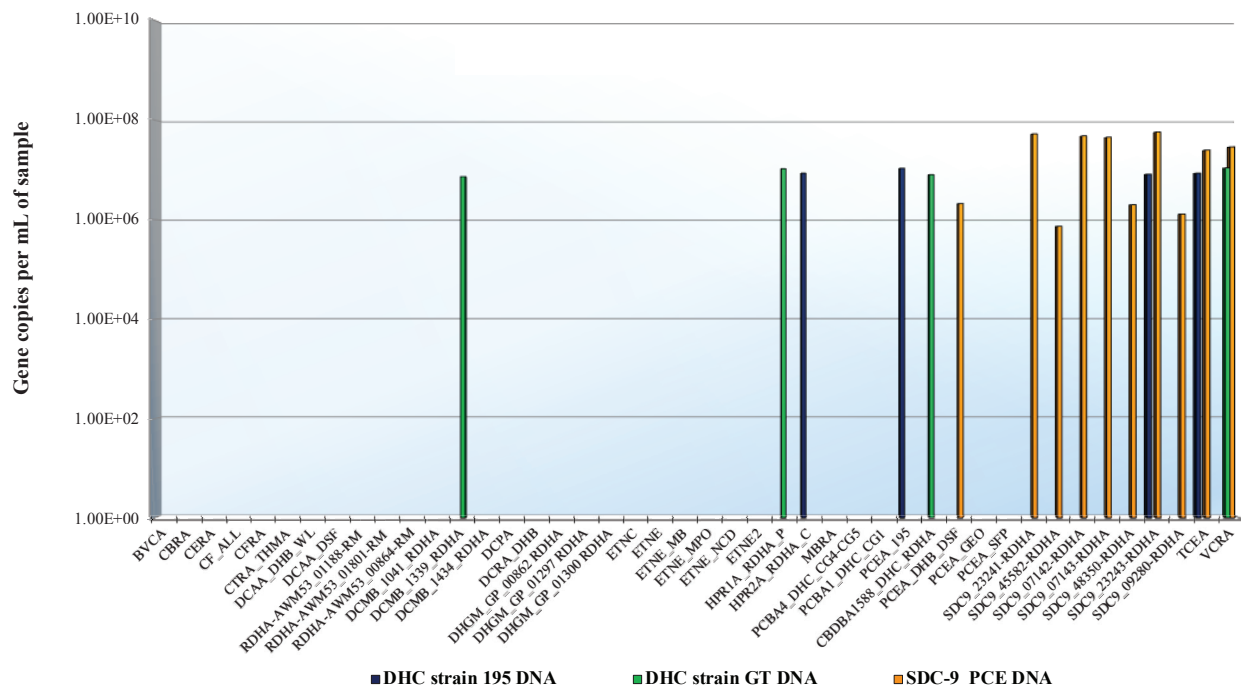


Figure 18. Enumeration of RDase genes in DNA samples from *Dhc* strain 195, *Dhc* strain GT and the *Dhc*-containing consortium SDC-9 amended with PCE using the RD qChip v2. P, Pinellas subgroup; C, Cornell subgroup; V, Victoria subgroup.

The assays amplified *vcrA* and three putative RDase genes (locus tags DehalGT_0241, DehalGT_1389 and DehalGT_1312) with DNA obtained from *Dhc* strain GT cultures (Table 22, Figure 18). *Dhc* strain GT does not possess the *pceA*, *tceA* and *bvcA* RDase genes but harbors *vcrA* enabling this strain to dechlorinate TCE to ethene. The gene abundance of *vcrA* was determined to be 1.09E+07 gene copies/mL. This value matches with *Dhc* cell abundance of 1.15E+07 16S rRNA gene copies/mL measured for this sample (Figure 17), indicating that both genes occur as single copies on the *Dhc* genome, which is consistent with prior observations (Ritalahti et al., 2006; Sung et al., 2006b). In *Dhc* strain GT DNA, DehalGT_0241 was quantified in an abundance of 1.05E+07 gene copies/mL. Information about the substrate specificity of the corresponding RDase is lacking, but an ortholog of this RDase, *cbdbA187*, in *Dhc* strain CBDB1 was highly transcribed in the presence of 1,2,3-TCB (Wagner et al., 2009). DehalGT_1189 yielded amplicons with genomic DNA samples of *Dhc* strain GT and an ortholog of DehalGT_1189, *Dcmb_1339*, was expressed in *Dhc* DCMB5 cells. Another putative RDase, DehalGT_1312, was detected with *Dhc* strain 195 template DNA and it was reported that *cbdbA1588* (an ortholog to DehalGT_1312) was highly transcribed together with the *cbrA* gene in *Dhc* CBDB1 (Schippe et al., 2013). Consistent with recent metagenome analyses that detected *tceA* and *vcrA* in consortium SDC-9 (Dang et al., 2018; Kucharzyk et al., 2020), the qPCR results revealed the presence of *tceA* and *vcrA* genes when the consortium was grown with PCE. The SDC9_45582 and SDC9_09280

assays (both targeting *Desulfitobacterium* RDase genes with unknown substrate specificity) quantified nearly $1.0\text{E}+06$ gene copies/mL. The presence of *Desulfitobacterium* species was shown with an assay specific to 16S rRNA gene of *Desulfitobacterium* and the abundance of *Desulfitobacterium* cells was $1.80\text{E}+06$ cells/mL in PCE-grown SDC-9 biomass. These data indicate that not all *Desulfitobacterium* strains in the PCE-grown consortium SDC-9 possess the SDC9_45582 and SDC9_09280 RDase genes. The SDC9_48350 assay, targeting the *pceA* genes of *Dhb/Desulfitobacterium*, yielded amplicons and the gene abundance was $1.99\text{E}+06$ gene copies/mL. The other *pceA* assay targeting *pceA* genes of *Dhb/Desulfitobacterium* (PCEA_DHB_DSF assay) also gave positive amplification and $2.09\text{E}+06$ gene copies/mL were enumerated. The *pceA* gene and *Desulfitobacterium* cells were present at similar abundances suggesting that PceA RDase(s) belonging to *Desulfitobacterium* species play a role in reductive dechlorination of PCE and/or TCE to cDCE in the PCE-grown SDC-9 consortium.

The other *Dhc* RDase genes (SDC9_23241, SDC9_07142, SDC9_07143, SDC9_23243, reported in (Kucharzyk et al., 2020)) were also detected and quantified in the range of $4.51\text{E}+07$ to $5.74\text{E}+07$ gene copies/mL and the abundance of these RDases correlated with the *Dhc* cell abundance (i.e., $4.91\text{E}+07$ gene copies/mL for the same sample). These four RDase genes were also reported for *Dhc* strain UCH-ATV1 (Yohda et al., 2017), which has been detected in the metagenome of an enrichment culture grown with cDCE. This culture was derived from TCE-contaminated groundwater. The substrate specificity of the corresponding RDases remains to be elucidated.

Quantitative analysis of genes implicated in corrinoid uptake and modification

The qPCR results obtained with RD-qChip v2 for corrinoid uptake and modification genes in axenic *Dhc* cultures (strains 195 and GT) and consortium SDC-9 are depicted in Figure 19 and the list of assays for these genes is presented in Table 23. The qPCR assays targeting genes implicated in corrinoid uptake and modification, including *cbiZ*, *cobA*, *cobC*, *cobS*, *cobT*, *cobU*, *btuC*, *btuD* and *btuF*, yielded the expected amplicons with DNA samples from axenic *Dhc* cultures and consortium SDC-9. Of note, some of the assays were specific to genes of a *Dhc* subgroup (Figure 19), and the results were consistent with other qPCR assays with the resolution to distinguish between the *Dhc* subgroups. For example, strain 195 belongs to the Cornell subgroup and the assays designed for this subgroup should exclusively amplified DNA of *Dhc* strain 195, but not DNA of strains belonging to the Pinellas and Victoria subgroups. As demonstrated in Figure 19, the assays designed for the Cornell subgroup gave positive amplification with DNA from *Dhc* strain 195 whereas the assays designed for the Pinellas and Victoria subgroups did not yield amplicons. The corrinoid uptake and modification genes specific to strains of the Pinellas subgroup were only detected in DNA extracted from *Dhc* strain GT. These findings indicate that the assays are specific and can distinguish *Dhc* strains that belong to different *Dhc* subgroups.

The abundance of lower ligand activation and attachment genes *cobT*, *cobS* and *cobC* were determined to be $7.59\text{E}+06$, $1.07\text{E}+07$ and $8.56\text{E}+06$ per mL of culture suspension, respectively, for *Dhc* strain 195 template DNA. Two copies of these genes are present on the 195 genome and the abundance values were divided by two to represent the *Dhc* cell numbers. For *Dhc* strain GT, the *cobT*, *cobS* and *cobC* gene abundances were $1.05\text{E}+07$, $1.16\text{E}+07$ and $1.36\text{E}+07$ gene copies/mL, respectively. The *Dhc* 16S rRNA gene copy numbers per mL of culture suspension were $8.93\text{E}+06$ (strain 195) and $1.15\text{E}+07$ (strain GT) indicating the utility of these assays included in the RD-qChip v2 design.

Table 23. Assays targeting *Dhc* corrinoid uptake and modification genes and included on RD-qChip v2.

Assay	Target (genus, species, strain, or gene)	Gene locus tag		
		<i>Dhc</i> 195 ^a	<i>Dhc</i> GT ^a	SDC-9 ^{a, b}
BTUC_DHC_C	<i>Dhc</i> Cornell subgroup	DET0651	-	✓
BTUC_DHC_P_V	<i>Dhc</i> Pinellas and Victoria subgroups	-	DehalGT_0583	-
BTUD_DHC_C	<i>Dhc</i> Cornell subgroup	DET0652	-	✓
BTUD_DHC_P	<i>Dhc</i> Pinellas subgroup	-	DehalGT_0584	-
BTUD_DHC_V	<i>Dhc</i> Victoria subgroup	-	-	-
BTUF_DHC_ALL	<i>Dhc</i>	DET0650	DehalGT_0582	✓
CBIZ_DHC_P	<i>Dhc</i> Pinellas subgroup	-	DehalGT_0585	-
CBIZ_DHC_C_V	<i>Dhc</i> Cornell & Victoria subgroups	DET0653	-	✓
COBA_DHC_ALL	<i>Dhc</i>	DET1224	DehalGT_0964	✓
COBC_DHC_P	<i>Dhc</i> Pinellas subgroup	-	DehalGT_0590	-
COBC_DHC_C_V	<i>Dhc</i> Cornell & Victoria subgroups	DET0659	-	✓
COBS_DHC_P1	<i>Dhc</i> Pinellas subgroup	-	DehalGT_0589	-
COBS_DHC_P2	<i>Dhc</i> Pinellas subgroup	-	DehalGT_0589	-
COBS_DHC_C_V	<i>Dhc</i> Cornell & Victoria subgroups	DET0658		✓
COBT_DHB	<i>Dhb</i> strains DSM 9455, CF and DCA	-	-	-
COBT_DHC_ALL	<i>Dhc</i>	DET0657	DehalGT_0589	✓
COBT_DSf	<i>Desulfitobacterium hafniense</i> strains Y51, PCE-S, DCB-2 and <i>Desulfitobacterium dehalogenans</i> ATCC 51507	-	-	✓
COBU_DHC_C	<i>Dhc</i> Cornell subgroup	DET0660	-	✓
COBU_DHC_P	<i>Dhc</i> Pinellas subgroup	-	DehalGT_0591	-
COBU_DHC_V	<i>Dhc</i> Victoria subgroup	-	-	-

^a The qPCR results demonstrated that consortium SDC-9 exclusively harbors *Dhc* strains affiliated with the Cornell subgroup. The assays targeting functional genes of the Pinellas and Victoria subgroups did not yield amplicons (depicted with a minus [-] sign). Also, other qPCR assays that did not yield amplicons are depicted with a minus [-] sign for consortium SDC-9 and *Dhc* strains 195 and GT.

^b The genome(s) of *Dhc* strain(s) and other organisms in the SDC-9 consortium have not been reported and no gene locus tags have been assigned. Instead of a locus tag, the assays that yielded amplicons are depicted with a check mark sign (✓).

The *cbiZ* gene involved in cobalamin biosynthesis was quantified at 4.26E+07 gene copies/mL with the assay specific to *Dhc* Cornell subgroup when the SDC-9 consortium was grown with PCE. BtuFCD is a confirmed corrinoid ABC transporter (Men et al., 2014) and it has been reported that the BtuFCD transport system was highly expressed in growing *Dhc* cultures (West et al., 2013). The abundances of *btuF*, *btuC* and *btuD* were determined to be 4.72E+07, 4.64E+07 and 4.95E+07 gene copies/mL, respectively, in PCE-grown SDC-9 cultures.

The *Dhc* 16S rRNA gene-targeted assays indicated that consortium SDC-9 harbors exclusively *Dhc* of the Cornell subgroup and members of the Pinellas or Victoria were absent. This observation is also illustrated in Figure 19 where the assays related to corrinoid uptake and modification genes targeting the Cornell subgroup gave positive amplification for consortium SDC-9, but the assays specific to the Pinellas and Victoria subgroups did not. These results corroborate the specificity of the assays included in the RD qChip v2 design.

The *cobT* assay specific to some *Dehalobacter* strains did not exhibit any amplification with DNA of PCE-grown consortium SDC-9 (Table 23). The *Dehalobacter* 16S rRNA gene-targeted assay did not yield amplicons with SDC-9 DNA either. Therefore, the absence of amplification with SDC-9 DNA as template for the *cobT*_DHB assay provides additional evidence that *Dehalobacter* is not a part of the SDC-9 microbial community when the culture is grown with PCE as electron acceptor. On the other hand, the assay specific to the *Desulfitobacterium cobT* (*cobT*_DSF) enumerated 1.04E+06 gene copies/mL in consortium SDC-9 culture suspension. This result is consistent with the qPCR data obtained for other *Desulfitobacterium* biomarker genes, including the 16S rRNA gene (1.80E+06 gene copies/mL) and the *pceA* gene (2.09E+06 gene copies/mL). The lower abundance of *cobT* (compared to the 16S rRNA gene and *pceA* gene abundance data) determined with the *cobT*_DSF assay can be explained with the *cobT* allele diversity observed in the genus *Desulfitobacterium*. The *cobT*_DSF assay is specific to *cobT* gene of *Desulfitobacterium hafniense* strains Y51, PCE-S, DCB-2 and *Desulfitobacterium dehalogenans* ATCC 51507, but would miss *cobT* of other known *Desulfitobacterium* species.

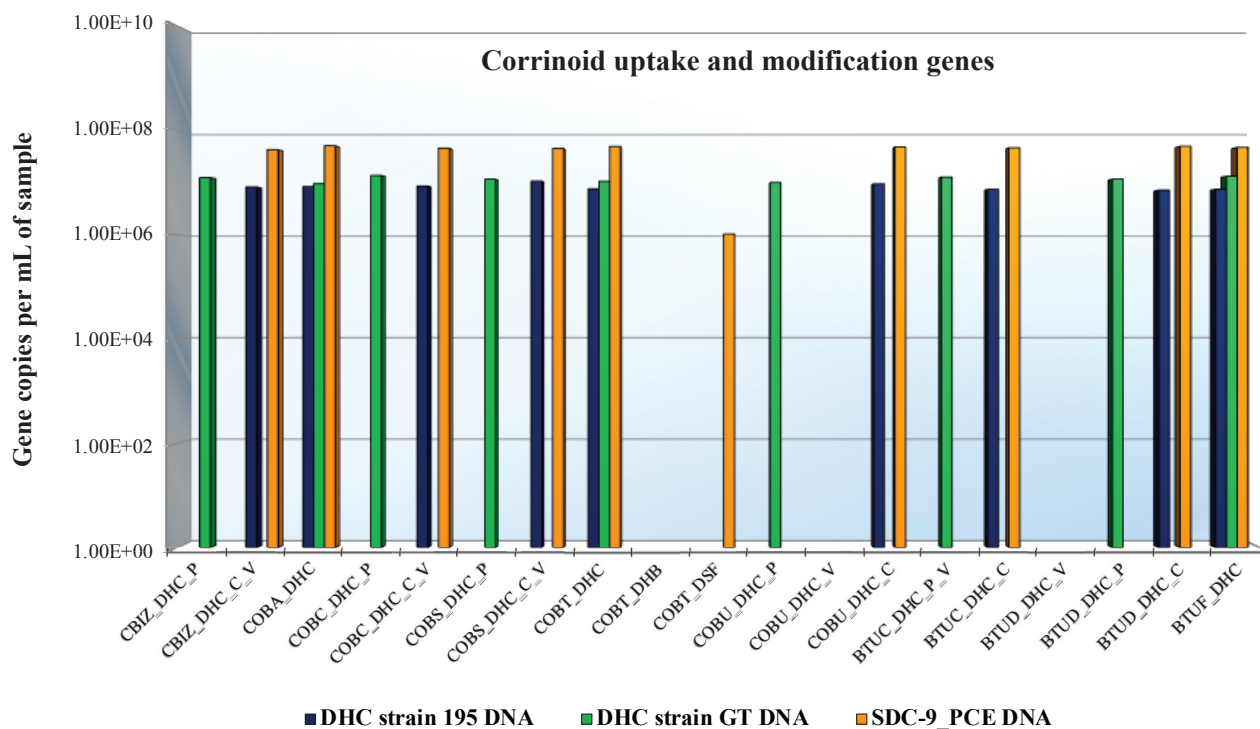


Figure 19. Enumeration of genes implicated in corrinoid uptake and modification using DNA samples from axenic *Dhc* cultures and PCE-grown consortium SDC-9 using the RD qChip v2. P, Pinellas subgroup; C, Cornell subgroup; V, Victoria subgroup.

Quantitative analysis of genes implicated in electron transfer to the chlorinated electron acceptor

The qPCR results for genes related to electron transport in *Dhc* and formate dehydrogenase (*fdhA*) genes of *Dhgm* strains are presented in Figure 20. The list of assays is shown in Table 24. qPCR assays targeting the *Dhc* hydrogenase genes *hycE*, *echE*, *hymB*, *hymC*, *vhuA* and *hupL* were detected in DNA extracted from *Dhc* strains 195 and GT cultures and from consortium SDC-9. The abundances of the hydrogenase genes were in comparable ranges as other single copy target genes (e.g., RDase genes) that occur on *Dhc* genomes.

The assays specific to formate dehydrogenase genes found on *Dhgm* genomes did not yield amplicons with template DNA from axenic *Dhc* cultures and consortium SDC-9. These observations confirm the specificity of the hydrogenase gene-targeted assays, and further demonstrate the absence of *Dhgm* in consortium SDC-9.

The FDHA_DHC and HUPL_DHC assays detected and enumerated the *fdhA* and *hupL* genes in all axenic *Dhc* cultures and consortium SDC-9. *Dhc fdhA* abundances of 8.62E+06 and 1.12E+07 gene copies/mL were determined in strain 195 and strain GT cultures, respectively. In consortium SDC-9 grown with PCE, 5.24E+07 *fdhA* gene copies/mL were measured. The *hupL* gene abundances were 9.60E+06 and 1.18E+07 gene copies/mL in strain 195 and strain GT cultures, respectively. In SDC-9 consortium, 5.34E+06 *hupL* gene/copies/mL were enumerated.

Table 24. Assays related to electron transport for *Dhc* and formate dehydrogenase genes specific to *Dhgm lykanthroporepellens* strain BL-DC-9 and/or *Dhgm formicexedens* strain NSZ-14 included on RD-qChip v2.

Assay	Target (genus, species, strain, or gene)	Gene locus tag		
		<i>Dhc</i> 195	<i>Dhc</i> GT	<i>Dhc</i> in SDC-9
CISM_DHC	<i>Dhc</i>	DET0187	DehalGT_0247	✓ ^a
ECHE_DHC	<i>Dhc</i>	DET0867	DehalGT_0746	✓
HUPL_DHC	<i>Dhc</i>	DET0110	DehalGT_0141	✓
HYCE_DHC	<i>Dhc</i>	DET1571	DehalGT_1366	✓
HYMB_DHC	<i>Dhc</i>	DET0146	DehalGT_0174	✓
HYMC_DHC	<i>Dhc</i>	DET0147	DehalGT_0175	✓
VHUA_DHC	<i>Dhc</i>	DET0615	DehalGT_0550	✓
FDHA1_DHGM_BLDC9	<i>Dhgm</i> strains BL-DC-9 and NSZ-14	- ^b	-	-
FDHA2_DHGM_BLDC9	<i>Dhgm</i> strain BL-DC-9	-	-	-
FDHA3_DHGM_BLDC9	<i>Dhgm</i> strains BL-DC-9 and NSZ-14	-	-	-

^a The genome(s) of *Dhc* strain(s) in consortium SDC-9 have not been reported and no gene locus tags have been assigned. Instead of locus tag, the assays that yielded amplicons are depicted with a check mark sign (✓).

^b The assays that did not yield amplicons are depicted with a minus [-] sign.

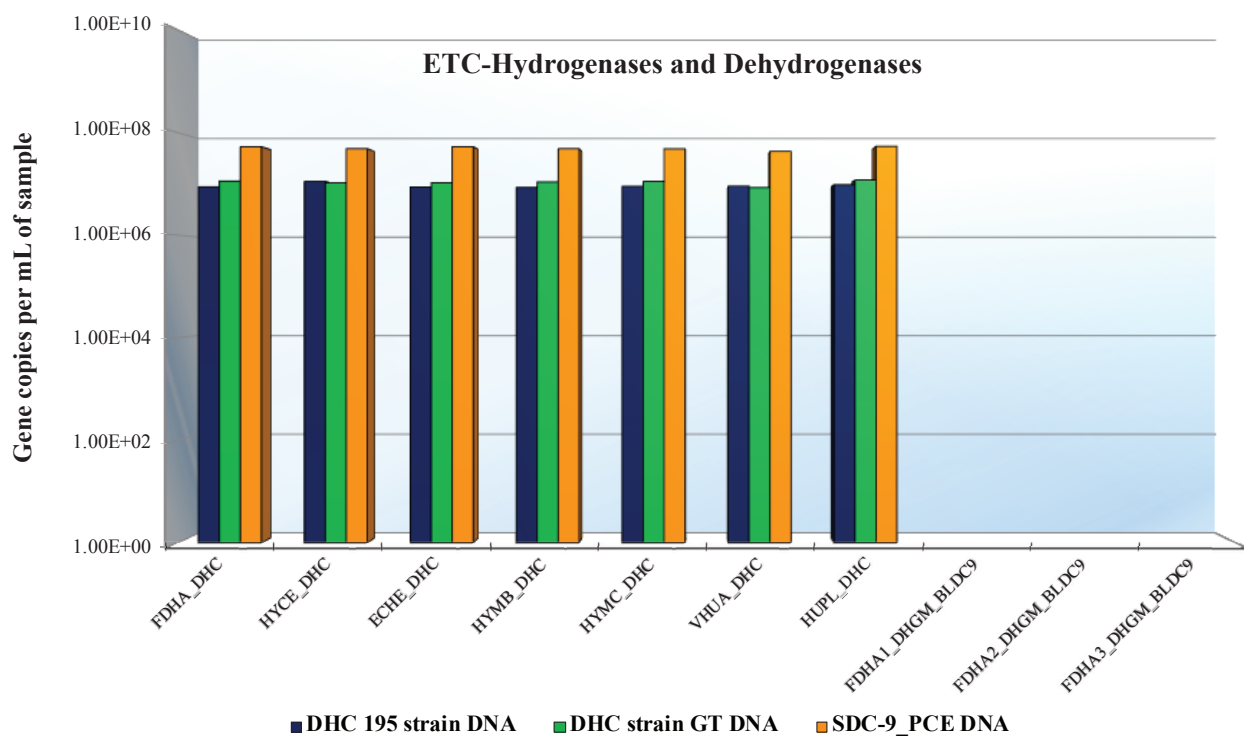


Figure 20. Enumeration of genes encoding hydrogenases and a component of the CISM complex using DNA samples from axenic *Dhc* cultures and consortium SDC-9 grown with PCE.

The assays targeting *Dhc* hydrogenase genes and the *fdhA* gene encoding CISM included on the RD-qChip v2 were validated. Transcriptomic studies reported high expression of *Dhc fdhA* and *hupL* genes in *Dhc*-containing laboratory cultures, and proteomic analysis corroborated high expression of FdhA and HupL (Heavner et al., 2018; Kublik et al., 2016; Morris et al., 2007). Proteomic workflows also measured *Dhc* FdhA and RDases (e.g., TceA) in groundwater collected from contaminated sites (Figure 30) (Solis et al., 2019), suggesting that the measurement of these biomarker proteins is feasible in environmental matrices such as groundwater.

These observations lead to a refined concept how *Dhc* activity can be monitored and measured. Figure 21 depicts a conceptual model of electron flow in *Dhc*. The Ni-Fe hydrogenase HupL oxidizes hydrogen and the electrons are channeled through a protein complex, which includes CISM, an iron-sulfur molybdoenzyme encoded by *fdhA*. All *Dhc* share this machinery independent of the specific type of chlorinated compound used as electron acceptor. In other words, the measurement of components of this machinery informs about general *Dhc* activity without providing information about specific reductive dechlorination activity. Information about specific reductive dechlorination activity is provided through measurement of RDases, which is feasible in groundwater (Solis et al., 2019). The *fdhA* genes of *Dhc* are highly conserved and the CISM protein can be easily measured with proteomics, including groundwater samples (Solis et al., 2019).

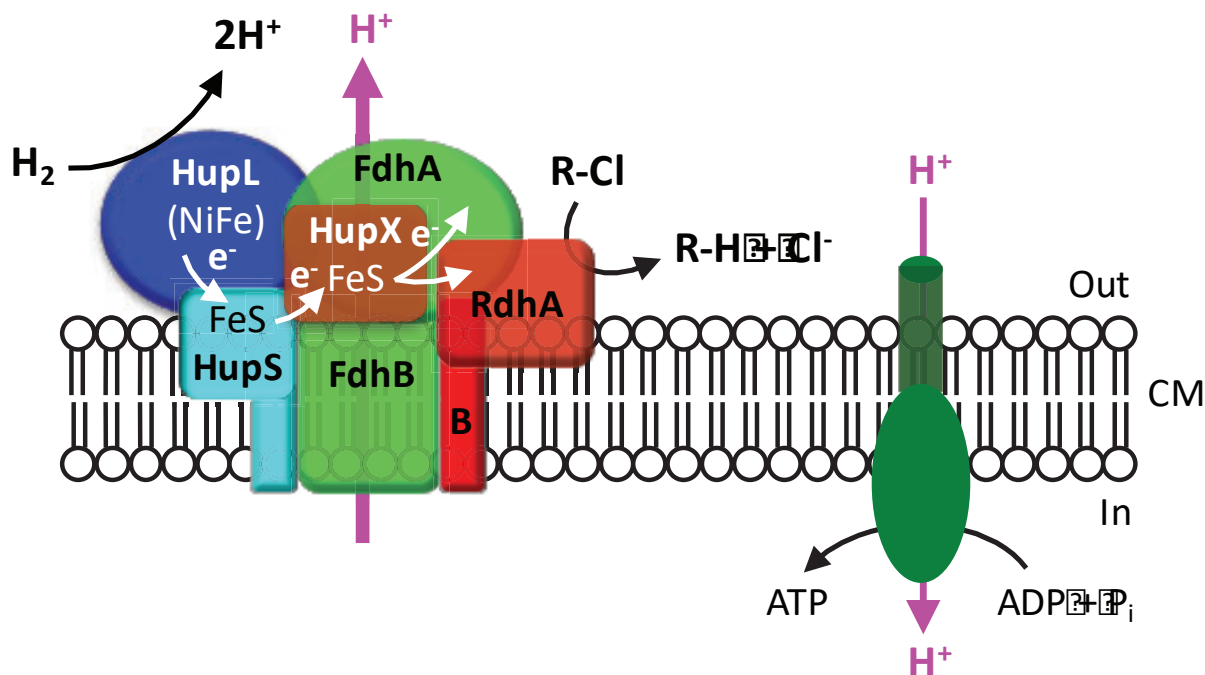


Figure 21. Conceptual model connecting hydrogen oxidation catalyzed by the HupL hydrogenase with reductive dechlorination catalyzed by a specific RDase.

This process is coupled to proton translocation and energy conservation. Electron flow proceeds through a protein complex that includes CISM (FdhA). The figure was modified based on published information (Heavner et al., 2018; Kublik et al., 2016; Morris et al., 2007).

Figure 22 illustrates this concept in greater detail. The quantitative assessment of CISM (FdhA) provides information about the machinery. The presence of *fdhA* transcripts and/or FdhA proteins indicates that *Dhc* are actively dechlorinating; however, this biomarker analysis does not provide information about the specific chlorinated compound that *Dhc* is utilizing as electron acceptor. If additional measurements determine the expression of specific RDases, the combined analysis informs about general and specific activity. This two-pronged approach is powerful because it measures two independent activity biomarkers thus increasing confidence in the interpretation of the data. Importantly, the measurement of the machinery (i.e., CISM) can serve as a measure of *Dhc* reductive dechlorination activity, even though RDases with assigned function cannot be detected.

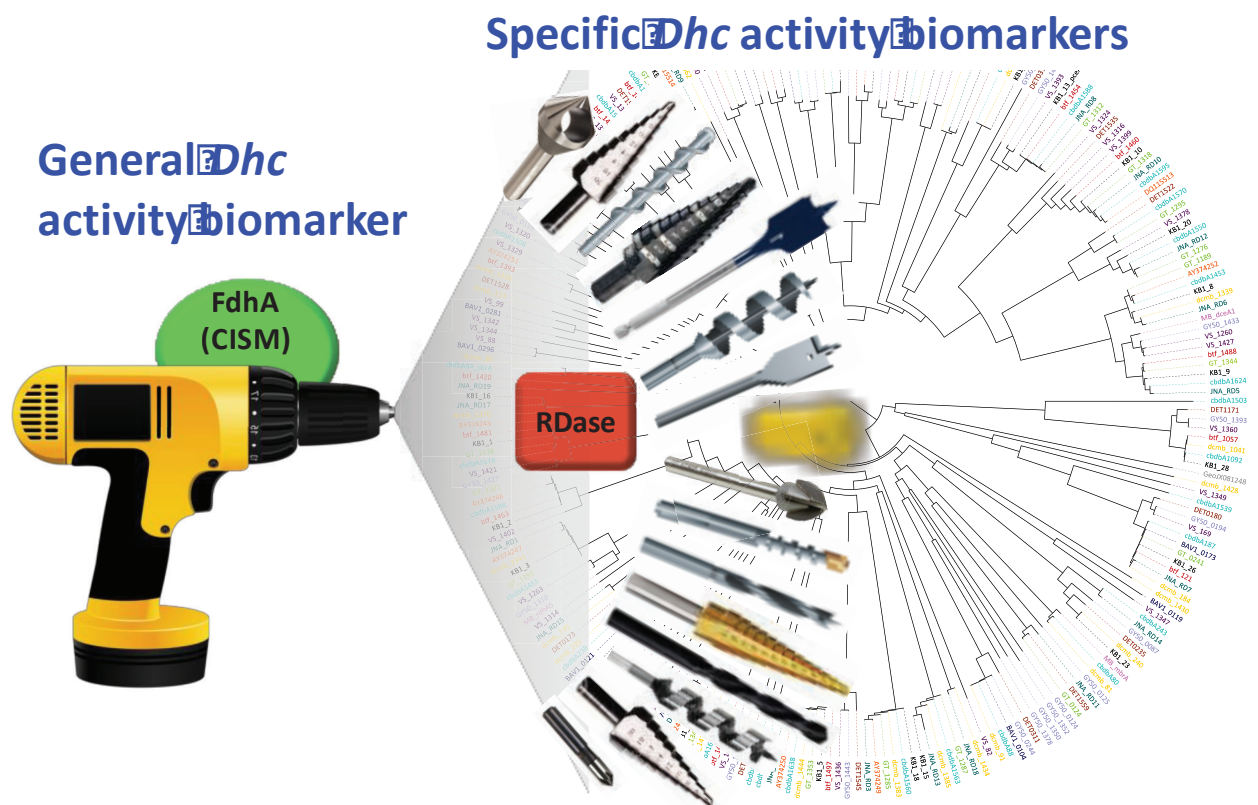


Figure 22. Cartoon illustrating the measurement of the general reductive dechlorination activity biomarker FdhA (CISM) and specific RDases.

The meaningful interpretation of RDase measurements requires information about the substrate range of the RDases, which is available for only a few RDases. The analysis of a general activity biomarker (i.e., the conserved *Dhc* electron transfer machinery) can provide activity information without knowledge of specific RDase function.

Quantitative analysis of genes implicated in dinitrogen (N₂) fixation in *Dhc* strains of the Cornell subgroup

Dhc strains of the Cornell subgroup possess *nifH*, *nifK* and *nifD* genes that encode functional nitrogenase (Kaya et al., 2019; Lee et al., 2009). In contrast, *Dhc* strains of the Pinellas and Victoria subgroups only possess the *nifH* gene (i.e., lack *nifD* and *nifK*) and cannot fix dinitrogen. Complete and functional *nif* operons bestow the ability to fix dinitrogen when fixed nitrogen is not available, and the presence of Cornell subgroup *Dhc* strains influence the outcomes of bioremediation at sites where fixed nitrogen is a limiting nutrient (Kaya et al., 2019; Lee et al., 2009). The abundance results for *Dhc* genes related to nitrogen fixation is illustrated in Figure 23 and the assays are listed in Table 25. As shown in Figure 23, the qPCR assays targeting *nifH*, *nifK* and *nifD* specific to the Cornell subgroup were detected in *Dhc* strain 195 and consortium SDC-9. With *Dhc* strain 195 template DNA, *nifH*, *nifK* and *nifD* were observed in abundances of 1.01E+07, 9.34E+06, and 1.10E+07 gene copies/mL, respectively. *Dhc* strain GT lacks *nifK* and *nifD* and no amplification of these genes was observed with strain GT template DNA. Only *nifH* was amplified (1.27E+07 gene copies/mL) but the *nifH* of Pinellas-type *Dhc* differs in sequence from the Cornell-type *nifH*. The assays on the RD-qChip v2 assaying dinitrogen fixation were validated successfully and the specificity of the assays was confirmed. These assays are useful to determine the presence and abundance of *Dhc* strains of the Cornell subgroup with the ability to fix dinitrogen. This information is useful at sites where fixed nitrogen limitations may limit contaminant degradation (Kaya et al., 2019; Lee et al., 2009).

Table 25. Assays targeting genes related to dinitrogen fixation included on RD-qChip v2.

Assay	Target (genus, species, strain, or gene)	Gene locus tag		
		<i>Dhc</i> 195	<i>Dhc</i> GT	<i>Dhc</i> in SDC-9
NIFD_DHC_C	<i>Dhc</i> Cornell subgroup	DET1155	-	✓ ^a
NIFD_DHV	<i>Desulfovibrio vulgaris</i>	-	-	- ^b
NIFH_DHC_C	<i>Dhc</i> Cornell subgroup	DET1158		✓
NIFH_DHC_P	<i>Dhc</i> Pinellas subgroup		DehalGT_1362	-
NIFK_DHC_C	<i>Dhc</i> Cornell subgroup	DET1154	-	✓

^a The genome(s) of *Dhc* strain(s) in SDC-9 consortium have not been reported and no gene locus tags have been assigned. Instead of locus tags, the assays that yielded amplicons are depicted with a check mark sign (✓).

^b The assays that did not yield amplicons are depicted with a minus [-] sign.

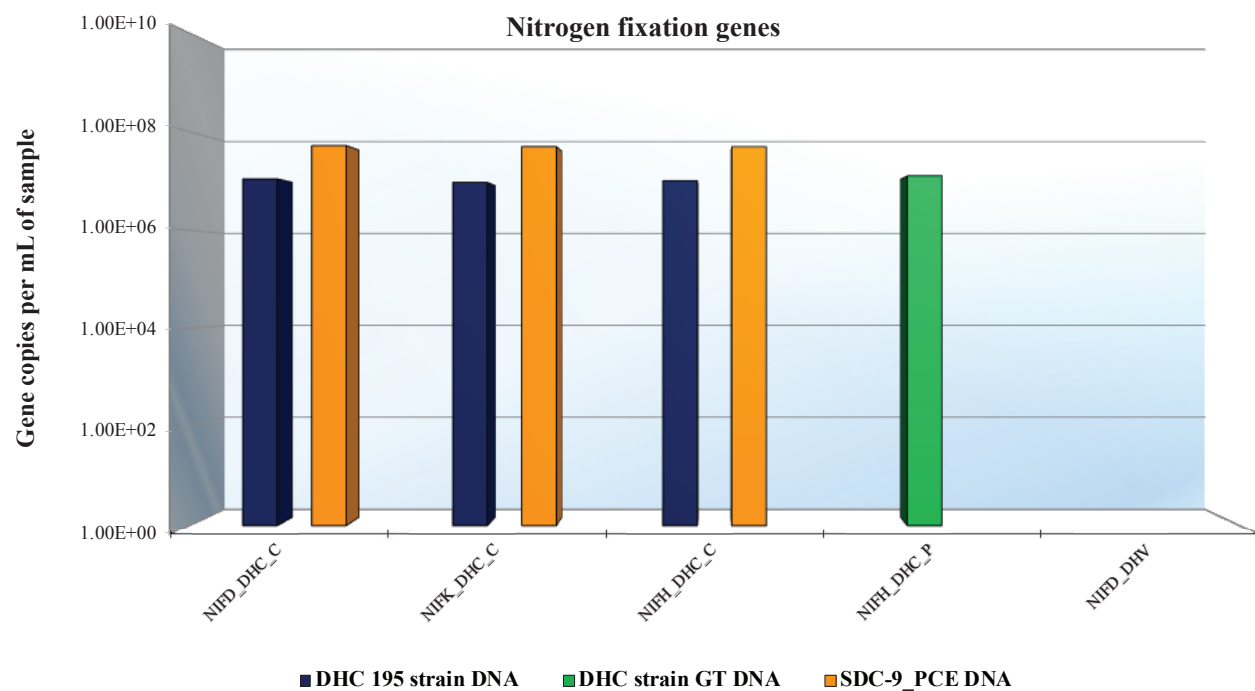


Figure 23. Enumeration of biomarker genes for dinitrogen fixation using DNA from axenic *Dhc* cultures and consortium SDC-9 grown with PCE.
P, Pinellas subgroup; C, Cornell subgroup; V, Victoria subgroup.

Internal and external control assays

Included in the design of the RD-qChip v2 were one internal and one external control assays. Control qPCR assays targeted *rpoB* (encoding the beta subunit of RNA polymerase), which serves as a housekeeping gene specific to *Dhc* (i.e., internal control), and the luciferase (*luc*) gene, which does not occur in groundwater (i.e., the external control). These assays allow normalization of gene and transcripts abundances in samples. As illustrated in Figure 24, the *rpoB* gene of *Dhc* was detected in all samples and abundances of $1.05\text{E}+07$, $1.16\text{E}+07$ and $5.06\text{E}+07$ gene copies/mL were determined in cultures of *Dhc* strain 195 and strain GT and in consortium SDC-9, respectively. As expected, no amplification occurred with the *luc* assay because *luciferase* mRNA was not added into any DNA sample as an external control.

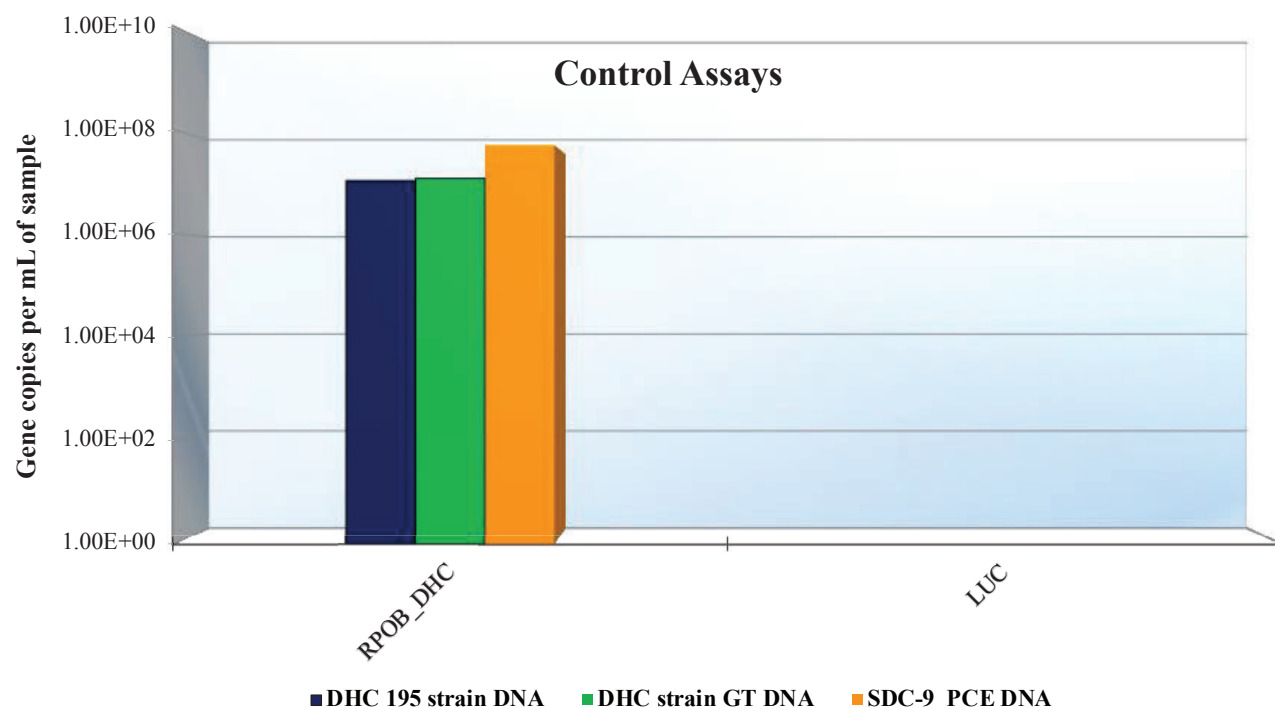


Figure 24. Enumeration of the internal control gene *rpoB* and the external control gene *luc* using DNA samples from axenic *Dhc* cultures and consortium SDC-9 grown with PCE. As expected, the LUC assay did not show any amplification.

The experimental efforts with laboratory cultures, including consortium SDC-9, demonstrated that the qPCR assays included in RD-qChip v2 are specific and generate highly reproducible results. The RD-qChip is a promising high-throughput tool for enumerating reductive dechlorination biomarker genes, and should find broad application for site assessment and implementing bioremediation monitoring regimes. RD-qChip results support adaptive site management decision making to ensure that the most promising technologies are being implemented to meet site-specific clean-up goals.

Cost comparison of regular versus high-throughput qPCR

Costs are associated with the application of MBTs. Even though the costs are low in relation to the overall investment of the remedial investigation and site cleanup, site owners and practitioners are often reluctant to spend (additional) money on the application of MBTs. This is unfortunate and short-sighted because the information MBTs provide can guide site management decision-making, avoid unproductive efforts, and can substantially shorten the active phase of remedial efforts, which would result in substantial cost-savings. qPCR is a standard MBT applied for site assessment prior to technology implementation but also relevant for monitoring of active and passive bioremediation. The design of sound monitoring plans and the rigorous implementation of monitoring regimes are crucial for successful bioremediation, so that the effects of treatment can be documented and linked to the microbiology, and corrective measures can be implemented based on scientific evidence. Therefore, qPCR monitoring should occur regularly (e.g., quarterly) during the active phases of remedial efforts and continue with lower frequency during the passive phase.

A major advantage of the QuantArray[®] system is the ability to analyze many target genes simultaneously in a parallel format. The analysis of a broader suite of biomarkers has obvious benefits as it provides refined information about the biodegrading microbial community. This knowledge enables better decision-making and is a relevant step in moving bioremediation from an empirical approach to a scientific approach with predictable outcomes. The successful and broad application of MBTs will enable precision bioremediation, which embraces the concept of remedial decisions, treatments, and monitoring regimes being tailored to the specific contaminated site, recognizing that each site is unique and that a “one size fits all” approach is neither cost-effective nor goal-oriented. Precision bioremediation aims at meeting cleanup goals in the most efficient manner while avoiding unnecessary investments and consumption of resources (energy, biostimulation amendments, etc.), and minimizing the ecological footprint of the treatment. Better remedial practice hinges on the rigorous application of MBTs. While upfront investment for MBT application and analysis is required, the potential for substantial overall cost-savings is large because MBT-informed site management decision-making can reduce remediation time frames and accelerate the path toward site closure.

A cost comparison was performed between the regular qPCR approach in 96-well plates and the high-throughput OpenArray[®] qPCR approach. Also included in the cost comparison was qPCR performed in 384-well plates, which relies on the same steps as regular qPCR. Table 26 shows the cost for a single qPCR assay performed using the regular qPCR approach in 96-well and 384-well formats and the RD-qChip v2. The cost-savings using the high-throughput qPCR approach are substantial, reducing the cost per qPCR assay from about \$4 to about \$0.60. The power of the high-throughput qPCR approach lies in its ability to analyze many targets in a parallel format (i.e., many samples can be analyzed simultaneously), which is ideal when used for contaminated site monitoring regimes. With a single RD-qChip v2 array plate, 102 gene targets and up to 24 samples can be analyzed simultaneously. The QuantStudio[®] can run up to four array plates at the same time, bringing the total number of qPCR assays per 2-hour instrument run to a theoretical maximum of 12,288. Since each array plate includes 384 controls assays, the actual number of qPCR assays for target genes is 2,688 per array plate.

The cost for a single RD-qChip v2 array plate (as of March 2020) is about \$1,500 and a minimum of 10 plates must be included in a single order. This substantial upfront investment requires that the qPCR assays are carefully designed and validated, as was done for RD-qChip v2. The \$1,500 cost per array plate requires efficient usage of the array plate’s capacity (i.e., 24 samples), because

assays without template DNA would not yield data and the array plates are single use only (i.e., cannot be run a 2nd time).

Table 26. Cost comparison of traditional qPCR versus high-throughput qPCR.

	96-well plate	384-well plate	OpenArray Plate (RD-qChip)
# of reactions	2,688 (112×24)	2,688 (112×24)	2,688 (112×24)
Reaction volume	20 µL	10 µL	33 nL
Cost for			
Primers/rxn ^a	\$0.005	\$0.005	Manufacturer includes primers and probes with array plates ^h
Probe/rxn ^b	\$0.146	\$0.146	
Mastermix/rxn ^c	\$1.972	\$0.986	\$0.005
Plate or chip/rxn ^d	\$0.059	\$0.015	\$0.571
QIAgility tips/rxn ^e	\$0.365	\$0.365	\$0.006
Accufill tips/rxn ^f	-	-	\$0.017
Labor/rxn ^g	\$1.354	\$0.339	\$0.029
Cost/rxn	\$3.901	\$1.856	\$0.628

^a Assumptions: Primers: \$0.37/base, average length 20 bp, 250 µL of 100 µM stock solution at a cost of \$7.4 per primer. Final primer concentration per assay is 900 nM → 2,775 rxns/\$14.8 (i.e., 0.005 \$ per assay)

^b Assumptions: Probe: follow format for primers (\$350/probe, 250 nM final concentration in assays, shipped is a volume of 60 µL with 100 µM probe concentration, enables 2,400 rxns).

^c Applied Biosystems™ TaqMan™ Universal PCR Master Mix (2X), no AmpErase™ UNG (Cat. No: 4364341), 10 mL (500 rxns for 96-well plate, 1,000 rxns for 384-well plate); Applied Biosystems™ TaqMan™ OpenArray™ Real-Time PCR Master Mix (Cat. No: 4462164), 5 mL (enough to run 41 array plates).

^d Applied Biosystems™ MicroAmp™ Optical 96-Well Reaction Plate (Cat. No: 4316813, 28 plates needed to perform 2,688 rxns), MicroAmp™ Optical 384-Well Reaction Plate (Cat. No: 4326270, 7 plates needed to perform 2688 rxns), Life Technologies custom-designed OpenArray Plate (RD-qChip) in a 112 assays x 24 samples format (1 plate needed to perform 2,688 rxns).

^e Qiagen, 50 µL Conductive Filtered Tips (Cat. No: 990512)

^f Applied Biosystems™ OpenArray™ AccuFill™ System Tips (Cat. No: 4458107), Applied Biosystems™ QuantStudio™ 12K Flex OpenArray™ Accessories Kit (Cat. No: 4469576)

^g \$26/hour is assumed for labor calculation. 96-well plate: 140 hrs needed to run 2,688 rxns (5 hours to prepare and run one 96-well plate, 28 plates are needed for 2,688 assays); 384-well plate: 35 hrs needed to run 2,688 rxns (5 hours to prepare and run one 384-well plate, 7 plates are needed for 2,688 assays); RD qChip: 3 hrs needed to prepare and run 2,688 rxns.

^h The list of primer and probe sequences is sent to Life Technologies and the company assembles the array plates with the custom primer/probe design. In this project, we also ordered all primers and probes to perform qPCR in 96- and 384-well plates for validation studies. This additional cost was not included in the cost/rxn calculation because this was a one-time expense for validation of the approach.

Task 2: Proteomics – Develop proteomics pipeline and validate with defined samples

Global proteomics analyses of pure cultures of *Dhc* for development of a LC-MRM-MS approach for biomarker protein monitoring

In-silico peptidome comparison provides preliminary support for the development of a *Dhc* biomarker monitoring approach through LC-MRM-MS targeted proteomics

Dhc strains co-exist with other bacteria in contaminated groundwater aquifers. This scenario creates a biological challenge for the desired use of *Dhc* peptides that can be used to inform the presence of *Dhc*-specific protein biomarkers. Therefore, to explore the specificity of the *Dhc* peptidomes (i.e., the predicted suite of peptides generated from the proteolytic digest) in a broad biological context, an *in silico* comparison between several *Dhc* proteomes and the proteomes of other bacteria that have been isolated from groundwater, soil or aquifer materials was conducted. Figure 25 shows the results from the Peptidome Analysis tool of Unipept 3.2.

As observed, members of the *Dhc* shared higher percentages of similarity in their peptidomes, with the most dissimilar strains being 195 and FL2, which clustered together at 47% similarity. This comparison also resulted in an average of 2% similarity between the peptidomes of *Dhc* strains 195 and VS to other bacteria, with a maximum of 4% similarity between these strains and *Dhgm lykanthroporepellens* strain BL-DC-9, while the remaining *Dhc* strains had less than 1% similarity with other bacteria. Overall, this *in silico* analysis suggests there is potential for species-level targeted proteomic analyses of *Dhc* proteins in environmental samples, including contaminated groundwater.

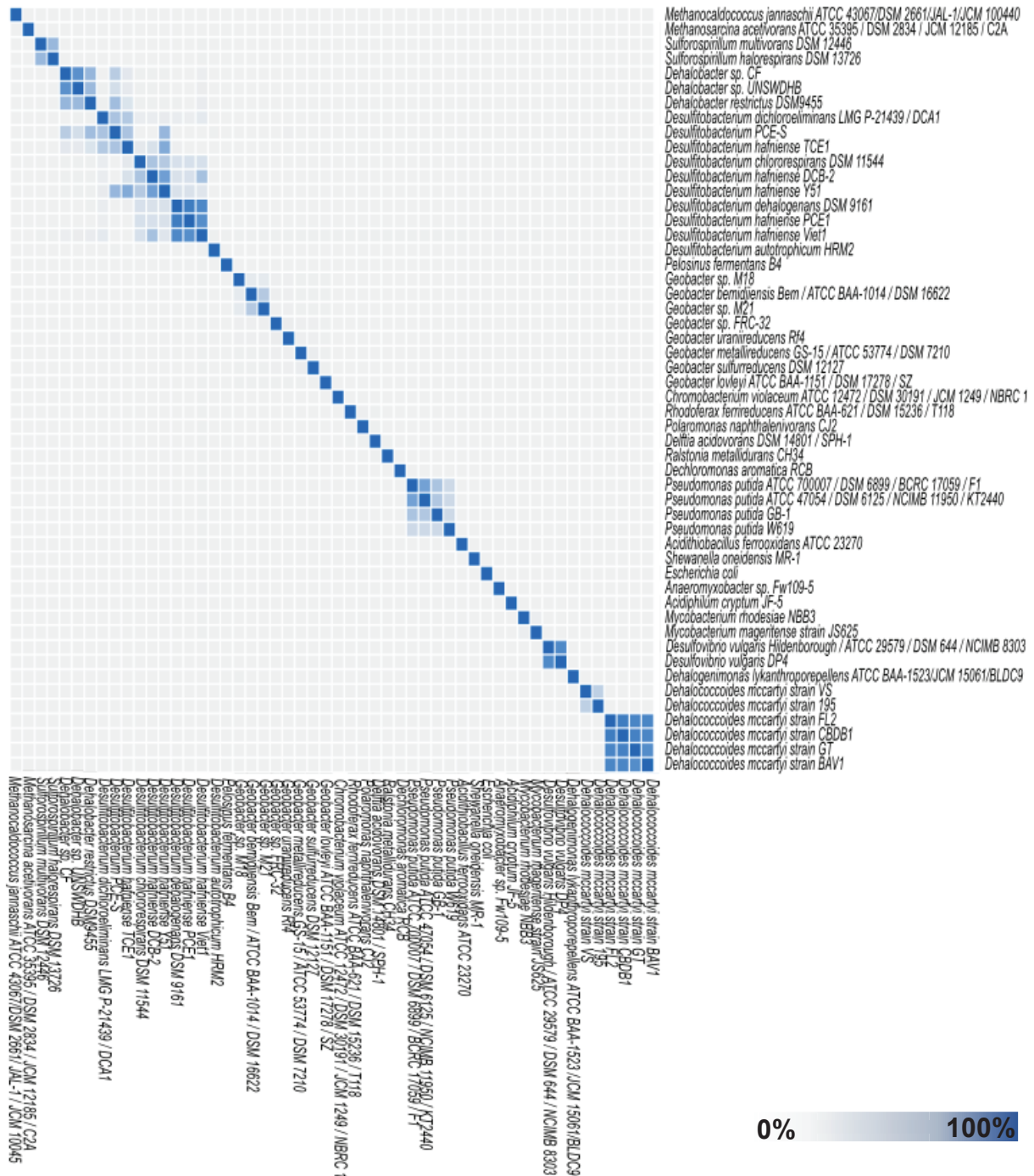


Figure 25. Peptidome similarity matrix calculated with the Unique Peptide Finder application of Unipept 3.2. Similarity matrix calculated with the Unique Peptide Finder application of Unipept 3.2 (<https://unipept.ugent.be/>) between the tryptic peptidomes of the axenic *Dhc* strains BAV1, GT, and FL2 and other bacteria that have been isolated or identified in groundwater or aquifer materials. The *E. coli* proteome was also added as distant comparison. More than 65% of peptidome similarity was observed amongst *Dhc* strains BAV1, FL2, GT and CBDB1.

Global proteomics of pure cultures of *Dhc* inform biomarker expression profiles and peptide selection for targeted proteomics

Designing of the MRM-MS assay

Designing an MRM-MS assay requires prior knowledge of the target proteins and peptides, which provides combinations of unique peptide precursor and fragment ion pairs (transitions) that facilitate their detection and identification at high sensitivity. As proteins can vary in their amino acid compositions, it is not uncommon that targeted proteins in LC-MRM-MS methods can yield tens to hundreds of peptides upon proteolytic digestion (Lange et al., 2008). Therefore, to explore the diversity of peptides produced from the tryptic digestion of proteins expressed by pure cultures of *Dhc* strains 195, FL2 and BAV1, high mass accuracy and resolution data from global proteomics runs of these tryptic digests was collected. This step provided an initial list of tryptic peptides derived from proteins that have been selected in previous LC-MRM-MS studies to inform about *Dhc* presence, general activity, and specific reductive dechlorination activity (Table 27) (Adrian et al., 2007b; Morris et al., 2007; Rowe et al., 2012; Schiffmann et al., 2014a; Werner et al., 2009).

Table 27. *Dhc* protein biomarkers used as initial targets. Protein accessions numbers in the proteome databases used for each strain are presented. Records marked “N/A” indicate the protein is not present in the respective proteome. *Dhc* protein biomarkers selected and monitored as initial targets in this study have been found in published targeted proteomics studies using tryptic digests of axenic *Dhc* cultures or mixed cultures containing *Dhc*.

Target biomarker [abbreviation]	Biomarker description	Protein accession		
		Strain 195 ^a	Strain FL2 ^b	Strain BAV1 ^a
60 kDa chaperonin [GroEL]	Housekeeping protein. Informs presence of <i>Dhc</i> .	Q3Z6L3	demc_1274	ABQ17815
Formate dehydrogenase, alpha subunit (iron-sulfur molybdo-enzyme subunit) [FdhA]	General marker of active dechlorination process.	Q3ZA14	demc_808	ABQ16756
Trichloroethene reductive dehalogenase [TceA]	Process specific marker of active dechlorination (TCE→VC)	Q3ZAB8	demc_738	N/A
Vinyl chloride reductive dehalogenase [BvcA]	Process specific marker of active dechlorination (DCEs, VC → Ethene)	N/A	N/A	ABQ17429
Elongation factor Tu [EF-TU]	Housekeeping protein. General activity/ presence of <i>Dhc</i> .	Q3Z7S9	demc_108	ABQ17463
Ribosomal protein L7/L12 [rpL7/L12]	Housekeeping protein. General activity/ presence of <i>Dhc</i> .	Q3Z7T6	demc_114	ABQ17470
BNR/Asp-box repeat domain protein [S-layer]	Structural protein. Presence of <i>Dhc</i> .	Q3Z6N3	demc_1296	ABQ17793

^a Proteins of *Dhc* strains 195 (ID. UP000008289) and BAV1 (ID. UP000002607) were downloaded from the Uniprot database.

^b The IGS Annotation Engine was used for structural and functional annotation of the *Dhc* strain FL2 sequences (<http://ae.igs.umaryland.edu/cgi/index.cgi>, Reference: PMID:21677861) and the web-based tool Manatee was used to view and download protein annotations (<http://manatee.sourceforge.net/>).

Global proteomics analyses resulted in proteome coverages of 59%, 57% and 60% for *Dhc* strains 195, FL2 and BAV1, respectively. The reported percentages were higher than the 44% obtained when a published raw spectral dataset of *Dhc* strain CBDB1 (PRIDE database ID: PXD003081) (Goris et al., 2016; Schiffmann et al., 2014b) was re-processed with the peptide/protein database search strategy used in this study, but similar to percentages published before for proteomics measurements of *Dhc* strains 195 and DCMB4 (Men et al., 2012; Pöritz et al., 2013; Schiffmann et al., 2014a; Schiffmann et al., 2014b; Werner et al., 2009). Overall, the analytical dynamic range of the proteome measurements spanned ~5 orders of magnitude, with all the targeted proteins ranking amongst the top 50% abundant proteins (Figure 26A). Each biomarker protein was also found with similar log2 normalized MS1 intensities within each strain dataset (Figure A-73). The resulting percentages of sequence coverages and number of peptide spectrum matches per targeted protein also demonstrated comparable efficiencies of tryptic digestion achieved between strains 195, FL2 and BAV1 (Figure 26C & Figure 26D). The complete protein identification data are listed in Supplemental Table S1, Excel Document.

The expression of FdhA proteins observed in cultures of *Dhc* strains 195, FL2, and BAV1 is in agreement with prior studies that have observed FdhA in comparable abundances relative to RDases and hydrogenases in actively dechlorinating *Dhc* pure and mixed cultures (Adrian et al., 2007b; Morris et al., 2007; Morris et al., 2006; Schipp et al., 2013; Türkowsky et al., 2018). Recent studies of the Fdh complex (i.e., the iron-sulfur molybdoenzyme complex I [CISM]) of *Dhc* strain CBDB1 revealed a tight spatial association between FdhA and the RDase CbrA (ID CbdbA194) (Zinder, 2016). Supported by *in vitro* dehalogenation activity assays, these observations suggest that FdhA serves an integral role in the respiratory chain of *Dhc* (i.e., FdhA may serve as an electron-channeling module between the Hup hydrogenase and the RDase (Kublik et al., 2016), and as such, can serve as a general biomarker of *Dhc* dechlorination activity (Figure 22).

RDase are biomarkers for active dechlorination and can provide additional information regarding specific chlorinated compounds that undergo reductive dechlorination. The types of chlorinated compounds dechlorinated by various RDases makes the *Dhc* group functionally diverse (Adrian et al., 2007b; He et al., 2005; Sung et al., 2006b; Taş et al., 2010). The TceA RDase in the proteomes of strains 195 and FL2 and the BvcA RDase in the proteome of strain BAV1 were observed amongst the top five most abundant proteins in their global proteomics dataset, respectively (Supplemental Table S1, Excel Document).

In total, 617 protein groups (> 85% amino acid sequence) were common between all three strains analyzed in this study and the dataset from strain CBDB1 used as comparison (Figure 26B). These protein groups encompass homologues of the targeted GroEL, EF-TU, rpL7/L12 and FdhA proteins. Putative S-layer proteins of strains BAV1 and CBDB1 clustered together at 89% sequence identity, whereas the annotated S-layer proteins of strains 195 and FL2 were independent from these and each other. For the RDases, the TceA homologues clustered at 99% identity, while RDase BvcA was found amongst the 61 unique protein groups of strain BAV1. These observations reveal that candidate peptide sequences from protein biomarkers can target multiple *Dhc* strains or can be potentially used as strain-specific targets when monitoring mixed cultures or environmental samples.

In addition to TceA homologues and BvcA, other 18 RDase homologues were also identified in these shotgun proteomics measurements, albeit at lower abundances (MS1 intensities) (Figure 28A-Figure 28 C). The identification of multiple RDases in actively dechlorinating cultures of *Dhc* is related to the multiple sets of RDase (*rdhA*) genes annotated in single *Dhc* genomes (i.e., 17 RDase genes in strain 195, 24 RDase genes in strain FL2, and 11 RDase genes in strain BAV1

(Türkowsky et al., 2018). The co-expression of RDases by single *Dhc* strains has been reported before and it has been hypothesized as a mechanism of adaptation to use naturally occurring and anthropogenic organohalogenes (Löffler et al., 2015; Yang et al., 2017c). Hence, to provide insight into the diversity of the expressed proteins and better validate our RDase biomarker selection, we evaluated the phylogenetic relationships between the RDases present in the proteomes of *Dhc* strains 195, FL2 and BAV1.

The phylogenetic analysis indicated that the selected TceA homologues from strains 195 and FL2 are closely related with each other but were independent from other expressed RDases, including the targeted BvcA and the other second most abundant RDases in each dataset (Figure 28). These results are important, as they highlight the uniqueness of TceA and BvcA as biomarkers for dechlorination of chlorinated ethenes. Moreover, the substrate ranges of TceA homologues and BvcA are known, while the participation of other RDases in reductive dechlorination reactions or other physiological roles remains to be proven experimentally (Türkowsky et al., 2018). The higher expression and sequence coverages obtained for the TceA and BvcA RDases also provided on average four times more the number of tryptic peptides obtained from other expressed RDases, which was helpful for the development of the targeted assay during designing process of targeted proteomics LC-MRM-MS approach for biomarker protein monitoring. The selection of peptide candidates for MRM used empirical rules (Gallien et al., 2011) to determine those peptides that could have higher chances of performing well under LC-MRM-MS conditions and at the same time indicate the presence of *Dhc* biomarker proteins in groundwater.

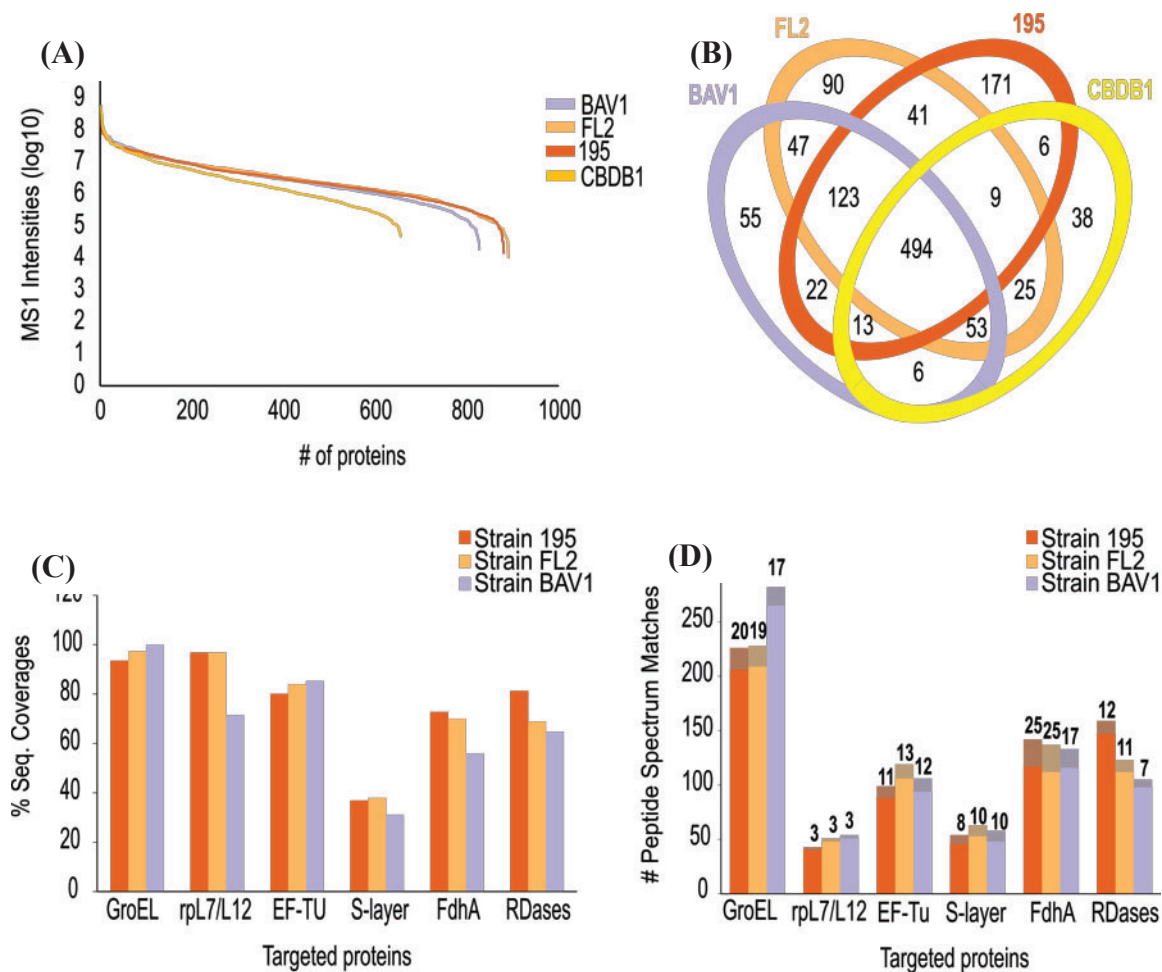


Figure 26. Protein abundance, clusters, coverages and unique peptides. Examples of validation by internal standards. (A) Extracted ion chromatogram (XIC) of the FdhA peptide ALGIVYLD SQAR (653.361 m/z ++) identified in a technical replicate of groundwater sample 33NA4. The peak group observed at 42.2 mins is the real signal from the peptide. Its identity was confirmed not only by comparing the fragmentation profiles from the same peptide in pure cultures of *Dhc* but also by comparison to the signal of a spiked internal standard observed at 42.3 mins and having a log2 fold change in raw AUCs of 7 compared to the endogenous signal. (B) XIC of the signals observed from the S-layer peptide AGIIDVPATADDATK (729.377 m/z ++) in a technical replicate of groundwater sample 97. In this case, an intense peak group was observed at 22.8 mins. However, the fragmentation profile of this signal compared to the resulting profiles in pure cultures did not meet the identity criteria; furthermore, a spiked internal standard showed the true signal originating from the targeted peptide at 34.0 mins, while the endogenous peak group of before was still observed at 22.9 mins.

Design of a targeted proteomics LC-MRM-MS approach for biomarker protein monitoring

Depending on the biological complexity of a sample, interfering signals from closely related peptide sequences or other completely unrelated sequences can result in mass pairs that are too close to be sufficiently differentiated out from the targeted peptides resulting in scenarios where multiple signals can be identified in a single MRM extracted ion chromatogram (XIC) (Gallien et al., 2011). Nonetheless, the advantage of developing this assay in axenic *Dhc* cultures is that targeted proteins were readily detectable and the XICs resulting from a bacterial isolate proteome

are expected to be less complicated compared to a community proteome. Therefore, most of the signals to be observed from the targeted peptides were expected to be intense peak groups, or traces of co-eluting transitions that are grouped for each targeted peptide with low levels of interferences (Lange et al., 2008).

Adaptation of global proteomics results to targeted proteomics

Signal quality evaluation of peak groups by LC-MRM-MS was conducted on 79, 81 and 66 peptides from the targeted proteins identified in the global proteomics datasets of cultures of *Dhc* strains 195, FL2 and BAV1, respectively. In total, 29 peptides and 142 transitions from the digest of strain 195, 22 peptides and 107 transitions from strain FL2 and 17 peptides and 83 transitions from strain BAV1, were selected. Examples of the type of signals chosen and discarded are shown in Figure 27. From this selection process, the top five transitions ranked by contribution to total area under the curve (AUC) per peptide were preserved, resulting in a total of 55 peptides (unique and shared between strains) equivalent to 270 transitions.

The analysis of shared peptides in protein homologues also supported the differentiation of target specific peak group signals from other interfering peptides having close transition m/z values, as peak group signals originating from the same targets in LC-MRM-MS runs tend to have similar retention time profile, shape and fragmentation profiles (Reiter et al., 2011). Figure A-74 shows the MRM chromatographic traces of peptide YFGASSVGAIK found in homologues of the TceA protein expressed by *Dhc* strains 195 and FL2. In the case of the tryptic digest of strain FL2, other low intensity peak groups like the one indicated with red arrowheads were also observed at all amounts of sample tested. However, when the same peptide was analyzed in strain 195, these peak groups were not consistently identified thus pointing out to the true signal derived from the peptide of interest.

The remaining candidates were then submitted to *in silico* analysis on an individual basis employing the Tryptic Peptide Analysis tool of Unipept, with the goal of determining protein sequences in the UniProt database that would generate the peptide upon digestion and discard peptides matching ≥ 100 Uniprot entries and found in several bacterial species.

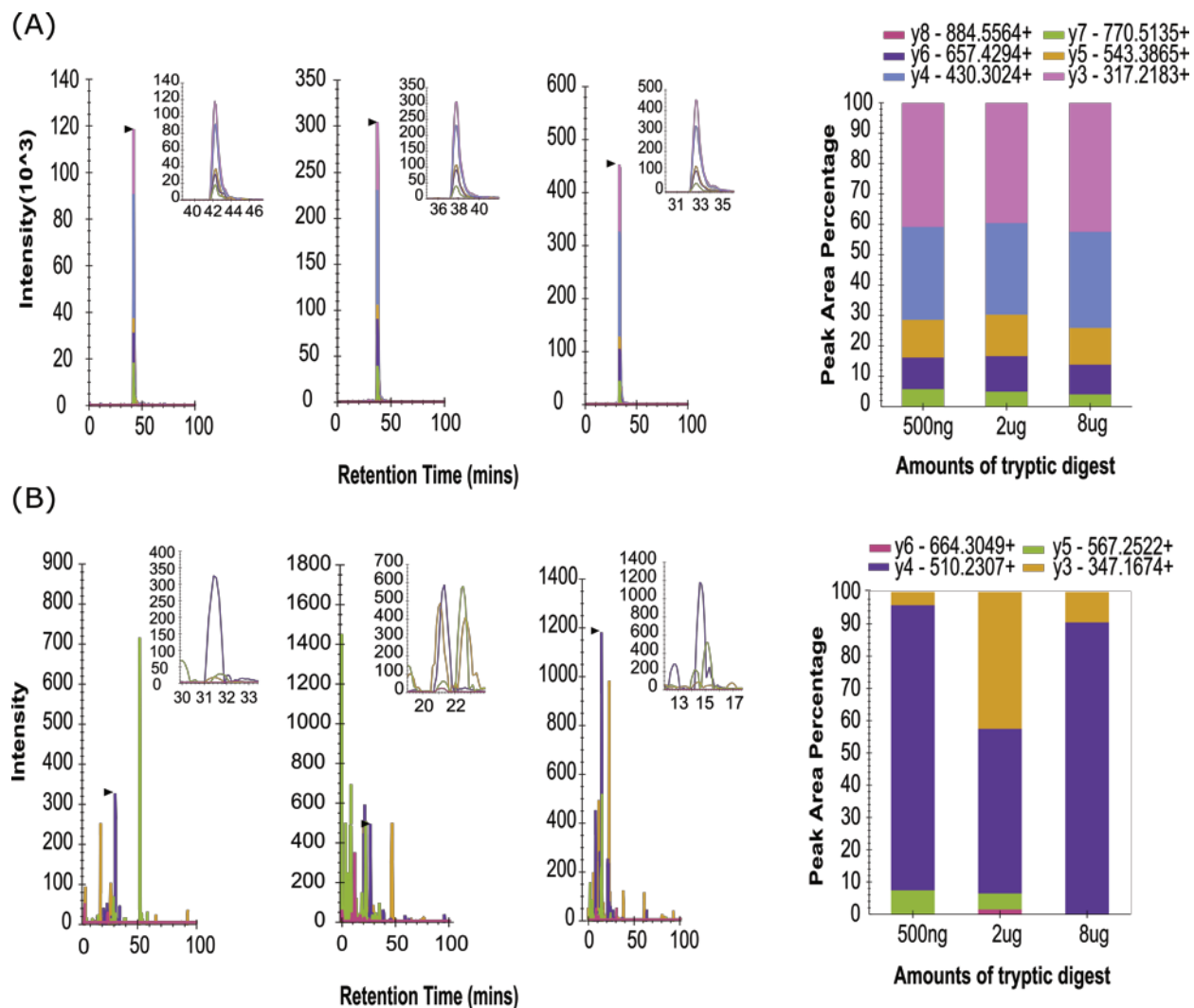


Figure 27. Best peak groups by Skyline for the GroEL peptides. XICs of the best peak groups that were automatically selected by Skyline for the GroEL peptides (A) GNLNILAVK (471.292 m/z ++) and (B) APGYGDR (368.174 m/z ++) identified in runs of 500ng, 2 μ g and 8 μ g (left to right) from the tryptic digest of a *Dhc* strain FL2 culture. The inserts in each XIC are the magnifications of the peaks marked by arrowheads. Each transition (precursor $\rightarrow$ fragment ion pair) is identified by different colors. The images on the right side show the relative contribution of each fragment ion to each peptide peak marked with arrowheads. In this case, peptide APGYGDR was removed from further analysis.

***In silico* specificity of selected peptides**

Essential for targeted proteomics experiments are control measures to ensure that the selected peptides uniquely identify the protein(s) of interest (Lange et al., 2008). The microbial diversity, and hence the diversity of proteins, in particular the presence of other organohalide-respiring bacteria thriving in the environments where *Dhc* is found (Löffler et al., 2013a; Yan et al., 2016; Yi et al., 2012) creates a challenge for the selection of unique peptides. Initially, a similarity comparison was done between the peptidomes of several *Dhc* strains versus the peptidomes other organohalide-respiring bacteria commonly found in groundwater aquifers or sediments, and observed the potential of developing a species-level targeted proteomic assay for *Dhc* and its application to contaminated groundwater; however, this was a simplified view, and due to the similarity of peptidomes between *Dhc* strains, the possibility of finding unique peptides targeting multiple *Dhc* strains was real. Due to this reason, sequence specificities of each selected peptide candidate were further assessed individually with the Tryptic Peptide Analysis tool of Unipept 3.2 and Protein BLAST searches. Peptides were deemed as *Dhc*-specific when they were not found in any other bacterial protein sequence available in UniProt and NCBI *nr* databases, and when multiple *Dhc* strains shared the candidate peptide sequence by means of both *in silico* searches. Peptides were considered semi-specific when they were found in proteins derived from related organohalide-respiring bacteria, and as non-specific when they were found in proteins of non-organohalide-respiring bacteria. Compiled results from these *in silico* searches are presented in Supplemental Table S2, Excel Document.

Out of the seven peptides selected from the housekeeping chaperonin GroEL, peptide DGVITIEESR was the only one non-specific to the *Dhc*, a surprising result considering that homologues of this protein are found in diverse classes of bacteria (Hayer-Hartl et al., 2016). From the targeted EF-TU proteins, peptide TTLTAATR was found in more than 100 UniProt entries, as was the case of the remaining peptide ELTSLGLK from the ribosomal protein L7/L12. Due to these observations, the three aforementioned peptides were removed from the analysis, which resulted in the complete loss of the rplL7/L12 protein from the assay.

Peptides selected from TceA and BvcA RDases were neither unique to *Dhc* strains nor did they provide strain-level specificity. In fact, they are part of protein sequences from other bacteria living in specialized ecological niches. For example, peptide YFGASSVGAIK from the TceA RDase, can be produced from a tDCE RDase subunit A protein expressed by *Dhgm* sp. strain WBC-2, while peptide LEILQ GK (465.268 *m/z*) can be found in “uncharacterized proteins” from *Bacteroidetes* bacterium GWA2_40_15 and *Frigoribacterium* sp. RIT-PI-h. The chosen BvcA peptides also matched RDase sequences found in microorganisms annotated as “bacterium” or “uncultured bacterium”. One possible explanation for these observations could be related to horizontal gene transfer, which has been proposed for RDases genes (Krajmalnik-Brown et al., 2007; Regeard et al., 2005).

Although the diversity of unique RDases has been described as a factor that can complicate their use as general biomarkers of *Dhc* reductive dechlorination activity in groundwater in a targeted proteomics setup (Heavner et al., 2018), the selected peptides can still function as bioindicators of homologous proteins even present in different bacterial species. Here, we decided to keep semi-specific *Dhc* RDase peptides as their detection still provides valuable information about dechlorination activity. For example, when combined with 16S rRNA gene-targeted qPCR measurements, it could confirm the identity of the species of microorganism(s) performing active reductive dechlorination. On this note, a recent publication discovered a *Dhgm* sp. that was able to dechlorinate TCE to ethene, suggesting that the contribution of *Dhgm* in the reductive

dechlorination of chlorinated solvents in contaminated aquifers may be greater than is currently recognized (Yang et al., 2017c).

Candidate peptides from the annotated FdhA (general biomarker of *Dhc* activity) and S-layer (structural housekeeping) proteins were specific to *Dhc* and in certain cases provided strain level resolution. For example, the *in-silico* analysis demonstrated that the FdhA peptides GTELISVDCR and SELEVISSLFSR were specific to *Dhc* strain 195, while peptide TDNNTNYSYINAIK was specific to the FdhA of *Dhc* strain BAV1. All peptides of the S-layer protein were specific to a few *Dhc* proteomes stored in UniProt, a useful characteristic for environmental monitoring of certain *Dhc* strains.

While the predicted strain uniqueness of the selected S-layer peptides can compromise their detection in environmental matrices, due to their absence in the proteomes of other *Dhc* strains that could be abundant in a sample, the advantage of including other “general” peptides derived from common housekeeping proteins such as GroEL and found in multiple *Dhc* strains (i.e., GVDTLANTVR) can potentially remedy this situation. The same scenario is applicable to the detection of FdhA. Although three of the selected peptides were strain specific, others (i.e., ALGIVYLD SQAR) can be found in protein homologues present in multiple *Dhc* strains. Additionally, the detection of strain-specific FdhA peptides, combined with the identification of RDase peptides, can be used as supporting evidence of the participation of particular *Dhc* strains in active dechlorination processes.

Table 28. Final set of peptides selected in axenic *Dhc* cultures and then monitored in consortium BDI and groundwater samples. *In-silico* predicted specificities, based on the analysis conducted with UniPept, are shown (detailed information is presented in Supplemental Table S2). All cysteine residues were carbamidomethylated [+57.0].

Targeted biomarker protein	Selected peptides	Biomarker description
60 kDa chaperonin (GroEL)	^Δ GVDTLANTVR WGAPTVIDDGVTIAR ^Δ IETVAELLPALEK GNLNILAVK AQIEETESAFDR LEGDEATGVSIVR	Housekeeping protein. General activity/presence of <i>Dhc</i> .
Formate dehydrogenase alpha subunit (FdhA)	SWDWALGEIANK ^Δ ALGIVYLD SQAR ^Δ +GTELISVDC [+57.0]R SSEQNAASLLK TDTNTDYSYVNAIK +SELEVISSLFSR GSAGEYPVIC[+57.0]TTVR LSTASSLEALAASFGR SGSEIAFIGGLIK SGSEIAFTGGLIK +TDNNTNYSYINAIK SELEVISSLRSR VC[+57.0]AFFAATGK	General marker of active <i>Dhc</i> dechlorination processes.
TCE reductive dehalogenase (TceA)	ΦDVDDL SAGK ΦLEIELQ GK ^Δ ΦYFGASSVGAIK	Process specific marker of active dechlorination (TCE → VC)
VC reductive dehalogenase (BvcA)	ΦDLYLAWAK ^Δ ΦTPVPIVWEEVDK ^Δ ΦSTVAATPVFNSFFR	Process specific marker of active dechlorination (DCEs, VC → Ethene)
Elongation factor Tu (EF-Tu)	NSFPGDEIPIVR ΦILDSAEPGDAVGLLLR NSFPGDEIPVVR ^Δ ILDTAEPGDAVGLLLR	Housekeeping protein. General activity/presence of <i>Dhc</i> .
Surface layer (S-layer)	+YFGNQWNQTALC[+57.0]K +AGIIPAPTTASDAYK +VAYGTTTGTETTATTLK ^Δ FYDVGILEWNADK TAVYATAVYDDGDDTLVR TWYSADGLTFTK ^Δ AGIIDVPATADDATK VC[+57.0]YGLPTGTDIEATTLK YFGNQWNQPATC[+57.0]K	Structural protein. Presence of <i>Dhc</i> .

†Peptide found only in one or two *Dhc* proteomes.

Φ Peptide found in protein homologues of other bacterial species (i.e., *Dhgm lykanthroporepellens* strain BL-DC-9).

^Δ Unlabeled peptide standards available (>95% purity).

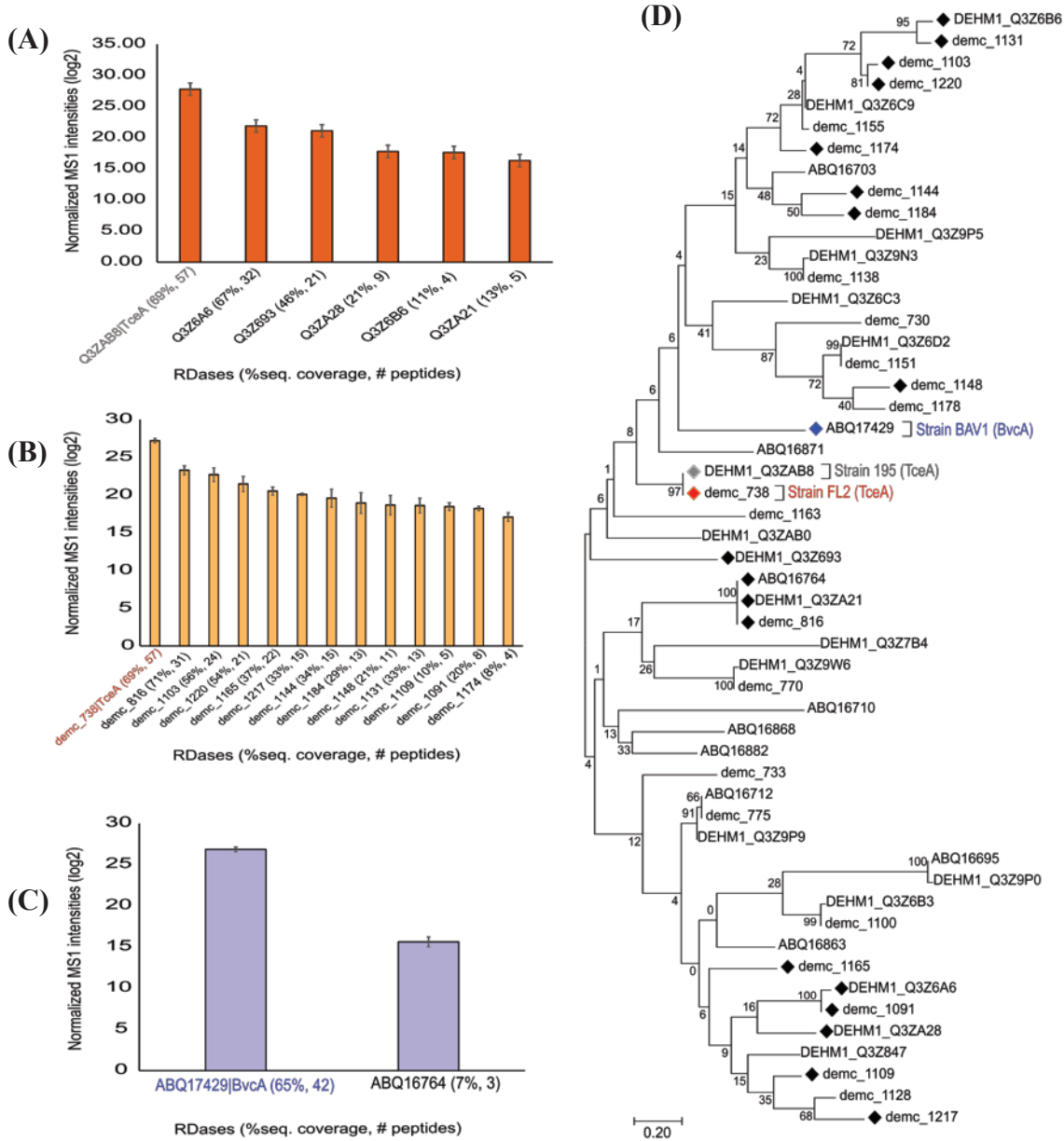


Figure 28. Relative abundances of other RDases identified in samples from axenic *Dhc* cultures using the global proteomics approach. Relative abundances of other identified RDases in global proteomics runs of tryptic digests of extracts from axenic *Dhc* strains 195, FL2, and BAV1 and their phylogenetic relationships with the targeted TceA (gray and orange) and BvcA (blue) RDases. Normalized log2 MS1 intensities of other RDase proteins identified in tryptic digests of (A) *Dhc* strain 195, (B) strain FL2 and (C) strain BAV1. Error bars were calculated based on 2 biological replicates. Numbers in parentheses are the percentage of sequence coverage and total number of peptides identified per protein, respectively. (D) Highest log likelihood (-3172.61) phylogenetic tree depicting the relationships among 52 RDases sequences in the proteomes of *Dhc* strains 195, FL2 and BAV1. Bootstrap values used to estimate the relative confidence scores in phylogenetic groups are shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The filled diamonds represent proteins identified by global proteomic analyses.

Application of the selected biomarkers for targeted proteomics analyses in a PCE-to-ethene dechlorinating consortium

The ability to detect any *Dhc* biomarker peptide in tryptic digests of groundwater samples was expected to be impacted by their biological complexities, as the chance of co-eluting peptides yielding fragments that are not distinguished from the monitored *m/z* transitions increases with the number of proteins present (Kruh-Garcia et al., 2014; Schiffmann et al., 2014a).

After selection of peptides and transitions in axenic *Dhc* cultures, their identification was initially assessed in tryptic digests of the dechlorinating consortium BDI. The BDI consortium harbors multiple *Dhc* strains, including strains BAV1, FL2, and GT (Fletcher et al., 2011; Yang et al., 2017a). The known microbial diversity of BDI allowed an easier validation of peptide identification with criteria that included, amongst others, the comparison of correlation scores for transition intensity ratios between the signals detected in samples to those observed in pure cultures or to samples spiked with 5 pmol of internal standards.

Through LC-MRM-MS analyses, 13 peptides were identified out of the 37 that were targeted. Among those, GroEL peptides with high representation in the proteomes of multiple *Dhc* strains were observed (Figure 29). Also detected was the more conserved GroEL peptide LEGDEATGVSIVR, which, according to the UniPept searches, is only present in the proteomes of *Dhc* strains 195, KBTCE2, CG4 and KBTCE3 (Supplemental Table S2, Excel Document). Interestingly, in relation to the identification of peptide LEGDEATGVSIVR, we also detected the EF-TU peptide NSFPGDEIPIVR, which is specific to the proteomes of the same *Dhc* strains as peptide LEGDEATGVSIVR, thus suggesting that these strains are part of the *Dhc* population in consortium BDI.

Active dechlorination activity was inferred through the presence of four FdhA peptides and one of the targeted TceA peptides. As with the identification of the GroEL peptide LEGDEATGVSIVR and the EF-TU peptide NSFPGDEIPIVR, the FdhA peptide GSAGEYPVICCTTVR was also found in the proteomes of strains 195, KBTCE2, CG4 and KBTCE3, which suggested the involvement of one or more of these strains in the dechlorination process. In addition, the detection of the TceA peptide YFGASSVGAIK, shared by *Dhc* strains 195 and FL2, provided additional evidence for the presence of strain 195-type bacterium in culture BDI.

The evidence provided by targeted proteomics about the existence of additional but not yet recognized *Dhc* strains in consortium BDI prompted us to explore the microbial diversity of this culture by means of high-mass-accuracy/high-mass-resolution global proteomics analyses. By assembling a proteome database of other known strains of *Dhc*, the BDI spectral data indeed revealed that organisms representing *Dhc* strain 195 were present in this culture, as we were able to detect unique peptides matching proteins specific to certain strains (*i.e.*, to the S-layer protein of *Dhc* strain 195). The complete list of protein identifications in BDI is presented in Supplemental Table S3, Excel Document.

Global proteomics analyses also revealed the absence of the BvcA enzyme in BDI, which was consistent with the targeted proteomics results. qPCR experiments showing that *Dhc* strain BAV1 carrying the *bvcA* gene was lost from consortium BDI after repeated transfers with PCE or TCE corroborated this result. The lack of *Dhc* strains expressing BvcA in BDI seems to be compensated by *Dhc* strains expressing VcrA (e.g., strain GT). VcrA was not targeted in the MRM assay, but expression levels of this enzyme were confirmed by global proteomics analysis of consortium BDI, where it may play a role in the dechlorination of cDCE to ethene. The involvement of *Dhc* strains expressing VcrA was also supported by the targeted detection of FdhA peptides matching to the proteomes of *Dhc* strains VS and GT (Figure 29).

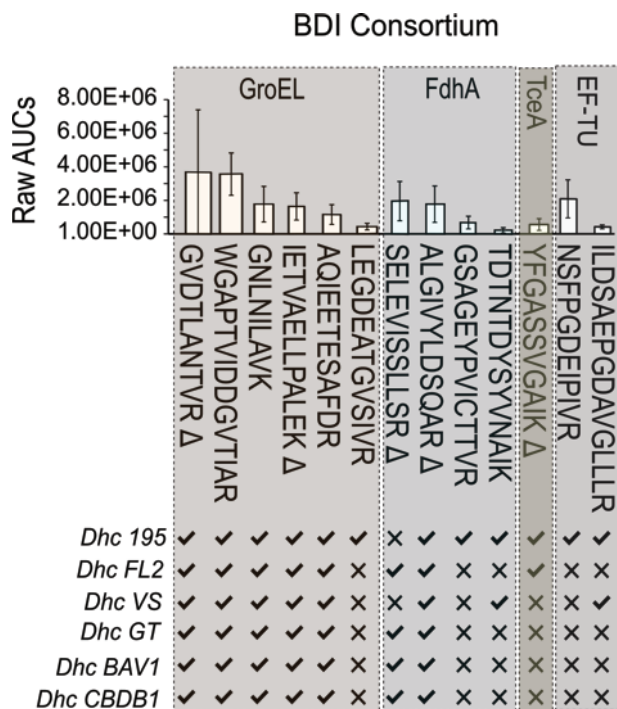


Figure 29. Identification of *Dhc* biomarker peptides by LC-MRM-MS in a tryptic digest of biomass of the PCE-to-ethene dechlorinating consortium BDI. The figure shows the average raw peak area under the curve (AUC) values of the targeted peptides identified in three technical replicate LC-MRM-MS runs. Error bars are the standard error of the mean. Peptides marked with Δ were identified with supporting evidence from spiked-in unlabeled standards. The inserts below the graph show the specificities of the peptides, determined *in-silico*, to the proteomes of the six most common isolates of *Dhc* bacteria. The complete list of other proteins and organisms that can produce the same peptide upon tryptic digestion are listed in the Supplemental Table S2, Excel Document.

Application of the selected biomarker panel for targeted proteomics analyses of groundwater impacted with chlorinated ethenes

Seven groundwater samples collected from various contaminated DoD sites impacted with chlorinated ethenes were analyzed by targeted proteomics. Amongst the identified contaminants were TCE, cDCE and VC. These compounds are substrates and intermediates of the anaerobic reductive dechlorination sequence carried out by *Dhc* that ultimately yield ethene as the end product (Figure 2).

qPCR measurements performed on groundwater samples M17, M18, 97, 116, and 29 (33NA4 samples for DNA extraction were not available) showed average total bacterial 16S rRNA gene copies/mL values ranging from $2.6\text{E}+07 \pm 1.4\text{E}+06$ in sample 116 to $9.8\text{E}+05$ copies/mL $\pm 4.9\text{E}+05$ in sample M18 (Figure 30A). qPCR measurements of 16S rRNA genes of relevant organohalide respiring bacteria (*Dhc*, *Dehalobacter*, and *Dhgm*) demonstrated the presence of *Dhc* bacteria in all samples, with the highest abundance of *Dhc* 16S rRNA genes quantified in sample M17. At the M17 sampling location, *Dhc* represented ~20% of the total bacterial 16S rRNA genes ($2.0\text{E}+05 \pm 1.7\text{E}+03$ copies/mL). Empirical information from bioremediation sites suggested that *Dhc* abundances exceeding $1\text{E}+6$ copies/L result in meaningful reductive dechlorination rates. Thus, the qPCR data suggested that M17 has a *Dhc* population sufficient for reductive dechlorination activity, whereas the *Dhc* abundance in the other samples was below the threshold. As discussed previously, the identification of *Dhc* genes does not necessarily indicate that *Dhc* is actively dechlorinating TCE or any other chlorinated ethene. Amongst the reasons for this observation are the lack of correlation between dechlorination activity and the abundance of *Dhc* 16S rRNA genes and the variable translation rates of RDase transcripts observed in pure and mixed cultures (Heavner et al., 2018; Rahm et al., 2006). Additionally, in groundwater samples, *Dhc* bacteria may be present but not contributing significantly to dechlorination processes due to inhibitory mechanisms (*i.e.*, the presence of perfluoroalkyl acids (Yin et al., 2019) or competition with other organohalide-respiring bacteria. Due to these factors, the identification of *Dhc* reductive dechlorination protein biomarkers would provide more definitive information about whether *Dhc* are actively involved in the dechlorination.

Analysis of the groundwater samples included in this study by targeted proteomics identified *Dhc* biomarker proteins and peptides only in groundwater samples collected from M17 and well 33NA4 (Figure 30B and Figure 30C). A few of the remaining samples were viscous and consisted of black oily, sticky material that complicated filtering in Sterivex cartridges and potentially limited DNA and protein recovery and subsequent measurements.

GroEL proteins were observed in both M17 and 33NA4 samples and were identified by peptides that are highly conserved across the proteomes of multiple *Dhc* strains, including those of the six isolates (Figure 30B and Figure 30C). Besides detection of GroEL in both M17 and 33NA4 samples, targeted peptides from the housekeeping EF-TU and structural S-layer biomarkers were also detected in groundwater M17 (Figure 30B). For example, the EF-TU peptide ILDTAEPGDAVGLLLR, which differs by a single threonine residue compared to the peptide identified in consortium BDI (Figure 30), and is present in multiple *Dhc* strains, demonstrated the utility of targeted proteomics to differentiate single amino acid changes in the sequences of the analytes. The additional detection of the S-layer peptide AGIIDVPATADDATK in sample M17, which is found in four *Dhc* proteomes (including strains GT and FL2), also suggested that specific *Dhc* strains were present in this sample.

Evidence of dechlorination activity was obtained by the detection of two and three FdhA peptides in samples M17 and 33NA4, respectively (Figure 30B and Figure 30C). Common between both samples was the detection of the FdhA peptides ALGIVYLD SQAR and SELEVISSLLSR, which can be found in 25 and 19 *Dhc* proteomes, respectively, of the 31 *Dhc* proteomes available in UniProt (as of July 2018). Peptide ALGIVYLD SQAR has been selected as MRM target for absolute protein abundance quantification in published reports examining pure and mixed *Dhc* cultures (Rowe et al., 2012; Werner et al., 2009), which also points to its high conservation amongst *Dhc* strains and robust characteristics for mass spectrometric analyses. In addition to the ALGIVYLD SQAR and SELEVISSLLSR peptides, the detection of the FdhA peptide

TDNDYSYVNAIK in groundwater sample 33NA4 suggested that organisms representing *Dhc* strains 195, KBTCE2, CG4 and KBTCE3 were involved in active dechlorination.

Supporting the FdhA observations in samples M17 and 33NA4 and hence the potential of active dechlorination, we also identified a TceA peptide in sample 33NA4 and a BvcA peptide in sample M17. For instance, the TceA peptide YFGASSVGAIK in sample 33NA4 (Figure 30C) suggested the involvement of *Dhc* strains expressing the TceA RDase (e.g., strains 195 and FL2) in the dechlorination reactions leading to the transformation of TCE to VC and ethene. Similarly, the BvcA peptide STVAATPVFNSFFR in sample M17 (Figure 30B) pointed to active transformation reactions of cDCE to ethene by strain BAV-type *Dhc*.

The data provided by LC-MRM-MS thus contrasted with the initial qPCR analysis, which detected *Dhc* 16S rRNA genes in all groundwater samples, but peptides of the targeted proteins were not identified in four of them (M18, 97, 116, or 129). This suggested that either the targeted proteins were not expressed in these samples, the proteins were too low in abundance to be detected by targeted proteomics, or the enzymatic digestion of the proteins in a sample could have produced a different set of peptides to the ones targeted. To provide insight into these issues, high-mass-accuracy and high-mass-resolution global proteomics data were also collected. For this purpose, the proteomes of six *Dhc* isolates and other bacteria that have been isolated from aquifers or sediment material contaminated with organic chlorinated compounds were combined into a database for MS spectra search (Supplemental Table S1, Excel Document).

Global proteomics revealed that the samples having the highest numbers of *Dhc* protein identifications were samples M17 (125 groups) and 33NA4 (38 groups), in which peptides from *Dhc* biomarkers were also detected by LC-MRM-MS (Figure 30D). Indeed, the *Dhc* dechlorination biomarkers BvcA for sample M17, TceA for 33NA4, and FdhA for both, were also identified in the global proteomics data.

The detection of TceA in sample 33NA4 and the absence of *Dhgm* proteins by global analyses suggested that the YFGASSVGAIK peptide detected before by targeted proteomics had a *Dhc* origin. We also observed that, except for S-layer proteins that were identified by a different set of peptides to the ones targeted in samples 129 and 116, all the other *Dhc* biomarkers were not detected by means of global proteomics analyses in samples 129, 116 and 97 (Supplemental Table S3, Excel Document), which largely agreed with the targeted proteomics results. In groundwater sample M18, *Dhc* GroEL, EF-TU, and S-layer proteins were identified but with a different set of peptides. The low numbers of *Dhc* protein groups detected in samples M18, 129, 116 and 97, which included proteins that are not directly involved in mediating dechlorination processes, in combination with the aforementioned *Dhc* 16S rRNA gene data, suggested that *Dhc* cells were present but not actively dechlorinating in these samples or expressing levels of proteins that fall below the detection limits of the proteomics approach.

Although these explanations need to be investigated in more detail and are considerations for future studies, targeted proteomics via LC-MRM-MS in the more economically accessible QqQ platform (compared to high mass resolution and accuracy instruments) offers an opportunity to monitor the expression of *Dhc* proteins in contaminated groundwater. When properly optimized, LC-MRM-MS approaches offer the ability to measure dozens of proteins of interest simultaneously, faster and with increased reproducibility and sensitivity over global proteomics methods (Gallien et al., 2011). Another advantage is the determination of absolute amounts of the target proteins in a sample. However, the first step on the application of quantitative MRM assays for environmental monitoring of *Dhc* bacteria requires the initial identification of biomarkers in

unenriched samples where low protein abundances and matrix effects can affect the viability of the assay.

Over the course of Task 2, it has been shown that by combining information from shotgun MS/MS spectra of axenic *Dhc* cultures to select peptide precursors and then testing their signals in MRM mode to further select those that ionized efficiently and were easily detected, *Dhc* peptides and proteins were detectable by LC-MRM-MS in contaminated groundwater from different sites. This approach did not consider any protein enrichment methods and was restricted to a 90 minutes LC gradient, with minimum optimization in the operational parameters. Future applications of targeted proteomics via LC-MRM-MS at groundwater sites should consider targeted peptide specificity in complex backgrounds and the ability to extract and digest proteins from different environmental samples collected using various sampling techniques. If available, the targeted capabilities of high mass accuracy and resolution mass spectrometers can lead to lower detection limits and better filtering of interfering ions and false signals in noisy chromatogram zones.

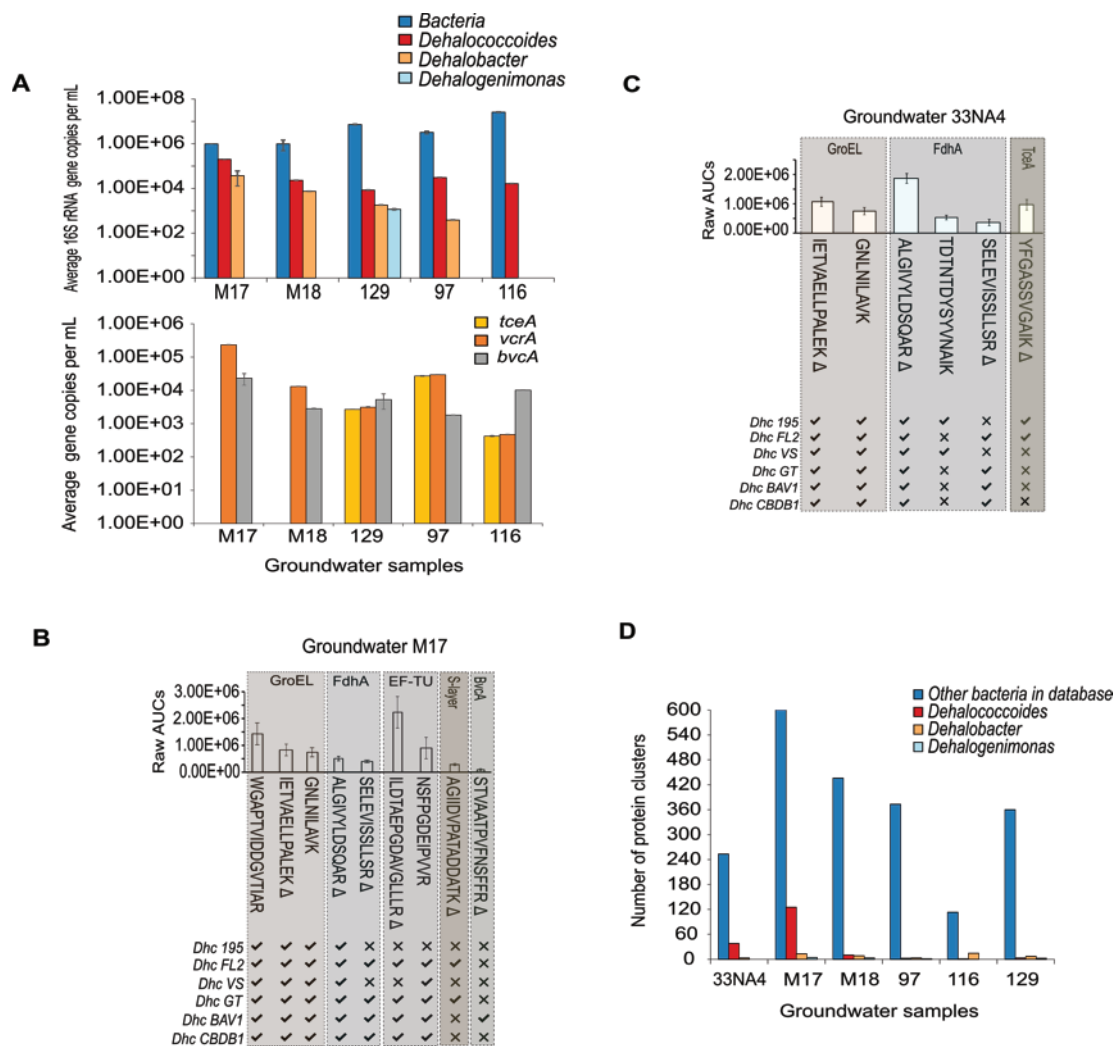


Figure 30. Identification of *Dhc* biomarker genes and proteins in groundwater samples. (A) qPCR measurements of Total Bacteria, *Dhc*, *Dhb* and *Dhgm* 16S rRNA gene copy numbers. Gene copy numbers of *tceA* and *bvcA* are also shown. Values are given on a log scale and each bar represents one DNA extraction quantified in triplicate. DNA-based analyses were not performed for sample 33NA4 due to limited availability of sample material. *tceA* genes were detected but not quantifiable in samples M17 and M18. (B-C) Average raw peak area under the curve (AUC) values of the targeted peptides identified in LC-MRM-MS runs of tryptic digests from groundwater samples M17 and 33NA4, respectively. Error bars are the standard error of the mean ($n=3$ technical replicates). Peptides marked with Δ were identified with supporting evidence from spiked-in unlabeled standards. The inserts below each graph show the specificities of the peptides, determined *in silico*, to the proteomes of six *Dhc* isolates. (D) Total number of proteins by bacterial genus analyzed by qPCR that were identified by global proteomics analyses of groundwater samples. A detailed list of proteins per sample is presented in Supplemental Table S1, Excel Document.

Task 3: Novel microbes

Reductive dechlorination of chlorinated ethenes in grape pomace compost microcosms and transfer cultures.

In anoxic grape pomace microcosms, PCE was reductively dechlorinated to ethene via TCE, cDCE, 1,1-DCE and VC as intermediates after a 300-day incubation period, and a similar dechlorination pattern was observed in transfer cultures (Figure 31). Following the addition of BES, an inhibitor of methanogenesis, cDCE was the dechlorination end product and VC and ethene were not formed. Without BES addition, the solids-free transfer cultures maintained the ability to produce ethene in defined, completely synthetic mineral salt medium.

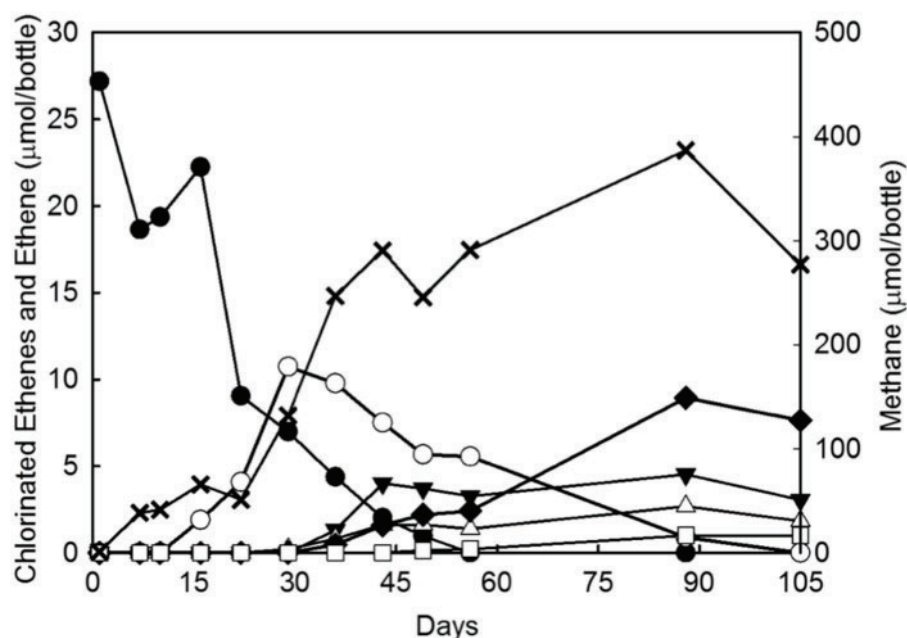


Figure 31. PCE dechlorination in an enrichment culture derived from a Grape Pomace microcosm without the methanogenesis inhibitor BES. The culture demonstrated the potential of PCE degradation to innocuous ethene. Solid circles, PCE; open circles, TCE; solid inverse triangles, cDCE; open triangles, 1,1-DCE; solid diamonds, VC; open squares, ethene; crosses, methane. The data points represent measurements in one dechlorinating culture vessel and a replicate incubation vessel showed similar dechlorination activity.

Comparison between PCE- and VC-fed enrichment cultures

In order to identify the population(s) responsible for the observed dechlorination activity, DNA was extracted from ethene-producing PCE- and VC-fed enrichment cultures for 16S rRNA gene amplicon sequence analysis. No evidence for the presence of *Dhc* 16S rRNA gene sequences was obtained, a finding supported by targeted PCR analysis, which failed to detect the *Dhc* 16S rRNA gene and the *tceA*, *bvcA*, and *vcrA* RDase genes implicated in reductive dechlorination of chlorinated ethenes (data not shown). Instead, *Dhgm* 16S rRNA gene fragments dominated the sequence pool, and represented 43.9% and 46.1% of all bacterial sequences in the PCE-fed and in VC-fed cultures, respectively (Figure 32).

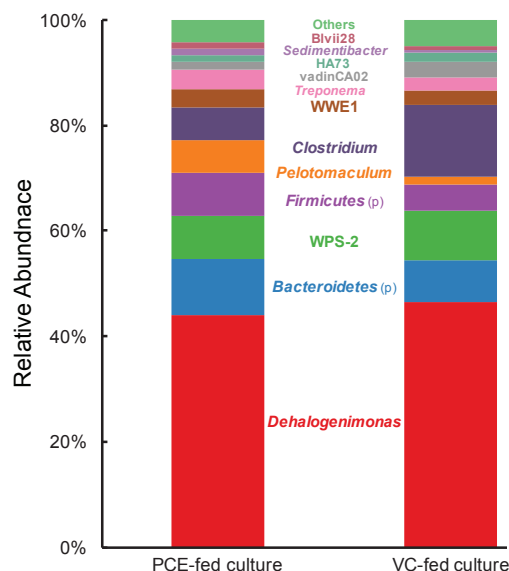


Figure 32. Community structure of PCE-fed and VC-fed grape pomace enrichment cultures as revealed by 16S rRNA gene amplicon sequencing.

The relative abundances of operational taxonomic units (OTUs) representing bacteria are shown at the genus level. Rare groups representing less than 1% of total bacterial community were categorized as “Others”. OTUs representing the phyla Bacteroidetes and Firmicutes could not be classified at the genus level. WPS-2, WWE1, vadinCA02 and HA73 represent uncultured bacterial groups.

Also detected were sequences of not-yet-cultured bacteria of the WWE1 and WPS-2 candidate divisions, which represented 3.6% and 8.3% of the PCE-fed and 2.6% and 9.4% of the VC-fed communities, respectively. Sequences assigned to the genera *Clostridium*, *Pelotomaculum*, *Treponema*, *Sedimentibacter*, as well as the unclassified groups HA73 of *Dethiosulfovibrionaceae* and vadinCA02 of *Synergistaceae*, were all present at >1% abundances in both PCE- and VC-fed communities.

Growth of *Dhgm* coupled with VC-to-ethene reductive dechlorination

In solids-free enrichment cultures, VC dechlorination to ethene commenced after a lag phase of about 20 days and stoichiometric amounts of ethene were produced over a 50-day incubation period (Figure 33).

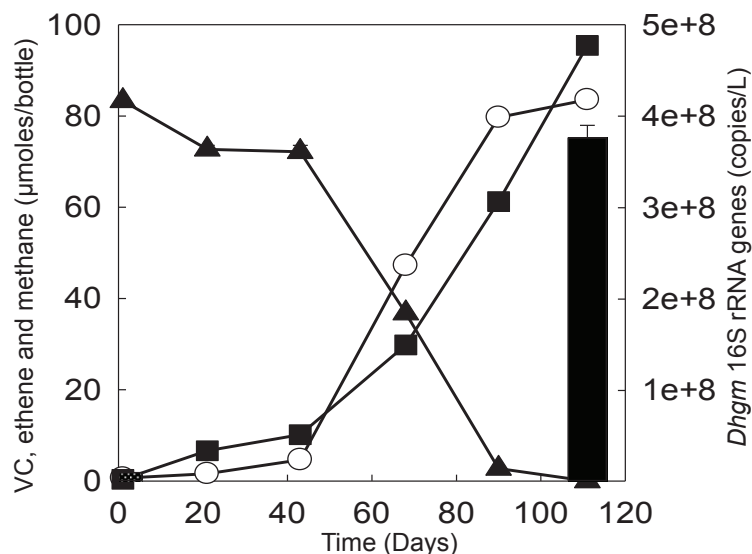


Figure 33. VC-to-ethene reductive dechlorination in the enrichment culture harboring strain GP (triangles, VC; squares, ethene; open circles, methane; solid bar, *Dhgm* 16S rRNA gene copies). Data points show the averages of duplicate cultures and the error bars show one standard deviation. If no error bars are shown, the standard deviations were too small to be illustrated.

Quantitative real-time PCR (qPCR) results demonstrated that the *Dhgm* 16S rRNA gene copies/mL increased from $1.02 \pm 0.12\text{E}+07$ (cells transferred with the inoculum) to $3.76 \pm 0.14\text{E}+08$ (a 37-fold increase) following complete VC degradation. The growth yields of the *Dhgm* population in culture GP were in the range reported for VC-dechlorinating *Dhc* strains and up to 2 orders of magnitude higher than those reported for *Dhgm lykanthroporepellens* strain BL-DC-9 grown with chlorinated propanes (Yang et al., 2017c). Following seven repeated transfers to fresh medium with VC as electron acceptor, culture GP maintained the ability to dechlorinate TCE, 1,1-DCE and cDCE to ethene, and $1.66 \pm 0.44\text{E}+09$, $7.21 \pm 0.45\text{E}+08$, and $1.72 \pm 0.01\text{E}+09$ cells per μmol of chlorinated electron acceptor consumed were produced. The VC-enriched culture GP lost the ability to dechlorinate PCE, and other potential chlorinated electron acceptors including CT, 1,2-DCA, 1,2,3-TCP and 1,2-D were not dechlorinated.

Metagenome analyses

A comparative analysis of taxonomic ranks derived from the metagenomic sequences of culture GP, the *Dhc*-containing consortia ANAS, KB-1, Donna II, and two non-dechlorinating microbial communities revealed that the metagenomes from dechlorinating and non-dechlorinating cultures did not produce distinct clusters (Figure 34A). Instead, the metagenomes were dispersed and the first principal component explained more than three times the variation observed between communities containing dechlorinating and non-dechlorinating members than principal component two (Figure 34A).

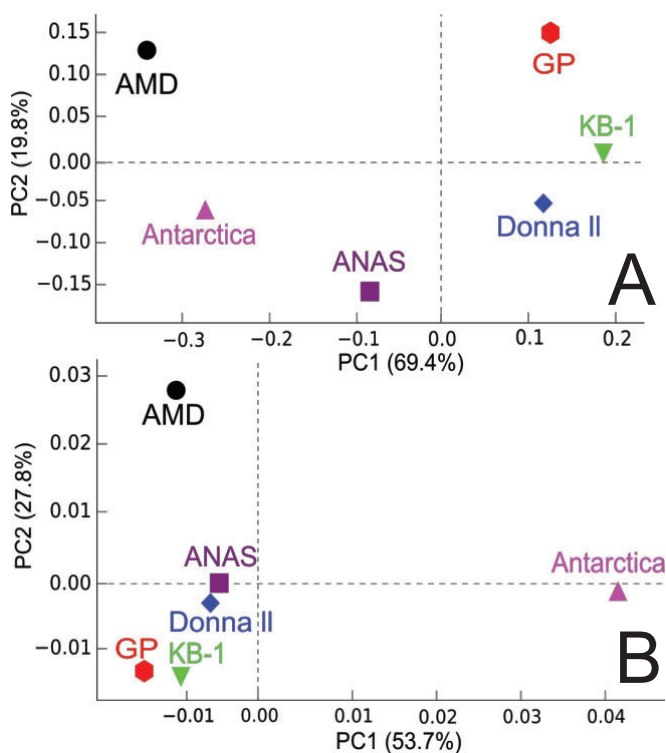


Figure 34. Principle component analysis of taxonomic profiles at the phylum level.

Principal component analysis of taxonomic profiles at the phylum level (panel A) and functional profiles (panel B) of metagenomes from the dechlorinating consortia ANAS, Donna II, KB-1 and GP. Included in the analysis were the metagenome datasets from two non-dechlorinating microbial communities (AMD and Antarctica). For the functional comparison (panel B), the metagenomic sequences were classified into SEED categories and the distribution of SEED categories was compared.

The separation between samples observed along the first principal component axis can be attributed to differences in the relative abundances of taxonomic ranks representing the phyla Chloroflexi, Proteobacteria, Actinobacteria, and Cyanobacteria among metagenomes containing dechlorinating and non-dechlorinating taxa (Table 29). For example, the average relative abundance of sequence reads representing Proteobacteria within non-dechlorinating metagenomes

was $37.4 \pm 9.90\%$, but were lower ($13.6 \pm 4.90\%$) in the metagenomes of the dechlorinating consortia. Sequences derived from members of the Chloroflexi had higher relative abundances in dechlorinating communities, whereas sequences representing the phyla Actinobacteria and Cyanobacteria had higher average relative abundances in metagenomes derived from the non-dechlorinating communities (Table 29).

Table 29. Relative abundances of the dominant phyla in the metagenomes of four dechlorinating consortia and the two non-dechlorinating microbial communities.

Included in the analysis were the dechlorinating consortia KB-1, ANAS, Donna II and GP and the non-dechlorinating microbial mixed cultures AMD and Antarctica (Yang et al., 2017c).

	Dechlorinating consortia	Non-dechlorinating communities
Actinobacteria	$2.1 \pm 0.8\%$	$9.2 \pm 3.0\%$
Proteobacteria	$13.6 \pm 4.9\%$	$37.4 \pm 9.9\%$
Chloroflexi	$36.4 \pm 12.2\%$	$2.6 \pm 1.4\%$
Cyanobacteria	$1.0 \pm 0.2\%$	$3.5 \pm 0.6\%$

In contrast, an ordination of the relative abundances of functional categories detected in the metagenomes indicated that the dechlorinating communities were more similar to each other than to the non-dechlorinating communities (Figure 34B). Specifically, the relative abundance of sequences assigned to branched chain amino acid biosynthesis ($0.17 \pm 0.05\%$ in dechlorinating versus $0.06 \pm 0.02\%$ in non-dechlorinating metagenomes), formate metabolism ($0.11 \pm 0.01\%$ versus 0.00%), iron transport ($0.15 \pm 0.04\%$ versus $0.02 \pm 0.03\%$), and reductive dechlorination ($0.76 \pm 0.44\%$ versus 0.00%) were enriched in the metagenomes of dechlorinating consortia. Further efforts to characterize the VC-dechlorinating culture GP relied on assigning genes from the assembled metagenome to the SEED (www.theseed.org) functional groups. More than 50% of the coding sequences from assembled contigs could not be assigned to a SEED functional category, indicating the presence of many genes with unknown functions in the VC-dechlorinating mixed culture GP. Among assigned SEED functional categories, genes encoding the metabolism of carbohydrates, amino acids and derivatives, proteins, DNA, and cofactors/vitamins/prosthetic groups/pigments were highly represented in the assembled reads.

Draft genome of the VC-dechlorinating *Dhgm* population

Binning of the metagenome sequences allowed the assembly of 16 contigs ranging in size between 1.0 kbp and 666.8 kbp ($N50 = 277.2$ kbp), and a draft genome of the organohalide-respiring *Dhgm* population was obtained. The draft genome had a size of 2.02 Mbp with a G+C content of 52%. CheckM analysis indicated that the genome was 94% complete (144 single copy marker genes detected) with 0% contamination and no strain heterogeneity (default 90% amino acid identity cutoff). Average contig coverage ranged between 7.5 and 505-fold, with an average genome coverage of 276-fold. Prokka annotation of the genomic bin predicted a total of 2,099 genes including three ribosomal RNAs (5S, 16S and 23S rRNA), 2,036 coding DNA sequences (CDS), 14 non-coding RNA sequences and 46 transfer RNAs. Pairwise sequence comparisons demonstrated that the 16S rRNA gene sequence representing the *Dhgm* sp. strain GP shares 99.3%, 96.9%, 96.0% and 95.3% sequence identities with *Dhgm formicexedens* strain NSZ-14 (Key et al., 2017), *Dhgm alkenigignens* strain IP3-3 (Key et al., 2016), *Dhgm* sp. strain WBC-2 (Molenda et

al., 2016), and *Dhgm lykanthroporepellens* strain BL-DC-9 (Moe et al., 2009), respectively. Phylogenetic analysis based on concatenated 5S-16S-23S rRNA gene alignments supported *Dhgm* sp. strain GP's affiliation with the *Dhgm* genus (Figure 35).

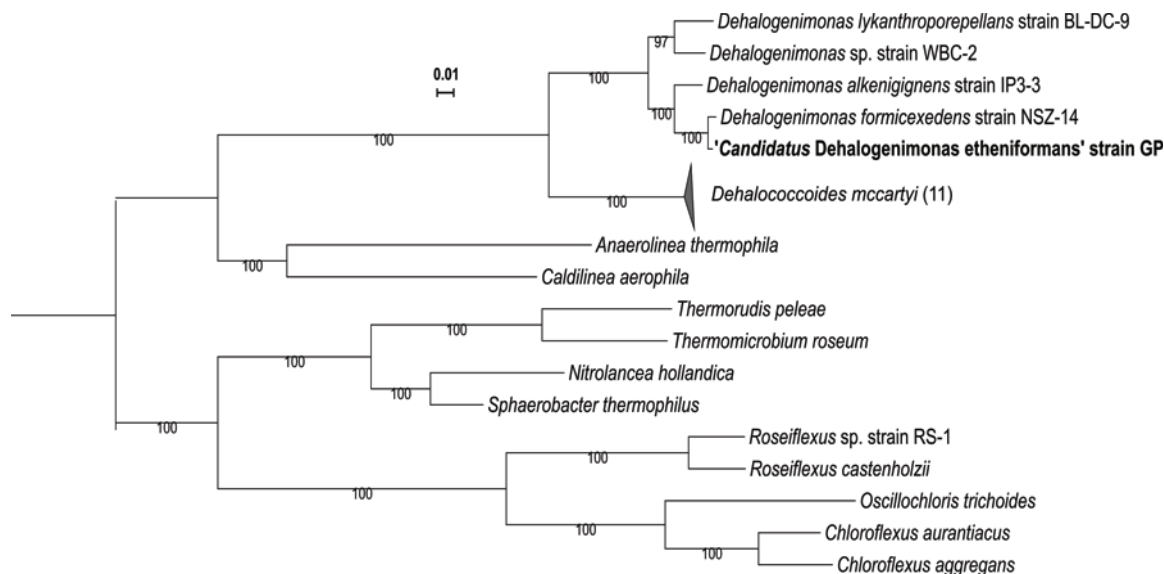
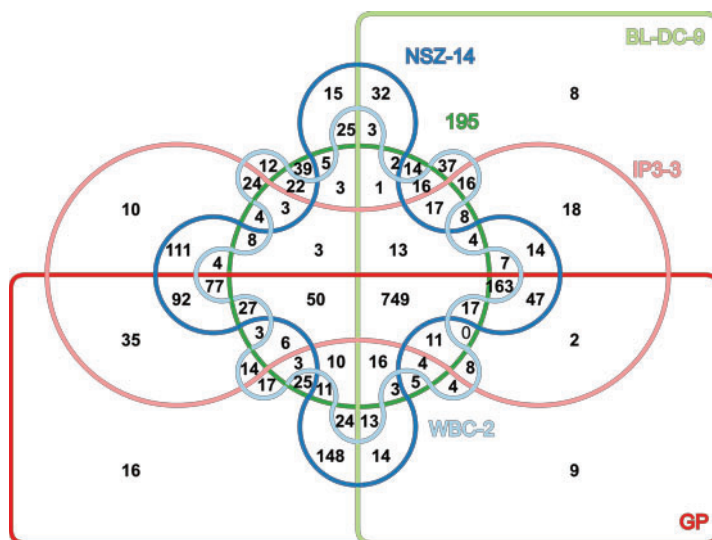


Figure 35. Midpoint rooted maximum likelihood phylogenetic tree showing the relationship of 'Candidatus Dehalogenimonas etheniformans' to other members of the Chloroflexi based on concatenated 5S-16S-23S rRNA genes.

The closest relatives of 'Candidatus Dehalogenimonas etheniformans' were *Dhgm formicexedens* strain NST-14T and *Dhgm alkenigignens* strain IP3-3. The scale bar indicates 0.01 nucleotide substitution per site.

A total of 2,021 orthologous gene clusters were shared between the genomes of *Dhgm* strains NSZ-14, IP3-3, WBC-2, BL-DC-9 and GP and the genome of *Dhc* strain 195, and 1,921 gene orthologous clusters were shared by at least two genomes. *Dhgm* sp. strain GP shared 1,454, 1,294, 1,159, 1,047, and 915 orthologous clusters with *Dhgm* strains NSZ-14, IP3-3, WBC-2, BL-DC-9 and *Dhc* strain 195, respectively (Figure 36).

Figure 36. Edwards' Venn diagram of orthologous gene clusters shared between *Dhc* and *Dhgm*. From the genomes of *Dhc* strain 195 and *Dhgm* strains NSZ-14, IP3-3, WBC-2, BL-DC-9 and GP, a total of 1,580, 2,064, 1,839, 1,655, 1,659 and 2,036 annotated coding sequences, respectively, were included in the analysis. These sequences were compared and clustered using a Markov cluster algorithm with an expect (E) value of $1e-5$ and an inflation value of 1.5.



Although *Dhgm* sp. strain GP shares

99.3% 16S rRNA gene sequence similarity with strain NSZ-14, the genome-aggregate average nucleotide identity (ANI) between both organisms is only 80.59%, well below the 95% ANI that corresponds to the 70% DNA–DNA hybridization standard that is frequently used for species demarcation (Löffler et al., 2013b), suggesting strain GP represents a distinct species.

Genes for *de novo* corrin ring biosynthesis were absent in the genome, but genes implicated in corrinoid salvage and modification (i.e., *cobA*, *cbiP*, *cbiB*, *cobU*, *cobT*, *cobC*, *cobS* and *cbiZ*) were detected. Similar to observations made with *Dhc*, the *Dhgm* genome possesses genes encoding two distinct cobinamide (Cbi-)-salvaging pathways: the bacterial pathway relying on *cobU/cobP* genes and the archaeal *cbiZ* pathway. Genes coding for the corrinoid ABC transporter *BtuFCD* and the dual-function cobalt/nickel transporter system *cbiMNQO* were also identified.

The function of a putative heterodisulfide reductase gene cluster (*hdrABC*) in the genome of *Dhgm* strain GP is unclear but may be involved in electron bifurcation systems (e.g., HdrABC-MvhADG of *Methanothermobacter marburgensis*, HdrABC-FlxABCD of *Desulfovibrio vulgaris* Hildenborough). In the electron bifurcation system of *Desulfovibrio vulgaris* Hildenborough, the HdrABC complex is responsible for transferring electrons (released from hydrogen or NADH + H⁺ oxidation catalyzed by the FlxABCD complex) to oxidized ferredoxin and a second electron acceptor small protein (e.g., DsrC) (Ramos et al., 2015). While orthologs of genes encoding HdrABC were observed across all available *Dhgm* genomes, none were detected in any sequenced *Dhc* genome, suggesting *Dhgm* and *Dhc* may employ different electron transfer systems. It is also worth mentioning that the *Dhgm* draft genome harbors two genes in a single operon, which encode proteins sharing greater than 39% and 54% amino acid identity to *arsD* and *arc3* of *Escherichia coli*, suggesting *Dhgm* strain GP experienced arsenic selection pressure and is capable of detoxifying arsenicals.

Detection and abundance of *Dhgm* at sites impacted with chlorinated solvents

For contaminated site assessment, monitoring, and remediation treatment decision making, the quantitative measurement of *Dhc* biomarker genes has become routine practice. In 1,173 groundwater samples collected from 111 chlorinated solvent-impacted sites, 16S rRNA genes of both *Dhc* and *Dhgm*, *Dhc* only, and *Dhgm* only were detected in 849, 79, and 97 samples, respectively. In 148 samples (<13%), neither *Dhgm* nor *Dhc* 16S rRNA genes were detected. In 812 samples with quantifiable *Dhgm* and *Dhc* cell numbers, *Dhgm* outnumbered *Dhc* in the majority (65%) of samples and the *Dhgm/Dhc* ratio was above 1 in 524 samples (Figure 37). Considering that a number of these samples were influenced by bioaugmentation with *Dhc*- (but not *Dhgm*-) containing consortia, *Dhgm* likely outnumber *Dhc* in even more of the sampling locations prior to bioremediation treatment. The known *Dhgm* genomes indicate a strict organohalide-respiring energy metabolism, and it is very likely that the presence of *Dhgm* implies that these bacteria contribute to *in situ* reductive dechlorination. Thus, the contribution of this organismal group to attenuation of chlorinated solvent contaminants is probably far greater than is currently acknowledged.

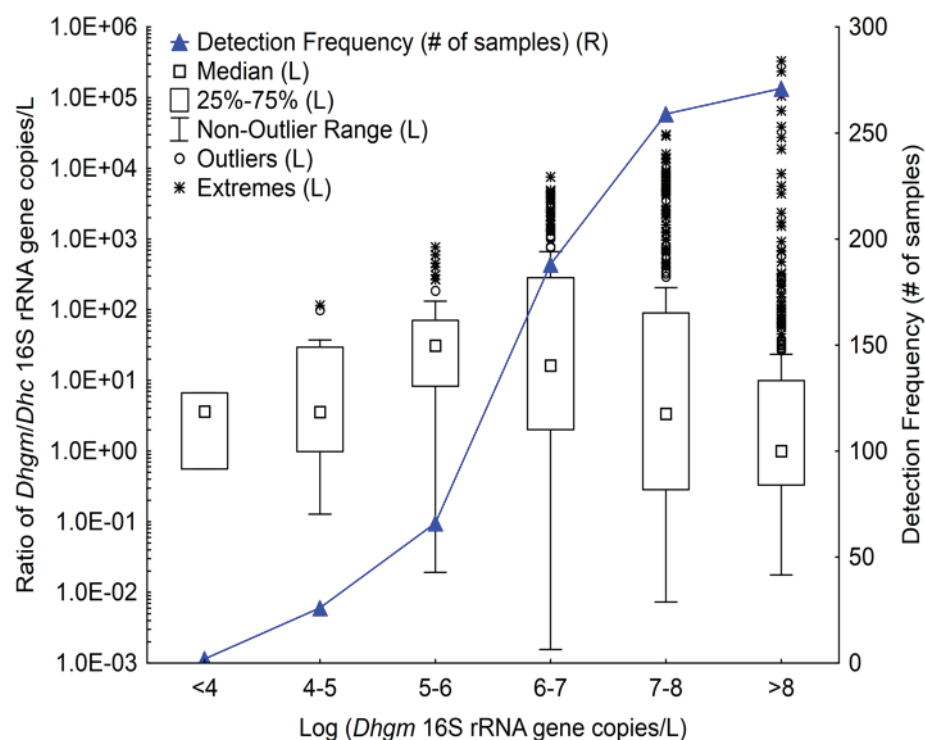


Figure 37. Distribution of *Dhgm* and *Dhc* in 1,173 groundwater samples collected from 111 chlorinated solvent-impacted sites.

Boxes represent the upper (75th) and lower (25th) quartiles and whiskers depict the non-outlier range. Outliers have *Dhgm/Dhc* ratios greater than the upper quartile *Dhgm/Dhc* ratio plus 1.5 times the difference between upper and lower quartile *Dhgm/Dhc* ratios, or less than the lower quartile *Dhgm/Dhc* ratio minus 1.5 times the difference between upper and lower quartile *Dhgm/Dhc* ratios. *Dhgm/Dhc* ratios greater than the upper quartile plus 2 times the difference of the quartiles or less than the lower quartile minus 2 times the difference between the quartiles were defined as extremes. Box plots were created using Statistica v12.0 (StatSoft, Inc., Tulsa, OK).

Among the organohalide-respiring Chloroflexi, *Dhc* have received most attention because of their ability to detoxify priority pollutants (He et al., 2005; Wang et al., 2014), their demonstrated relevance for *in situ* bioremediation (Löffler et al., 2006; Stroo et al., 2012), and the availability of representative isolates (Adrian et al., 2000; He et al., 2003b; Maymó-Gatell et al., 1997) and bioaugmentation consortia (Adrian et al., 2016; Löffler et al., 2013a). To date, metabolic VC-to-ethene reductive dechlorination has been exclusively linked to the presence and activity of *Dhc* strains, and the value of monitoring *Dhc* 16S rRNA genes and the *Dhc* RDase genes *tceA*, *vcrA* and *bvcA* for supporting contaminated site management decisions has been demonstrated (Stroo et al., 2012). At sites, where VC disappearance was observed but *Dhc* were not detected, VC degradation was attributed to other processes, including abiotic reactions mediated by mineral phases such as magnetite (He et al., 2015) or aerobic microbial VC oxidation (Coleman et al., 2002; Mattes et al., 2010). The discovery of non-*Dhc* populations carrying novel VC RDase genes indicates that a broader diversity of microorganisms contributes to anaerobic VC metabolism and detoxification. This relevant observation demonstrates that the absence of known *Dhc* biomarker genes should not be used as an argument that the microbial reductive dechlorination process is not driving contaminant removal.

Task 4: Novel biomarkers

Identification of dcpA as a biomarker for 1,2-D-to-propene reductive dechlorination

cDNA libraries identify the 1,2-D RDase gene

Using template cDNA derived from the RNA pool of the 1,2-D-grown cultures RC and KS, PCR with the degenerate RDase-targeted primers B1R and RR2F yielded amplicons of approximately 1,500 bp in length (Figure 38). No amplification occurred when RNA prior to the reverse transcription (RT) step was used as the PCR template, confirming that all genomic DNA had been removed from the RNA pool (Figure 38).

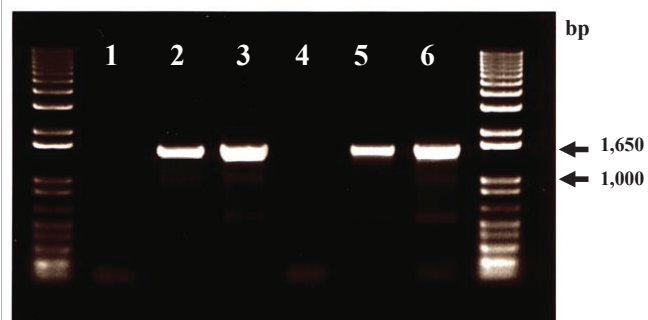


Figure 38. PCR amplification of the 1,2-D RDase gene for cDNA library construction. cDNA from 1,2-D-grown and propene-producing RC and KS cultures was used as template with the degenerate primers RRF2 and B1R. Lanes 1-3 correspond to samples from culture RC and lanes 4-6 to samples from culture KS. Lanes 1 and 4 are no reverse transcriptase reaction controls to demonstrate the absence of genomic DNA in

the RNA samples; lanes 2 and 5 show amplification using cDNA as template, and lanes 3 and 6 correspond to positive controls performed with genomic DNA from the respective cultures. The 1Kb Plus Ladder from Invitrogen is shown on both sides of the gel.

Among 200 *E. coli* clones screened from the B1R and RR2F amplicon-derived clone libraries of cultures RC and KS, 12 and 10, respectively, had vectors carrying cDNA fragments of the expected size of ~1,500 bp. Sequence analysis of the 10 KS cDNA library clones with an insert of the correct size revealed a single, 1,486-bp sequence. The sequence included the nearly complete RDase A gene and a partial RDase B gene, indicating that these genes were cotranscribed. An open reading frame corresponding to the RDase B gene start was found 18 nucleotides (nt) downstream of the RDase A gene TAA stop codon and included 35 nt of the adjacent RDase B gene. Analysis of the 12 RC cDNA library clones revealed eight inserts with the same 1,486-bp insert found in the KS cDNA library clones, and four clones had a 1,589-bp insert. The 1,589-bp insert consisted of 1,472 bp of the partial RDase A gene, 19 nt of intergenic region, and 98 bp of a partial RDase B gene. The 1,486-bp insert cloned from both *Dhc* strain RC and strain KS genes showed 90% sequence identity to a putative RDase gene (gene tag Dehly_1524) found in the genome of the 1,2-D-dechlorinating *Dhgm lykanthroporepellens* strain BL-DC-9. To rule out the presence of a *Dhgm lykanthroporepellens*-type population in the RC and KS cultures, PCR with primers targeting the *Dhgm lykanthroporepellens* 16S rRNA gene was performed; however, DNAs from cultures RC and KS failed to produce an amplicon, confirming that *Dhgm lykanthroporepellens* was not present in these cultures. The second, 1,589-bp insert found in four RC clones was 99.9% similar to the putative RDase gene RCRdA02 (accession no. EU266045). This gene was almost identical (98 to 99%) to the *Dhc* RDase genes DehalGT_1352 (accession no. CP001924), cbdbA1638 (accession no. AJ965256), and KSRdA02 (accession no. EU266035) and also demonstrated high similarity (96% nt identity) to FL2RdhA6 in *Dhc* strain FL2 (accession no. AY374250). Additionally, RCRdA02 shared 98 to 99% nt sequence identity with RDase genes retrieved from the KB-1 and TUT2264 dechlorinating mixed cultures (KB1RdhAB5 [accession no. DQ177510] and

TUT2264_rdhA2 [accession no. AB362921]) and from a contaminated site (FtL-RDase-1638 [accession no. EU137843]). At the nucleotide level, no gene in the *Dhgm lykanthroporepellens* strain BL-DC-9 genome shared similarity with RCRdA02.

Protein assays and LC-MS/MS analysis

After BN-PAGE separation of *Dhgm lykanthroporepellens* strain BL-DC-9 proteins, a gel slice representing the section below the 75-kDa marker demonstrated 1,2-D-dechlorinating activity (Figure 39A, slice 4). Coomassie staining revealed that this gel slice comprised multiple polypeptides (i.e., several visible bands) near the 75- to 55-kDa size marker, with a major band of 45.5 kDa (Figure 39A). Subsequent SDS-PAGE separation of the proteins eluted from this gel piece exhibited protein bands with masses of 75, 63, and 50 kDa (Figure 39B, slices N1, N2, and N3).

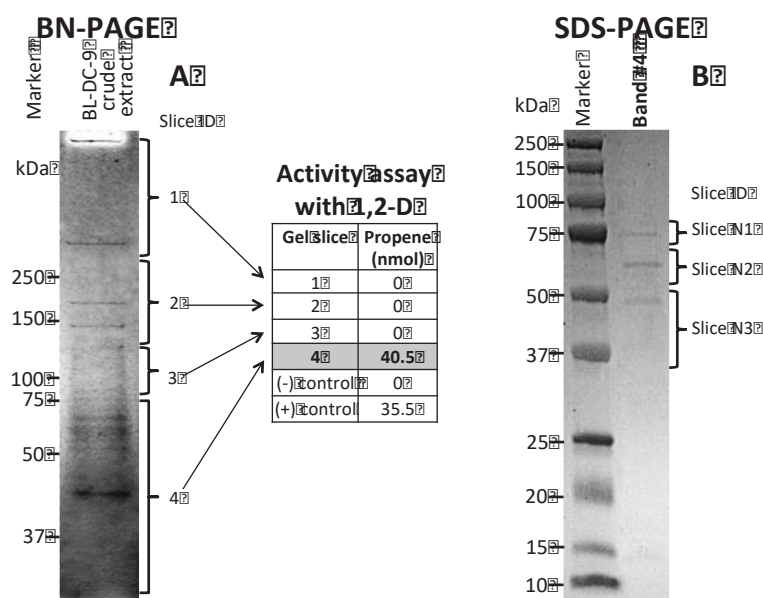


Figure 39. Activity assays following BN-PAGE separation of crude extracts of *Dhgm lykanthroporepellens* strain BL-DC-9 cells grown with 1,2-D.

(A) Coomassie-stained BN-PAGE showing the predominant proteins and the gel sections that were subjected to dechlorination activity testing with 1,2-D. For enzyme activity assays, gel slices from unstained lanes adjacent to the Coomassie-stained lanes were used. (B) Propene formation was observed only in gel slice 4, which was subjected to SDS-PAGE. Three bands were visualized by SDS-PAGE, and gel slices N1, N2, and N3 were further analyzed by LC-MS/MS. DcpA (Dehly_1524) was the only RDase detected in gel slice 4.

LC-MS/MS analysis of the proteins separated by SDS-PAGE identified 15 *Dhgm lykanthroporepellens* strain BL-DC-9 proteins (Table 30, data for slices N2 and N3). In the N1 gel section, protein levels were too low for confident identification, while in the gel section around 50 kDa (i.e., slice N3), Dehly_0337 (annotated as translation elongation factor Tu) and Dehly_1524 (annotated as an RDase) were the dominant proteins, based on peptide spectral abundances (Table 30). Other peptides associated with gel slice N3 included subunits of the nickel-dependent hydrogenases, encoded by Dehly_0929 and Dehly_0726 (Table 30). Among all the protein fragments recovered from slices N2 and N3, the highest coverage and spectral counts belonged to Dehly_1407, a chaperonin GroEL protein (Table 30, data for slice N2). Dehly_1524 was the only RDase associated with the gel slices (Table 30), corroborating that this enzyme catalyzes 1,2-D-to-propene dichloroelimination. The 1,2- RDase was designated DcpA, encoded by the *dcpA* gene.

Table 30. *Dhgm* strain BL-DC-9 proteins identified in gel slice 4 exhibiting 1,2-D-to-propene dechlorination activity following BN-PAGE. Gel slice 4 was further separated in SDS-PAGE into gel slices N1, N2, and N3. Proteins are listed in order of decreasing spectral counts; DcpA is indicated in bold letters. In the N1 gel slice, proteins levels were too low for confident identification and no data for N1 were included in the table.

Gel Slice	Gene ID	Protein Accession number	Protein Length	Sequence Coverage (%)	Distinct Peptides	Spectral Counts	Adjusted NSAF values	Protein Description
N2	Dehly_1407	YP_003759016	534	33.0	17	230	74691.4	Chaperonin GroEL
	Dehly_0935	YP_003758558	543	9.0	5	12	3832.4	DAK2 domain fusion protein YloV
	Dehly_0744	YP_003758371	526	7.0	3	12	3956.2	D-3-Phosphoglycerate dehydrogenase
	Dehly_1273	YP_003758885	610	6.7	3	13	3695.7	Hypothetical protein Phosphoribosylaminoimidazolecarboxamide Formyltransferase/IMP cyclohydrolase
	Dehly_1425	YP_003759034	511	7.0	3	10	3393.6	Arginyl-tRNA synthetase
	Dehly_1020	YP_003758642	557	3.9	2	10	3113.4	Formate--tetrahydrofolate ligase
	Dehly_0812	YP_003758435	588	4.9	2	8	2359.4	Glutamine synthetase
	Dehly_1485	YP_003759090	500	4.4	2	5	1734.1	Dihydroxy-acid dehydratase
	Dehly_0665	YP_003758293	555	4.3	2	4	1249.8	Translation elongation factor Tu
	Dehly_0337	YP_003757978	400	5.5	2	3	1300.6	Carboxyl transferase
	Dehly_0353	YP_003757993	515	6.6	2	2	673.5	
N3	Dehly_0337	YP_003757978	400	9.8	4	23	53241.2	Translation elongation factor Tu
	Dehly_1524	YP_003759128	482	6.6	3	14	26894.3	Reductive dehalogenase
	Dehly_0929	YP_003758552	423	6.9	2	3	6566.9	Nickel-dependent hydrogenase large subunit
	Dehly_1407	YP_003759016	534	6.4	3	3	5201.9	Chaperonin GroEL
	Dehly_0692	YP_003758320	437	3.9	2	2	4237.7	Diaminopimelate decarboxylase
	Dehly_0726	YP_003758353	480	3.5	2	2	3858.1	Nickel-dependent hydrogenase large subunit

* NSAF stands for Normalized Spectral Abundance Factor and is used for quantitative proteomic analysis by taking the MS/MS spectral counts of a matching peptide from a protein and dividing it by its length (number of amino acids) resulting in a Spectral Abundance Factor (SAF). The SAF is normalized against the sum of all SAFs in the sample, resulting in the NSAF value. Adjusted NSAF values allow for direct comparison of a protein's abundance between individual runs and samples.

Primer walking and characterization of *dcpAB* gene cassette

Since the degenerate primer pair B1R and RRF2 did not amplify the complete *dcpA* and *dcpB* gene sequences, the entire *dcpAB* genes from strains *D. mccartyi* RC and KS were obtained using primer walking approaches. The application of primers *dcp_up120F* and *dcpA-1449R* yielded ~1,569-bp PCR products and extended the sequence 89 bp upstream of the ATG start codon. The final and complete assembly of the *dcpA* gene sequences derived from *Dhc* strains RC and KS were nearly identical (99.8% nt sequence identity), and the translated protein sequence differed by only a single aa, at position 85 (i.e., a serine [S] in strain RC was replaced by a leucine [L] in strain KS). Inspection of the region upstream of the start codon identified the putative ribosome binding site (RBS or Shine-Dalgarno sequence) AGAGG, starting 9 nt upstream of the *dcpA* start codon, and a putative dehalobox (Gábor et al., 2006) was identified 67 nt upstream of *dcpA*. Primer walking procedures also extended *dcpB* through the TAA stop codon (an additional 184 bp). The final assembly of the *dcpB* genes of *Dhc* strains RC and KS revealed that both sequences shared 99% sequence identity and their corresponding proteins differed in only one aa, with glutamine (Q) replaced by a glutamic acid (E) in strain RC at position 11 (Figure 40). Therefore, the WYXW motif, which is conserved in other RDase B proteins, is present in the form WYEW in *Dhc* strain RC and in the form WYQW in *Dhc* strain KS and *Dhgm lykanthroporepellens* strain BL-DC-9 (Figure 40). The final assembly of the *dcpAB* gene cassette of *Dhc* strains RC and KS consisted of a 1,455-nt (484 aa) *dcpA* gene and a 219-nt (73 aa) *dcpB* gene separated by an 18-nt spacer.

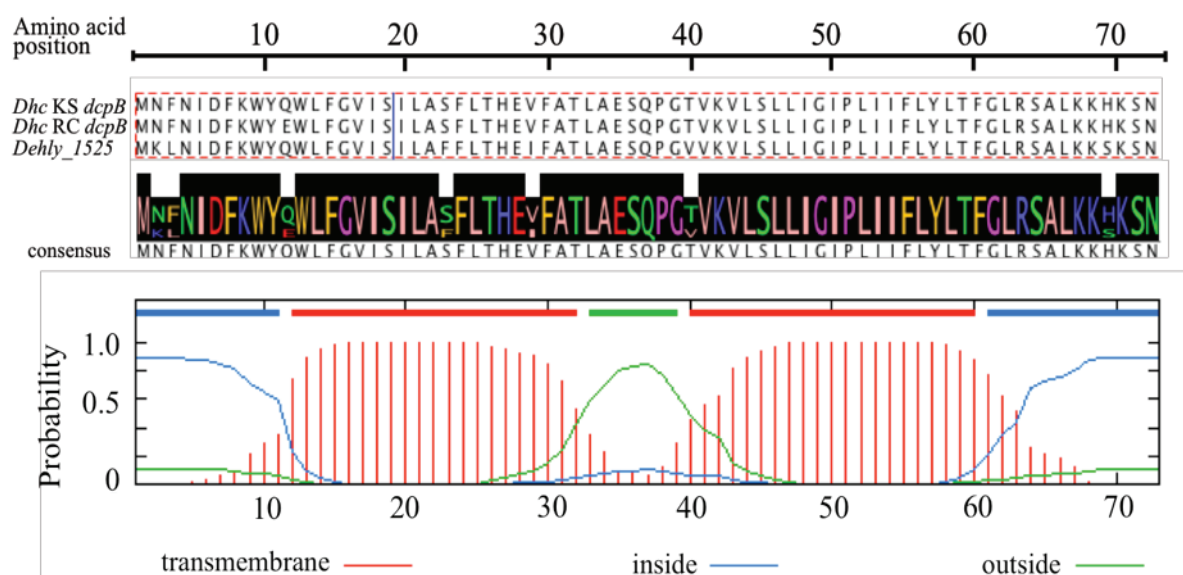


Figure 40. Characteristic features of DcpB.

The upper portion of Figure 40 shows the translated 73 amino acid-long sequence encoded by the *dcpB* genes of *Dhc* strains RC and KS and *Dhgm* strain BL-DC-9 (Dehly_1525) aligned with ClustalW and visualized in Jalview. The lower portion of Figure 40 represents the topology of this membrane-anchoring protein predicted by TMMOD, a Hidden Markov model program used to predict where proteins span the cell membrane (www.liao.cis.udel.edu/website/servers/TMMOD/). The deduced topology of the DcpB protein revealed two transmembrane regions between positions 12-32 and 41-61. Additional characteristics include two inside loops (i.e., facing the cytoplasm) at amino acid positions 1-11 and 62-73, and one outside loop (i.e., facing the periplasm) from positions 33-40.

Computational characterization of DcpA and DcpB

The Tat signal peptide RRDFMK, starting at position 9 and with a predicted peptide cleavage site between aa positions 30 and 31, was identified (Padilla-Crespo et al., 2014). The mature DcpA protein (i.e., after cleavage of the signal peptide) in *Dhc* strain RC and strain KS had a predicted isoelectric point (pI) of 5.99 and a molecular mass of 50.8 kDa. The corrinoid binding motif DXHXXG-X₄₁₋₄₂-SXL-X₂₄₋₂₈-GG, found in several corrinoid-containing enzymes from prokaryotes (38), was not present in DcpA, but a putative corrinoid binding sequence (DHXG-X₃₉-S-X₃₂-G) close to the C terminus was identified (Padilla-Crespo et al., 2014). The DcpA proteins of both *Dhc* strain RC and strain KS share two identical iron-sulfur cluster binding motifs (FCX₂CX₂CX₃CP and CX₂CX₃C) (Figure 6).

The topology of the DcpB protein revealed two predicted trans- membrane regions, spanning from positions 12 to 32 and 41 to 61 (Figure 40). Additional characteristics included two inside loops (i.e., facing the cytoplasm), from aa positions 1 to 11 and 62 to 73, and one outside loop (i.e., facing the periplasm), from positions 33 to 40 (Figure 40). Furthermore, a putative RBS (AGAGG) for initiation of translation was detected in the small 18-nt intergenic region separating *dcpA* and *dcpB*.

dcpA sequence similarity to other RDase genes

The *dcpA* genes of *Dhc* strains RC and KS and *Dhgm lykanthroporepellens* strain BL-DC-9 (Dehly_1524) shared 60% overall nt identity to the pseudogene DET0162, identified in the genome of *Dhc* strain 195, and an even greater sequence identity (66%; 260 of 395 nt) occurred near the 3' end. The DET0162 pseudogene is 1,464 nt long and has a point mutation that results in a premature stop codon leading to a truncated, 59-aa-long polypeptide. If completely translated, the gene would encode a putative RDase of approximately 486 aa with all the common RDase features, including the Tat RRDFMK motif near the N terminus and the FCX₂CX₂CX₃CP and CX₂CX₃C iron-sulfur cluster binding motifs near the C terminus. The 486-aa protein would have 45% aa identity to the DcpA proteins of strain RC and strain KS and 46% identity to DcpA of strain BL-DC-9. Located 129 bp downstream of the 3' end of the DET0162 pseudogene is a characteristic B gene (DET0163). This B gene also shared 55 to 56% nt sequence identity (44 to 47% aa sequence identity) with the *dcpB* genes of *Dhgm lykanthroporepellens* strain BL-DC-9 (Dehly_1525) and *Dhc* strains RC and KS.

The *Dhc* DcpA proteins shared 92% sequence identity and 95% sequence similarity with the *Dhgm lykanthroporepellens* strain BL-DC-9 DcpA protein (Dehly_1524). DcpA shared no more than 34% aa sequence identity with other *Chloroflexi* RDases and other RDase sequences deposited in databases. Inspection of the recently closed genome of the 1,2-D-to-propene-dechlorinating *Desulfitobacterium dichloroeliminans* strain DCA1 (LMG P-21439) (www.ncbi.nlm.nih.gov/genome/?term=txid871963) revealed no RDase with >18% aa sequence identity to DcpA. Recently, a nomenclature for RDases was proposed (Hug et al., 2013b), and DcpA clusters nearest to the RD_OG20 group, which is composed of BAV1_0104, cbdbA88, and DET0311. This ortholog group shares only 31 to 34% aa identity and no more than 45% similarity with DcpA, suggesting that the *Dhgm lykanthroporepellens* and *Dhc* DcpA sequences form a separate cluster.

dcpA-targeted PCR and qPCR

PCR with primers *dcpA*-360F and *dcpA*-1449R produced a single amplicon of the expected size of 1,089 bp when applied to genomic DNA from cultures RC and KS and *Dhgm* strain BL-DC-9. No amplicons were obtained with template DNA from *Dhc* strain GT, which cannot dechlorinate

1,2-D. qPCR standard curves generated with primers *dcpA*-1257F and *dcpA*-1449R using SYBR Green reporter chemistry exhibited linear amplification ranging from 1.7 to 1.7×10^8 16S rRNA gene copies per μL of template DNA (e.g., slope = -3.4 , y intercept = 36.6 and $R^2 = 0.999$). Melting curve analyses of amplicons generated with genomic DNA from *Dhc* culture RC, culture KS and *Dhgm* strain BLDC-9 showed a single, symmetric peak (suggesting a single PCR product) with average melting temperatures (T_m) of 78.5 ± 0.1 , 79.2 ± 0.1 , and 78.6 ± 0.1 , respectively (Padilla-Crespo et al., 2014). TaqMan qPCR assays with the same primer pair combined with probe *dcpA*-1426 also exhibited linear amplification over the same range of template DNA concentrations (e.g., slope = -3.5 , y intercept = 39.5 and $R^2 = 0.997$).

TaqMan qPCR demonstrated that *Dhc* cell numbers increased during cultivation with 1,2-D as electron acceptor. Cultures RC and KS produced $1.8 \pm 0.1 \times 10^7$ and $1.4 \pm 0.5 \times 10^7$ *Dhc* cells per μmol of Cl^- released. The enumeration of *dcpA* and *Dhc* 16S rRNA genes in replicate cultures of *Dhc* strains RC and KS demonstrated that both gene targets occurred in similar abundances. In KS cultures, $6.6 \times 10^7 \pm 0.4 \times 10^7$ *Dhc* 16S rRNA gene copies and $6.2 \times 10^7 \pm 0.4 \times 10^7$ *dcpA* genes were detected/mL of culture suspension. Culture RC produced $6.1 \times 10^7 \pm 0.2 \times 10^7$ 16S rRNA genes and $5.2 \times 10^7 \pm 0.3 \times 10^7$ *dcpA* genes/mL of culture. These findings indicate that both *Dhc* genomes harbored a single *dcpA* gene. The *dcpA*-targeted primers *dcpA*-1257F and *dcpA*-1449R were also used to quantify the *dcpA* transcript abundances in *Dhc* cultures RC and KS. The RT-qPCR results showed the upregulation of *dcpA* gene transcription in actively dechlorinating RC and KS cultures as compared to cultures that had consumed all 1,2-D (Figure 41).

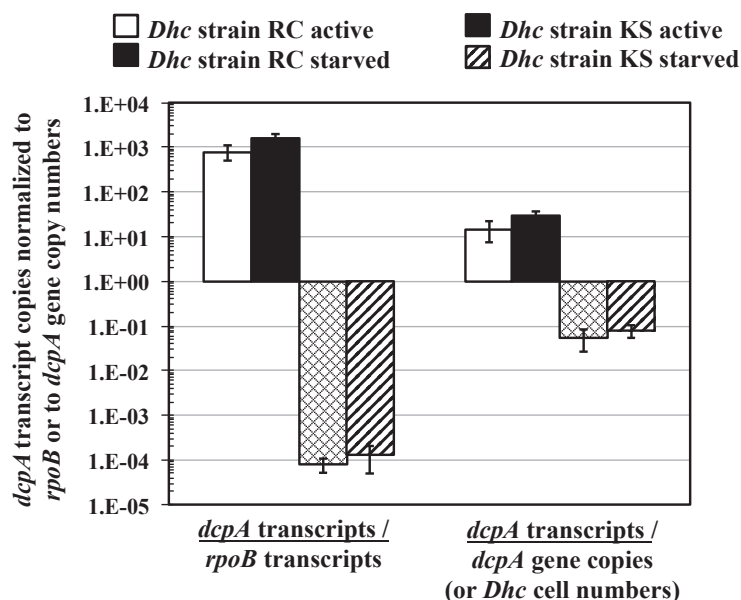


Figure 41. Relative transcript copy abundances in cells growing with 1,2-D. *dcpA* transcript levels were normalized to *rpoB* or to *dcpA* gene copy numbers.

Triplicate samples were analyzed, and the reported values represent the averages for at least three independent biological cultures. Error bars depict standard errors. Negative numbers represent downregulated target genes, while positive numbers represented upregulated genes. A ratio near unity (close to 1) indicates an insignificant change in the number of *dcpA* transcripts per *rpoB* transcript or the ratio of *dcpA* to *Dhc* gene copy numbers.

Application of *dcpA* PCR and qPCR assays to microcosm and environmental samples

Propene was detected in 1,2-D-amended microcosms established with five out of the 13 sample materials tested (Table 31). In contaminated sediments from Third Creek, TN, the *dcpA* gene increased from below the quantification limit to $5.6 \pm 0.1\text{E}+06$ copies/mL in propene producing sediment-free enrichment cultures. Nested PCR assays detected both *Dhgm* and *Dhc* 16S rRNA genes in the initial Third Creek sediment samples. qPCR assays indicated that *Dhc* and *Dhgm* populations also increased from below the quantification limit in the initial samples to $5.6 \pm 0.6\text{E}+06$ and $9.7 \pm 0.3\text{E}+03$ gene copies/mL of sediment-free enrichment culture, respectively. Nested PCR detected the *dcpA* gene in all aquifer and sediment samples that yielded 1,2-D-dechlorinating microcosms. The only microcosms with positive *dcpA* detection but without propene formation were established with aquifer materials from the site at Barra Mansa, Rio de Janeiro, Brazil. The *dcpA* gene was not detected in site materials collected from the Waynesboro site and in Ft. Pierce groundwater samples collected outside of a 1,2-D plume. Consistent with the absence of the *dcpA* gene, the microcosms established with these materials failed to dechlorinate 1,2-D (Table 31). Interestingly, three of four wells collected inside the 1,2-D plume at the Ft. Pierce site tested positive for *dcpA*, consistent with the detection of propene at these well locations.

Table 31. Site materials used for microcosm setup to evaluate 1,2-D reductive dechlorination activity and analyzed for the presence of *Dhc* and *Dhgm* 16S rRNA gene and the *dcpA* gene ^e

Sample designation	Sample location	Sample type	Major reported contaminants	Date of collection	Dechlorination end products	Presence of indicated gene		
						<i>Dhc</i> 16S	<i>Dhgm</i> 16S	<i>dcpA</i>
Microcosms								
Third Creek, TRS1	Third Creek, Knoxville, TN	Sediment	PCE, TCE, 1,1,1-TCA	Feb. 2011	Propene	+	+	+
Third Creek, TRS2	Third Creek, Knoxville, TN	Sediment	PCE, TCE, 1,1,1-TCA	Feb. 2011	Propene	+	+	+
Third Creek, TRS3	Third Creek, Knoxville, TN	Sediment	PCE, TCE, 1,1,1-TCA	March, 2011	Propene	+	+	+
Neckar River	Stuttgart, Germany	Sediment	None	May, 2011	Propene	+	+	+
Trester	Stuttgart, Germany	Solids ^a	None	May, 2011	Propene	+	+	+
001-ST-SO, 2.7-2.9 m	Barra Mansa, Brazil	Sediment	CF, CT	Aug. 2010	- ^b	+	ND	+
002-ST-SO, 5.7-5.8 m	Barra Mansa, Brazil	Sediment	CF, CT	Aug. 2010	- ^b	+	ND	+
Way-MW13D-12J811	Waynesboro, GA	Groundwater	1,2-D and 1,2-DCA	Aug. 2010	-	ND	ND	ND
FP1-MW46, 22-26 m	Ft. Pierce, FL	Sediment	None ^c	Aug. 2010	-	ND	ND	ND
FP2-MW49, 26-27 m	Ft. Pierce, FL	Sediment	None ^c	Aug. 2010	-	+	+	ND
FP3-MW49, 46-47 m	Ft. Pierce, FL	Sediment	None ^c	Aug. 2010	-	+	+	ND
FP4-MW47, 47-48 m	Ft. Pierce, FL	Sediment	None ^c	Aug. 2010	-	+	ND	ND
FP5-MW49, 95-98 m	Ft. Pierce, FL	Sediment	None ^c	Aug. 2010	-	ND	ND	ND
FP-MW33, 13-14 m	Ft. Pierce, FL	Groundwater	18,000 µg/L of 1,2-D	June, 2012	Propene	+	+	+
FP-MW26, 14-15 m	Ft. Pierce, FL	Groundwater	17,000 µg/L of 1,2-D	June, 2012	Propene	+	+	+
FP-MW20, 20-21m	Ft. Pierce, FL	Groundwater	810 µg/L of 1,2-D	June, 2012	Propene	+	+	+
DNA samples								
FP-MW-2S, 6-7 m	Ft. Pierce, FL, USA	DNA/Biobead	14,000 µg/L of 1,2-D	July, 2011	No Propene ^d	+	ND	ND
FP-MW-20, 20-21 m	Ft. Pierce, FL, USA	DNA/Biobead	810 µg/L of 1,2-D	Feb. 2011	Propene ^d	ND	ND	+
FP-MW-26, 14-15 m	Ft. Pierce, FL, USA	DNA/Biobead	17,000 µg/L of 1,2-D	Mar. 2011	Propene ^d	+	+	+
FP-MW-61, 20-21 m	Ft. Pierce, FL, USA	DNA/Biobead	140 µg/L of 1,2-D	May, 2011	Propene ^d	ND	+	+
FW-024	IFC site, Oak Ridge, TN	DNA/GW	Multiple	Feb. 2004	NT	+	ND	ND
FW-103	IFC site, Oak Ridge, TN	DNA/GW	Multiple	Feb. 2004	NT	+	+	+
FW-100-2	IFC site, Oak Ridge, TN	DNA/GW	Multiple	Aug. 2005	NT	+	ND	+
FW-100-3	IFC site, Oak Ridge, TN	DNA/GW	Multiple	Feb. 2004	NT	+	ND	+

^a Solid residue (trester) from wine making consisting mostly of grape skins.

^b Small amounts of 1-chloropropane (1-CP) or 2-chloropropane (2-CP) were detected in live microcosms but also in negative controls.

^c Ft. Pierce is contaminated with 1,2-D (up to 24,000 µg/liter), but the sediments tested here were from wells outside the plume area.

^d Dechlorination not tested on microcosm; data provided reflect field-site conditions. There was no propene detected in FP1-MW-2S.

^e PCE, tetrachloroethene; TCE, trichloroethene; 1,1,1-TCA, 1,1,1-trichloroethene; 1,2-DCA, 1,2-dichloroethane; CF, chloroform; CT, carbon tetrachloride; NT, not tested; ND, not detected; -, no dechlorination in microcosm after 90 days of incubation.

dcpA gene diversity

The primer pair *dcpA*-360F and *dcpA*-1449R retrieved nearly complete (~1 kb) *dcpA* amplicons with template DNAs extracted from seven distinct sample materials, and cloning and sequencing efforts generated 48 *dcpA* sequences (Figure 42). Distance analysis of the DcpA sequences (a total of 247 aa was analyzed) and high bootstrap values indicated that the sequences formed two distinct phylogenetic clusters. Cluster 1 included 33 environmental DcpA sequences most similar (93 to 95%) to *Dhgm lykanthroporepellens* DcpA (Dehly_1524), and cluster 2 comprised 15 DcpA sequences most similar (95 to 99%) to the DcpA proteins of *Dhc* strains RC and KS. All but one of the 48 DcpA sequences contained both of the characteristic iron-sulfur cluster binding motifs (FCX₂CX₂CX₃CP and CX₂CX₃C) (Padilla-Crespo et al., 2014). Overall, the translated environmental *dcpA* sequences differed by 5 to 7% from the DcpA gene of *Dhgm lykanthroporepellens* strain BL-DC-9 and by 1 to 8% from the DcpA genes of *Dhc* strains KS and RC. Interestingly, the deduced DcpA sequences from different continents (i.e., Europe and South America) shared >98% sequence identity, indicating that this RDase either is highly conserved or was recently disseminated.

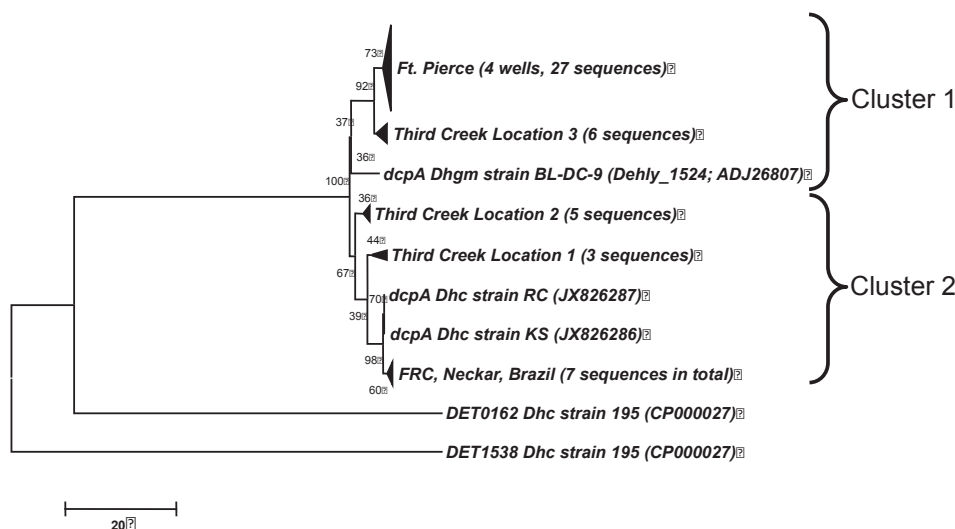


Figure 42. Phylogenetic tree of DcpA sequences. The neighbor-joining tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic relationships. All positions containing gaps and missing data were eliminated and a total of 247 aa positions were included in the final dataset. Evolutionary distances were computed using the number of differences method and the scale bar indicates the number of amino acid differences per sequence. Samples that clustered together were grouped and numbers in parentheses indicate the number of sequences of each group. The RDase DET1538 of *Dhc* strain 195 served as an outgroup to root the tree. Cluster 1 shares highest aa sequence identity to DcpA of *Dhgm* strain BL-DC-9 (93-95%), while Cluster 2 comprises sequences with higher sequence identity to DcpA of *Dhc* strains KS and RC (95-99%).

Dhgm lykanthroporepellens and *Dhc* populations have been implicated in 1,2-D-to-propene reductive dechlorination (Moe et al., 2009; Ritalahti et al., 2004). Since the *Dhc* 16S rRNA gene provides insufficient resolution to distinguish strains with the ability to transform 1,2-D from strains lacking this trait, a functional biomarker was sought to support site assessment (i.e., are 1,2-D-dechlorinating populations present?) and bioremediation monitoring (i.e., are 1,2-D-

dechlorinating populations active?). An integrated approach combining gene presence, transcription, and enzyme activity implicated *dcpA* in 1,2-D dichloroelimination to propene. *dcpA* was co-transcribed with the associated *dcpB* gene, indicating that poly-cistronic mRNA was generated (a feature shared with other *Dhc* RDase operons); a putative RBS preceded both the *dcpA* and *dcpB* genes (allowing for translation initiation at multiple sites); and a single chromosomal copy was identified in *Dhc* strains RC and KS as well as *Dhgm lykanthroporepellens* strain BL-DC-9. Also, the presence of transcriptional regulatory elements (e.g., a ribosomal binding site and a putative dehalobox box up-stream of the *dcpA* gene start codon) and the results of the experimental transcription studies (cDNA library and RT-qPCR analyses) suggest that *dcpA* gene activity is regulated, presumably by the presence of 1,2-D.

The gene most similar to *dcpA* is the pseudogene DET0162, identified in the genome of *Dhc* strain 195. This pseudogene may be a vestigial remnant of a functional gene that shared a common ancestor with *dcpA*. Interestingly, the DET0162 pseudogene has the RBS AGGAG and a possible dehalobox, suggesting that this gene is under regulatory control; however, this pseudogene has an alternate GTG start codon, which is less effective than ATG (Reddy et al., 1985). Transcriptional studies targeting the 19 RDase genes of *Dhc* strain 195 demonstrated that *pceA* (encoding a PCE RDase), *tceA* (encoding a TCE-to-VC RDase), and DET0162 were the only RDase genes upregulated during growth with PCE or TCE as electron acceptors (Fung et al., 2007). Similar results were reported with PCE-dechlorinating mixed cultures containing *Dhc* strain 195, where the DET0162 pseudogene and *tceA* were highly upregulated (Rahm et al., 2006). This pseudogene, along with *pceA*, exhibited high transcript levels in *Dhc* strain 195 cultures grown with 2,3-dichlorophenol (Fung et al., 2007). Although the transcript levels of the pseudogene significantly increased when PCE, TCE, or 2,3-dichlorophenol was provided as an electron acceptor to strain 195 cultures, the translated product was never detected (Fung et al., 2007), probably because of the premature stop codon and ensuing proteolysis. The original function of this pseudogene remains speculative.

Analysis of the *Dhc* RC cDNA clone library revealed two active RDase genes: RCRdA02 and *dcpA*. *Dhgm lykanthroporepellens* strain BL-DC-9 does not possess a homolog of RCRdA02 but does have a highly similar *dcpA* gene. Furthermore, the RCRdA02 gene has nearly identical orthologs in several *Dhc* strains that cannot grow with 1,2-D (i.e., strains CBDB1, FL2, and GT). These findings corroborate that *dcpA* is the 1,2-D RDase gene and also indicate that RCRdA02 is not directly involved in 1,2-D reductive dechlorination. Interestingly, the ortholog of RCRdA02 in strain FL2 (FL2RdhA6) was one of multiple RDase genes transcribed in strain FL2 cultures grown with TCE, cDCE (Waller et al., 2005), and tDCE, suggesting that RCRdA02 is not induced by a specific chloroorganic substrate. In previous studies, the RCRdA02 homolog KB1RdhAB5 was transcribed in consortium KB-1 exposed to TCE, cDCE, or VC (Waller et al., 2005), while in the enrichment culture TUT2264, the RCRdA02 homolog TUT2264_rdhA2 was highly transcribed in cultures spiked with PCE (Futamata et al., 2009). Additionally, transcripts of the FtL-RDase-1638 gene (99% nt identity to RCRdA02) were recovered in cDNA libraries established with RNAs extracted from chlorinated ethene-contaminated groundwater (Lee et al., 2008b). Another RDase gene, DET1545, which is 86% identical to RCRdA02 (and whose translated product is 94% identical and 97% similar to the protein encoded by RCRdA02), was highly expressed during the transition to stationary phase (Johnson et al., 2008) and in pseudo-steady state (Rahm et al., 2008a) in cultures containing *Dhc* strain 195. Overall, these findings suggest that the RCRdA02 gene is constitutively transcribed in metabolically active *Dhc* strains. The quantification of RCRdA02 transcripts in environmental samples may serve as an indicator of

general *Dhc* activity; however, RCRdA02 transcription appears not to be linked to specific reductive dechlorination reactions. Recent studies have demonstrated that stress conditions (i.e., oxygen exposure, heat, or starvation) influence RDase gene transcription, and RDase transcripts can be measured in *Dhc* cultures not exhibiting reductive dechlorination activity (Amos et al., 2008; Fletcher et al., 2011). These findings emphasize that *Dhc* RDase gene activity and/or transcript turnover is poorly understood, and regulatory controls have yet to be identified and verified. Nevertheless, transcriptional analysis applied to cultures amended with 1,2-D identified the RDase gene *dcpA*, which serves as a biomarker for 1,2-D dichloroelimination to environmentally benign ethene.

Previous studies investigated the environmental distribution of RDase genes with assigned functions (e.g., *bvcA*, *vcrA*, and *tceA*) in sample materials retrieved from aquifers contaminated with chlorinated ethenes (Krajmalnik-Brown et al., 2007; Müller et al., 2004). The recovered RDase sequences exhibited >95% and 98% identity with known *tceA* and *vcrA* genes, respectively, even in samples collected from geographically distinct locations (Krajmalnik-Brown et al., 2007; McMurdie et al., 2011; Müller et al., 2004; Sung et al., 2006b). Similar results were found in this study, where *dcpA* sequences retrieved from geographically distinct samples shared >98% sequence identity with *Dhc* strains RC and KS and the *Dhgm lykanthroporepellens* BL-DC-9 *dcpA* sequences. Of course, it is expected that similar RDase gene sequences would be recovered with PCR primers targeting conserved RDase gene motifs. Nevertheless, the primer pair *dcpA*-360F and *dcpA*-1449R amplified 1,089 bp-long *dcpA* gene fragments with identifiable sequence variability between the conserved primer binding sites, which was useful to improve the understanding of *dcpA* sequence diversity. This approach revealed two *dcpA* clades, both of which were captured with the *dcpA*-targeted PCR approach that was designed.

The utility of *dcpA*-specific nested PCR and qPCR assays for the sensitive detection and enumeration of *dcpA* genes, respectively, in environmental samples was demonstrated, and the presence and abundance of *dcpA* correlated with propene formation. Only in the samples from the Barra Mansa site in Brazil was *dcpA* detected but no 1,2-D dechlorination activity observed in the microcosms. Groundwater from this site contained up to 7,860 µg/L CF as well as carbon tetrachloride and 1,1,1-TCA, which have all been described as potent *Dhc* inhibitors (Duhamel et al., 2002). The microcosms did not produce methane, further supporting the hypothesis that carbon tetrachloride, CF, and/or 1,1,1-TCA affected microbial activity, including the 1,2-D-dechlorinating population(s). This observation suggests that 1,2-D bioremediation may require prior removal of inhibitory cocontaminants.

Although other 1,2-D RDases exist (e.g., *Desulfitobacterium dichloroeliminans* strain DCA1 does not harbor the *dcpA* gene but dechlorinates 1,2-D to propene), 1,2-D-respiring *Chloroflexi* organisms appear to be major contributors to this activity at the sites investigated, and the *dcpA*-targeted PCR assays augment the available toolbox for site assessment and bioremediation monitoring. Carbon stable isotope enrichment factors associated with 1,2-D dichloroelimination in cultures RC and KS harboring the *dcpA* gene have been determined (Fletcher et al., 2009), and a comprehensive MBT approach can now be applied to tackle 1,2-D-contaminated sites. The application of environmental molecular diagnostics promises to identify sites amenable to bioremediation and to achieve cleanup goals faster, leading to early site closures and realizing significant cost savings to the site owner(s), which will ultimately determine the value of these MBTs for remediation practice.

Identification of *cerA* as a novel biomarker for VC-to-ethene reductive dechlorination

'*Candidatus* Dhgm etheniformans' strain GP was identified as the TCE-to-ethene dechlorinating bacterium in mixed culture GP (Task 3). Growth experiments demonstrated that strain GP grew with VC as electron acceptor (Figure 43), but qPCR assays failed to detect the known VC RDase genes *tceA*, *vcrA* and *bvcA*, suggesting strain GP harbors a different (novel) VC RDase. The analysis of the metagenome-assembled genome of strain GP corroborated the absence of known VC RDase genes. Therefore, the goal was to identify the gene encoding the VC RDase in *Dhgm* sp. strain GP.

Protein profiling and identification of a novel putative VC RDase

The proteomics analysis identified three abundant putative RDases, prokka_00862, prokka_01300, and prokka_02004, in GP cultures grown with TCE, 1,1-DCE and VC, respectively (Figure 43).

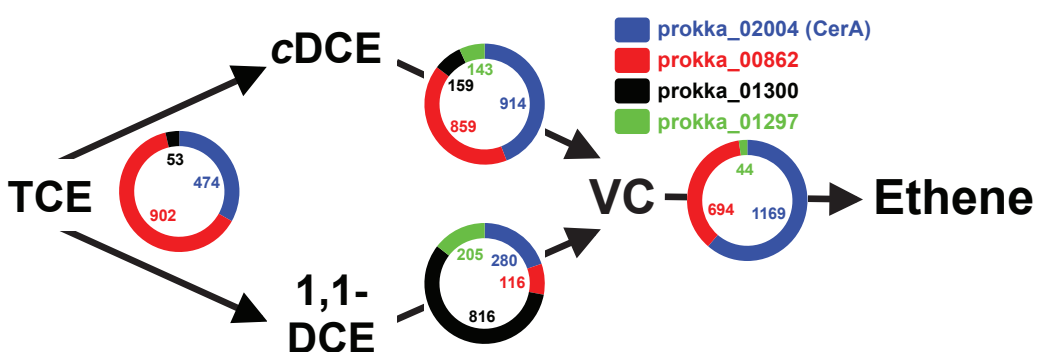


Figure 43. Relative RDase A protein abundances in mixed culture GP.

Shown are normalized spectral counts of peptides detected in cultures grown with TCE, cDCE, 1,1-DCE and VC as electron acceptors. In cDCE-grown cultures, prokka_00862 and prokka_02004 had similar measured abundances (i.e. spectral counts), suggesting equal expression. The abundances of the detected peptides indicated high expression of the prokka_02004 RDase in cells collected from VC-grown GP cultures, while a lower abundance of the prokka_02004 RDase was observed in TCE-, 1,1-DCE-, cDCE-grown GP cultures. The prokka_02004 abundance (i.e. normalized spectral counts) correlated with the amount of ethene formed in the cultivation vessels amended with different chlorinated ethenes, supporting the proposition that prokka_02004 has activity towards VC. Prokka_02004 grouped in a cluster comprising the characterized RDases TceA, BvcA and VcrA (Figure 6), and shared 83.3%, 56.1% and 49% amino acid similarity (76.2%, 42.1, and 34.9% amino acid identity) with the TceA, and the characterized VC RDases BvcA and VcrA, respectively. No peptides representing RDase B proteins encoded in the genome of *Dhgm* strain GP were detected.

Based on observations from both the phylogenetic (Figure 44) and proteomics analyses, prokka_02004 RDase likely represents a VC RDase and the gene encoding this enzyme was designated *cerA* (for chloroethene reductase gene).

grown with cDCE, 1,1-DCE and VC, albeit with significantly lower spectral counts than the major RDases. Previous studies revealed the expression of multiple RDases in organohalide-respiring *Chloroflexi* grown with polychlorinated benzenes as chlorinated electron acceptors, a phenomenon that is currently not understood (Pöritz et al., 2015; Schiffmann et al., 2014b). The putative RDase prokka_00862 was differentially expressed when culture GP was cultivated with TCE, 1,1-DCE, cDCE and VC (Figure 44). Phylogenetic analysis did not group prokka_00862 with the known VC RDases but affiliated this protein with the putative RDase DGWBC_1144 identified in *Dhgm* sp. strain WBC-2 (Figure 44). *Dhgm* sp. strain WBC-2 is responsible for transforming tDCE to VC in a mixed culture enriched with 1,1,2,2-tetrachloroethane (Molenda et al., 2016). During growth with tDCE, strain WBC-2 expressed four RDases (DGWBC_0411, DGWBC_1144, DGWBC_1574, and DGWBC_0279), and blue native polyacrylamide gel electrophoresis (BN-PAGE) analysis identified DGWBC_0411, designated TdrA, as the tDCE-to-VC RDase (Molenda et al., 2016). No specific activity could be assigned to DGWBC_1144 but strain GP expressed a related protein (prokka_00862), and these putative RDases may have functional roles in reductive dechlorination of chlorinated ethenes. Strain GP also possesses an RDase (prokka_01475) that shares more than 65% amino acid identity with the PCB RDases PcbA-CG4 and PcbA-CG5 of *Dhc* strains CG4 and CG5, respectively (Figure 44). *Dhgm* 16S rRNA gene sequences have been detected in PCB-dechlorinating enrichment cultures (Wang et al., 2013) and PCB-impacted marine sediment (Klaus et al., 2016), suggesting that prokka_01475 represents a potentially novel PCB RDase.

Identification of a general biomarker for *Dhc* and *Dhgm* activity

A characteristic feature of obligate organohalide-respiring bacteria is the presence of multiple hydrogenase genes. Gene clusters encoding a [Ni/Fe] hydrogenase complex (EC 1.12.2.1), a NAD-reducing hydrogenase complex (EC 1.12.1.2), a periplasmic [Fe] hydrogenase complex (EC 1.12.7.2) and an uptake hydrogenase complex (EC 1.12.99.6) were identified on the draft genome. Similar to the other three sequenced *Dhgm* genomes (i.e., strains BL-DC-9, IP3-3 and NSZ-14), which possess multiple genes annotated as formate dehydrogenase alpha subunit (FdhA), two genes encoding FdhA (EC 1.2.1.2) were identified on the genome of strain GP, whereas *Dhc* genomes harbor only one copy of the *fdhA* gene. The phylogenetic analysis of putative *Dhgm* and *Dhc* FdhA proteins suggested that *Dhgm* may possess two types: one related to the *Dhc*-type FdhA (53.3% amino acid identity), while the other is more similar to *Clostridium* FdhA proteins (up to 48.7% identity) (Figure 45). The *Dhc*-type FdhA has cysteine at the putative active site, while *Dhgm*-type FdhA has different amino acids (e.g., cycteine or selenocysteine) at the same site.

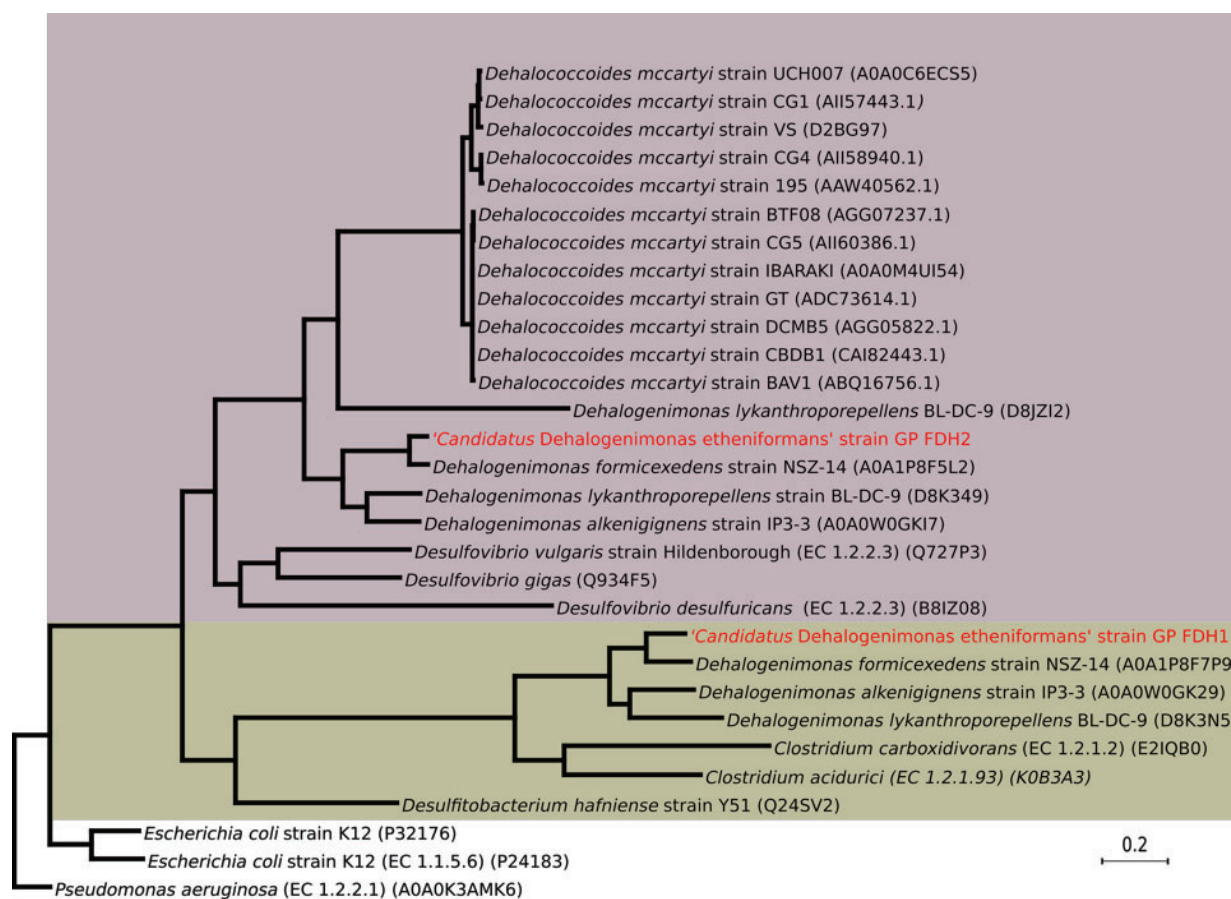


Figure 45. Phylogenetic analysis of formate dehydrogenase alpha subunit sequences encoding a complex iron sulfur molybdoenzyme (CISM).

CISM proteins encoded on *Dhc*, *Dhgm* and select other bacterial genomes are shown. All sequences were aligned using MUSCLE and the tree was built using FastTree in Geneious. The scale bar indicates 0.3 amino acid substitutions per site.

A total of 52 putative RDase genes were identified, 10 of which were associated with B genes that encode B proteins with 1 to 4 trans-membrane spanning helices. All putative RDase protein sequences had either a TAT signal peptide or a Sec signal peptide predicted by PRED-TAT (Bagos et al., 2010). One of the predicted RDases (prokka_01475) shared 36.8%, 67.6% and 65.8% identities with the three characterized PcbA RDases identified in *Dhc* strains CG1, CG4 and CG5, respectively (Wang et al., 2014).

Task 5: Effects of Perturbations

Bioremediation has emerged as a robust technology to treat groundwater aquifers impacted with chlorinated ethenes. At the majority of sites, bioremediation treatment increases contaminant degradation rates and promotes the formation of environmentally benign ethene. At some bioremediation sites, the rates of reductive dechlorination are inadequate to meet cleanup goals and detoxification (i.e., ethene formation) is not achieved. The exact reasons why bioremediation is less productive at some sites are not always clear, which limits the implementation of adaptive site management (ASM) strategies to overcome existing bottleneck(s). The causes for inadequate reductive dechlorination performance at some sites include unfavorable redox conditions (Amos et al., 2008), a lack of electron donor, and the presence of co-contaminants, or other compounds, that inhibit *Dhc*. For example, the presence of surfactants and chloroform had a strong inhibitory effect on *Dhc* reductive dechlorination activity (Amos et al., 2007b; Duhamel et al., 2002).

Biostimulation efforts focus on the addition of fermentable organics to establish redox conditions favorable for *Dhc* and to increase the flux of hydrogen, the key electron donor to fuel *Dhc* reductive dechlorination activity. Far less attention has been given to fixed nitrogen availability, and laboratory and field-scale efforts explored the effects of nitrogen limitations and the addition of ammonium on *Dhc* growth and reductive dechlorination activity.

Research to date has mostly focused on elucidating the geochemical conditions that promote *Dhc* reductive dechlorination activity and inhibitors of the dechlorination process have received less attention. The experimental efforts performed under this task explored inhibitory effects of the chlorofluorocarbon CFC-113, a co-contaminant in chlorinated ethene plumes at a number of sites, and N₂O, a common metabolite of microbial turnover of fixed nitrogen (e.g., nitrate, ammonium, nitrogen-containing organic compounds).

The recognition that *Dhc* are corrinoid auxotrophs (i.e., lack the ability for de novo synthesis of the corrin ring system) but the RDase enzyme systems strictly require corrinoid to perform reductive dechlorination, highlight the need for an improved understanding of the source of corrinoid in the contaminated subsurface. To this end, the research characterized the corrinoid requirements of *Dhc* and conducted experiments to elucidate the impact of geochemistry of the corrinoid pool.

Effects of CFC-113 on *Dhc* reductive dechlorination activity

The environmental fate of CFC-113 has not received much attention, mainly because the Maximum Contaminant Level (MCL) for CFC-113 is 1.2 mg/L,¹⁷ much higher compared to the MCLs established for chlorinated ethenes (i.e., 0.005 mg/L for PCE and TCE, 0.07 mg/L for cDCE, and 0.002 mg/L for VC) (USEPA, 2009). CFC-113 has been reported to undergo reductive dechlorination in microcosms established with landfill leachate, (Deipser et al., 1997; Lesage et al., 1992; Lesage et al., 1990), sewage sludge, (Balsiger et al., 2005) and aquifer materials (Balsiger et al., 2005). Several transformation intermediates, including 2,2-dichloro-1,1,1-trifluoroethane (HCFC-123a), 2-chloro-1,1,1-trifluoroethane (HCFC-133), 1-chloro-1,1,2-trifluoroethane (HCFC-133b), chlorotrifluoroethene (CTFE), and trifluoroethane (TFE) were detected (Figure 46). Zero valent iron (ZVI) (Archbold et al., 2012; Farrell et al., 2000; Muegge et al., 2009; Roberts et al., 1996) and naturally-occurring reactive mineral phases (Butler et al., 1999; Ferrey et al., 2004; Jeong et al., 2007; Lee et al., 2002a; Lee et al., 2002b; Lin et al., 2009; O'Loughlin et al., 2003) have been reported to mediate abiotic transformation of halogenated organic compounds. Specifically, ZVI has been demonstrated to degrade CFC-113 to HCFC-123a

and CTFE (Figure 46), (Archbold et al., 2012) and a permeable reactive ZVI barrier has been implemented for *in situ* remediation of CFC-113-contaminated groundwater (Muegge et al., 2009). Reactive mineral phases, such as mackinawite, (Butler et al., 1999; Jeong et al., 2007) green rust, (Lee et al., 2002b; O'Loughlin et al., 2003) magnetite, (Ferrey et al., 2004; Lee et al., 2002a) and manganese dioxide (Lin et al., 2009) also have been demonstrated to degrade certain halogenated compounds, but the reactivity of these mineral phases toward CFC-113 have not yet been documented. Reductive transformation of halogenated compounds can be catalyzed *in vitro* by microbial transition metal cofactors, such as cobamides (Co), coenzyme F430 (Ni), and hematin (Fe) (Burris et al., 1998; Im et al., 2014; Krone et al., 1989; Krone et al., 1991; Ochoa-Herrera et al., 2008). Reductive dehalogenases (RDases) require a cobamide as cofactor (Yan et al., 2013) and the reduced Co(I) form is a supernucleophile that catalyzes reductive dehalogenation reactions (Burris et al., 1996; Im et al., 2014; Krone et al., 1991; Ochoa-Herrera et al., 2008). Reduced hematin has been demonstrated to catalyze reductive transformation of CFC-113, and HCFC-123a and CTFE were detected as transformation products (Figure 46) (Lesage et al., 1992).

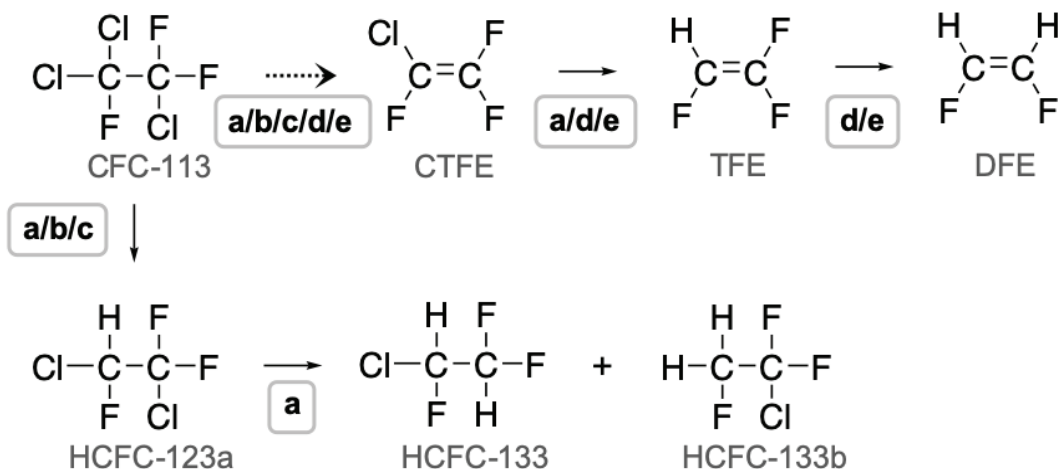


Figure 46. Summary of reported pathways and intermediates in CFC-113 degradation. (a) Microbial transformation reactions observed under anoxic conditions (Balsiger et al., 2005; Deipser et al., 1997; Lesage et al., 1992; Lesage et al., 1990). (b) Abiotic degradation by ZVI (Archbold et al., 2012) (c) Biomimetic degradation by hematin (Lesage et al., 1992; Miller et al., 2005). (d) Biomimetic degradation by vitamin B₁₂ (this project). (e) Co-metabolic degradation by corrinoid-producing homoacetogenic bacteria (this project). The dotted arrow indicates a dihaloelimination reaction and the solid arrows indicate a hydrogenolysis reaction.

The goal of the experimental efforts was to evaluate the effects of CFC-113 and its transformation products CTFE, TFE, and cDFE on reductive dechlorination of chlorinated ethenes and another groundwater contaminant, 1,2-D. We then comprehensively explored potential attenuation processes for CFC-113 including (i) microbial degradation under oxic and anoxic conditions, (ii) abiotic degradation mediated by reactive minerals, and (iii) corrinoid-catalyzed (biomimetic) transformation. The findings indicated that CFC-113 is a potent inhibitor of reductive dechlorination catalyzed by members of Dehalococcoidia (i.e., *Dhc* and *Dhgm*), but CFC-113 is not inert and cometabolic reductive dechlorination and defluorination leads to products that do not affect reductive dechlorination of chlorinated ethenes.

Inhibition of *Dhc* reductive dechlorination by CFC-113

Complete reductive dechlorination of TCE to ethene occurred in SDC-9 cultures without CFC-113 (Figure 47a). In the presence of CFC-113 as a co-contaminant, *Dhc* activity was severely inhibited in a concentration dependent manner (Figure 47b-d). TCE dechlorination stalled at cDCE in the presence of 7.6 μM CFC-113 (Figure 47b), and no ethene formation occurred even after extended incubation periods of 3 months. At higher CFC-113 concentrations, TCE dechlorination was also inhibited, and correspondingly less cDCE formed (Figure 47b-d).

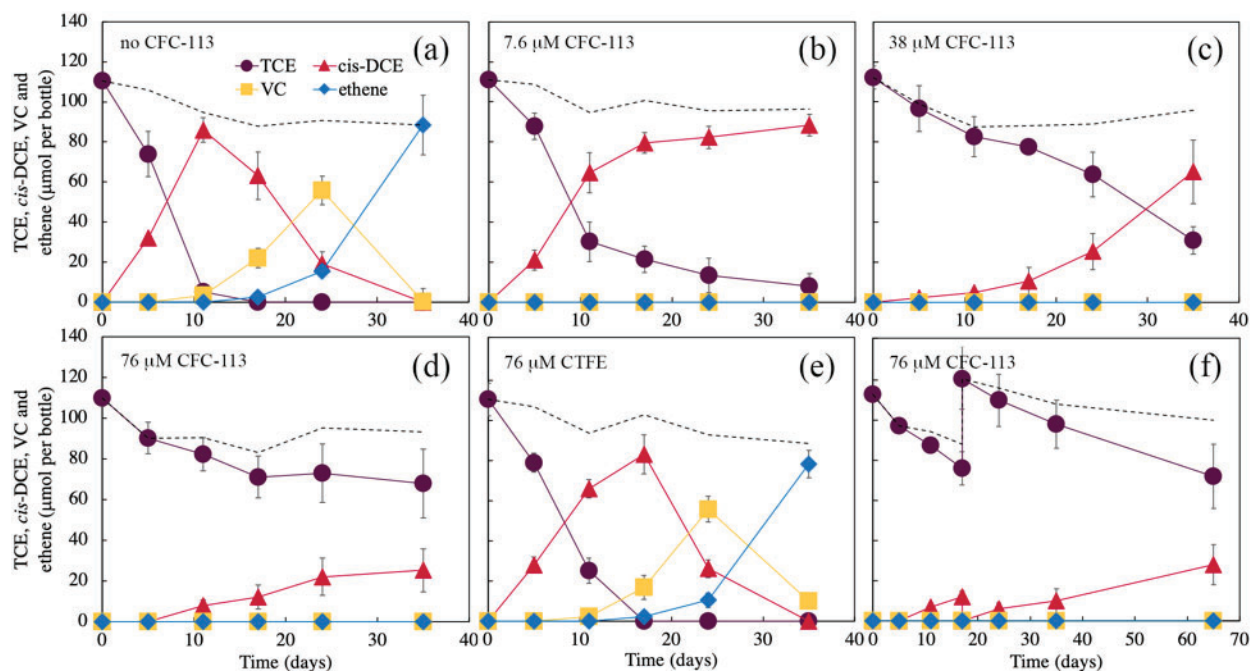


Figure 47. Inhibition of reductive dechlorination of TCE by the *Dhc*-containing consortium SDC-9. Triplicate SDC-9 cultures were supplied with (a) 0 μM , (b) 7.6 μM , (c) 38 μM , (d) 76 μM of CFC-113, or (e) 76 μM of CTFE in the aqueous phase, and (f) 76 μM of CFC-113 was removed from the culture after 17 days by flushing the headspace with $\text{N}_2\text{-CO}_2$ (80/20, v/v) and neat TCE was replenished. Error bars represent standard deviations of triplicate cultures and are not visible when smaller than the symbols. The dotted lines represent the total amount of chlorinated ethenes and ethene (i.e., the sum of TCE, cDCE, VC, and ethene).

TCE dechlorination rates declined from $0.30 (\pm 0.05)$ to $0.07 (\pm 0.02) \text{ d}^{-1}$ in the presence of $7.6 \mu\text{M}$ CFC-113, and decreased further with increasing CFC-113 concentrations, i.e., $0.03 (\pm 0.01)$ and $0.01 (\pm 0.00) \text{ d}^{-1}$ in the presence of 38 and $76 \mu\text{M}$ CFC-113, respectively (Figure 48).

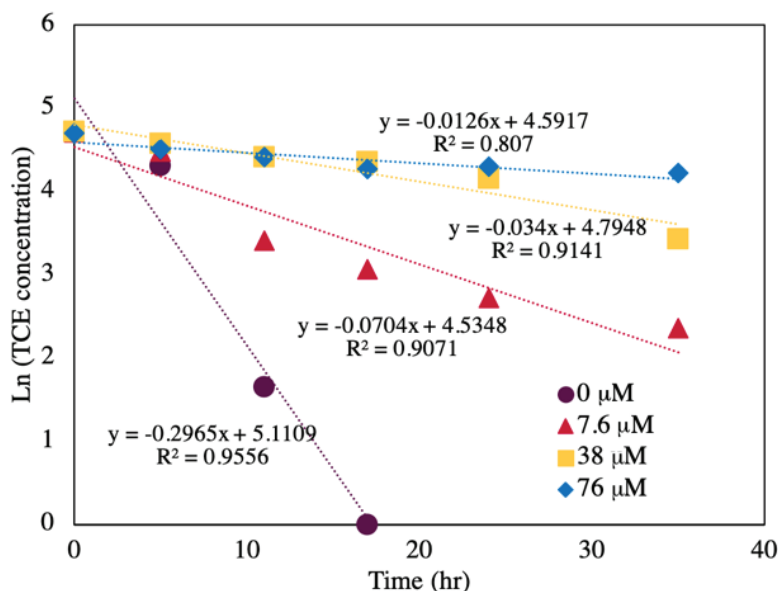


Figure 48. Natural log of TCE concentrations over time in the presence of different concentrations of CFC-113 and the corresponding linear regression curves.

The data were extracted from the measurements displayed in Figure 47.

When CFC-113 was removed from the cultivation vessels after 17 days by flushing the headspace with $\text{N}_2\text{-CO}_2$ (80/20, v/v), TCE dechlorination resumed, but further dechlorination to VC or ethene did not occur (Figure 47f). In incubation vessels amended with CFC-113 transformation products (i.e., CTFE, TFE, and cDFE), slightly lower or similar TCE dechlorination rates were observed (0.27 ± 0.02 , 0.30 ± 0.03 , and $0.31 \pm 0.03 \text{ d}^{-1}$, respectively) and complete dechlorination to ethene occurred (Figure 47e and Figure 49) indicating that these compounds had little or no inhibitory effect on reductive dechlorination of chlorinated ethenes.

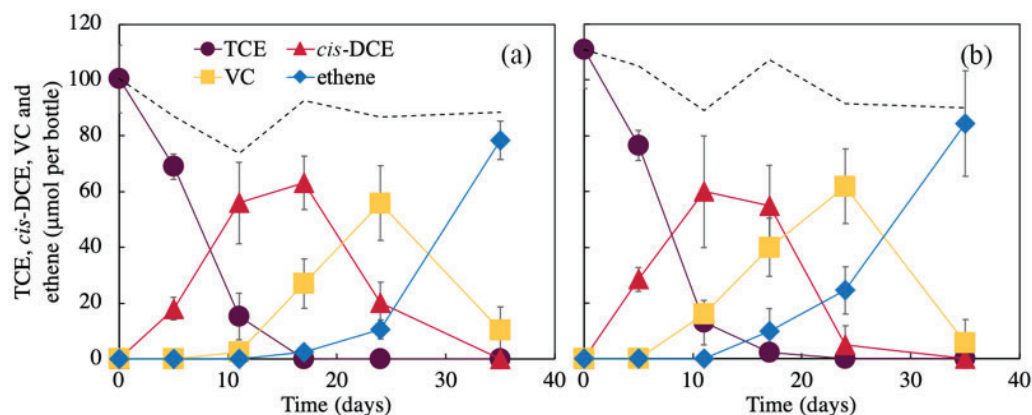


Figure 49. *Dhc*-containing SDC-9 consortium demonstrating reductive dechlorination of TCE.

A culture was spiked with either (a) TFE or (b) cDCE to reach an aqueous phase concentration of $76 \mu\text{M}$.

Reductive dechlorination of 1,2-D by strain BL-DC-9 was also significantly inhibited by CFC-113, and the 1,2-D degradation rate decreased from $0.12 (\pm 0.04)$ to $0.02 (\pm 0.01) \text{ d}^{-1}$ in the presence of $76 \mu\text{M}$ CFC-113 (Figure 50). Still, 1,2-D was stoichiometrically converted to propene over a 1-month incubation period, indicating that $76 \mu\text{M}$ CFC-113 impacted the rate but not the extent of reductive dechlorination of 1,2-D (Figure 50). Under any incubation condition, transformation of CFC-113, CTFE, TFE, and cDFE did not occur in SDC-9 cultures.

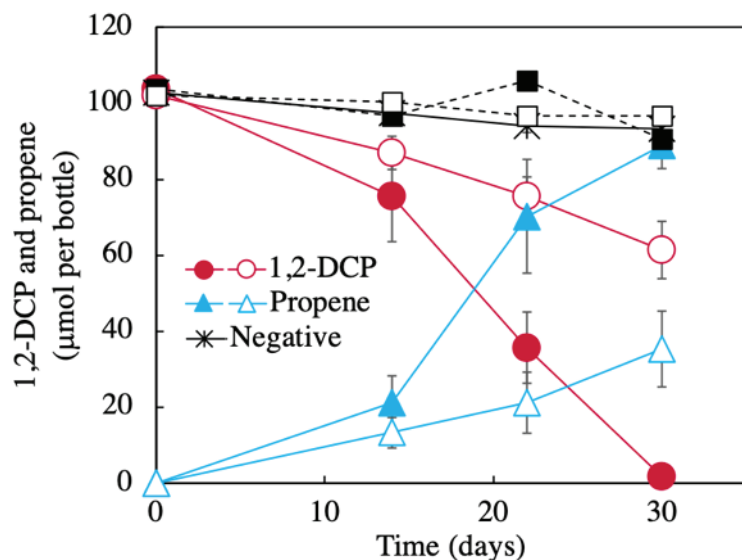


Figure 50. Reductive dechlorination of 1,2-D by *Dhgm lykanthroporepelens* strain BL-DC-9.

The graph shows dechlorination performance in the presence (open symbols) or absence (closed symbols) of $76 \mu\text{M}$ of CFC-113 (aqueous phase concentration). Error bars are not shown if they are smaller than the symbols. The dotted lines represent the sum of the 1,2-D and propene amounts in the absence (open squares) and presence (closed squares) of CFC-113, respectively.

Sediment microcosms

In microcosms prepared with sediment materials collected from three different locations, reductive dechlorination of CFC-113 was observed and up to 35% of CFC-113 was recovered as CTFE. A second dose of 5 mM lactate did not result in additional CTFE formation. In transfer cultures, the dechlorination activity was not sustained, and neither significant decreases of CFC-113 concentrations nor transformation intermediates were observed (Figure 51).

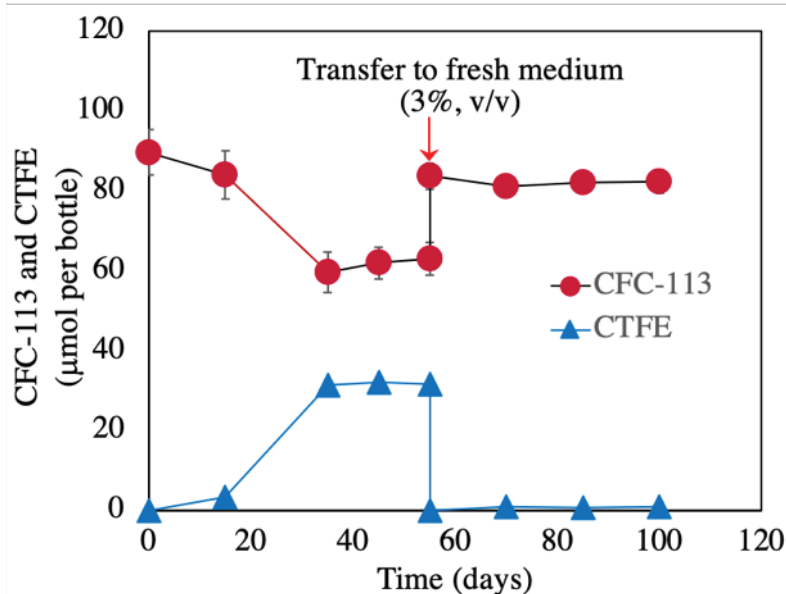


Figure 51. Concentration profiles of CFC-113 and CTFE in Third Creek sediment microcosms.

The red arrow indicates when the microcosms were transferred to fresh medium. Similar observations were made with the other sediment materials tested.

Abiotic degradation by reactive mineral phases

No CFC-113 transformation was observed in any of the incubations with reactive mineral phases including mackinawite, green rust, and magnetite and manganese dioxide (Figure 52). The reactivity of each reactive mineral phase was confirmed in positive control incubations with TCE, and the TCE degradation rate constants and detected products with the reactive mineral phases were consistent with a previous study that employed the same materials (Im et al., 2014).

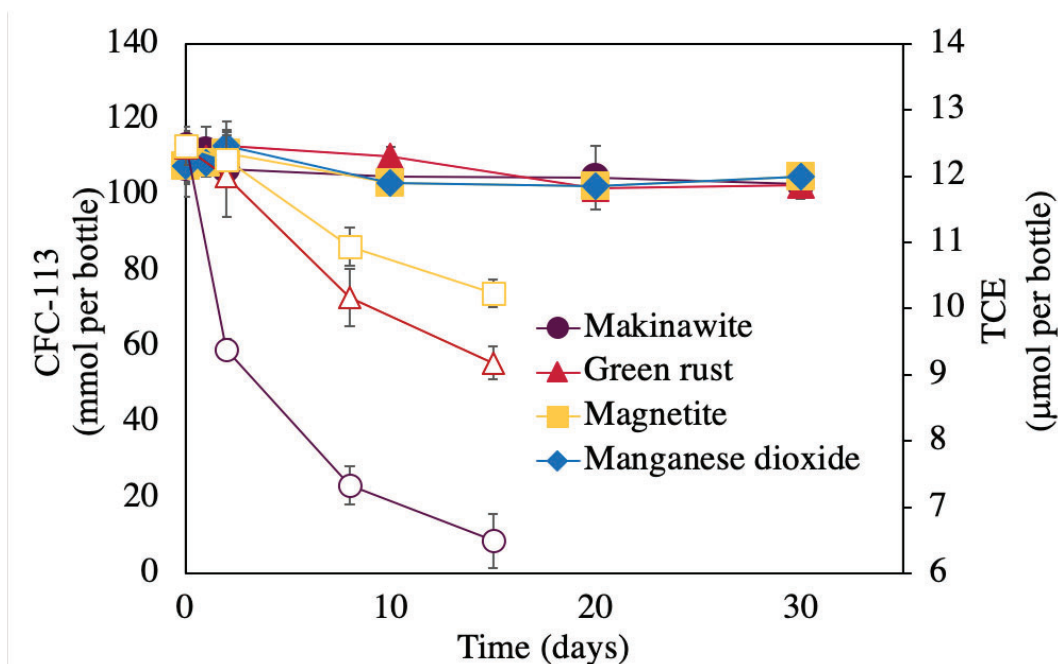


Figure 52. CFC-113 concentration profiles in abiotic incubations with reactive mineral phases including mackinawite, green rust, magnetite, and manganese dioxide. The open symbols represent TCE concentrations in the positive control incubations with mackinawite, green rust, and magnetite. To demonstrate the reactivity of manganese dioxide, 44 μM BPA was completely removed within 1 hour.

Biomimetic reductive defluorination by vitamin B₁₂

Figure 53 shows the biomimetic reduction of CFC-113 (Figure 53a) and CTFE (Figure 53b) mediated by 10 μM vitamin B₁₂ reduced with titanium(III) citrate. CFC-113 was reductively dechlorinated to CTFE, and CTFE was further dechlorinated to TFE (Figure 53a). Incubation of CTFE with the vitamin B₁₂ supernucleophile produced not only TFE but also cDFE, which is indicative of reductive defluorination (Figure 53b). The formation of 2.1 (±0.1) μmol cDFE coincided with the release of 1.8 (±0.2) μmol of inorganic fluoride, indicating stoichiometric reductive defluorination of TFE to cDFE. The rates of vitamin B₁₂-catalyzed degradation of CFC-113 and CTFE were compared with those of TCE, cDCE, and VC under the same reaction conditions.

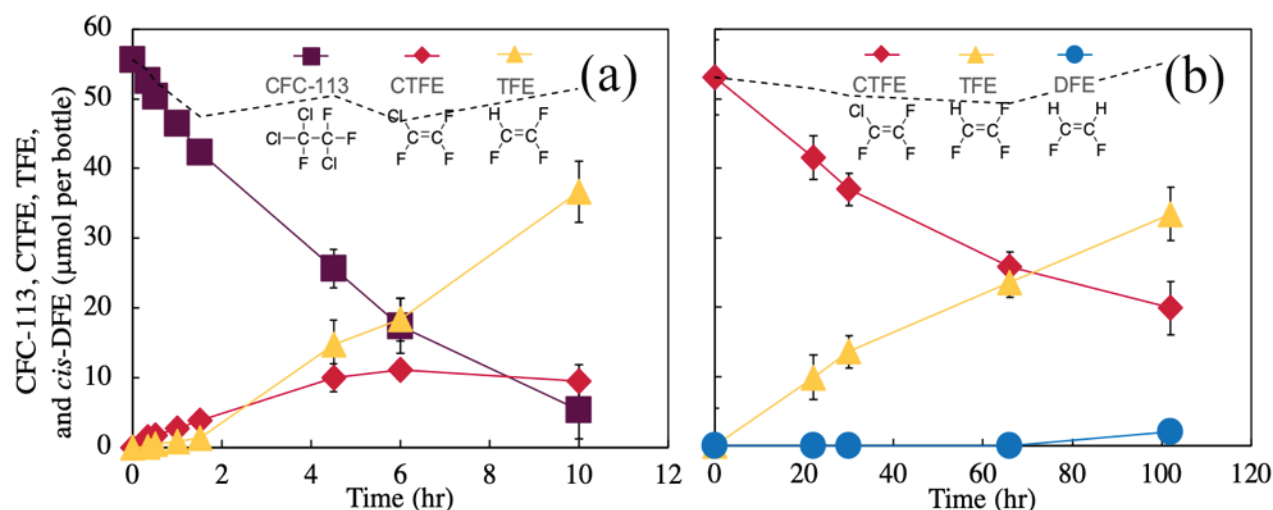


Figure 53. Biomimetic reductive dehalogenation of CFC-113 and CTFE by vitamin B₁₂ (10 μM) with titanium (III) citrate (5 mM) as the reductant.

(a) Reductive dechlorination of CFC-113 to CTFE and TFE. (b) Reductive dechlorination of CTFE to TFE and reductive defluorination of TFE to cDFE. The dotted lines represent the total chlorofluorohydrocarbons amounts in the incubation vessels (i.e., the sum of CFC-113, CTFE, TFE, and cDFE).

Table 32 summarizes the observed intermediates and the calculated reaction rates for the individual halogenated compounds. The dechlorination rate of CFC-113 (0.22 ± 0.01 d⁻¹) was 3.4 times faster than that of TCE (0.066 ± 0.009 d⁻¹), and 2.3 times faster than that of CTFE (0.10 ± 0.01 d⁻¹). The dechlorination rates of cDCE and VC were orders of magnitude slower than the observed rate for CFC-113 dechlorination.

Table 32. Biomimetic reductive dehalogenation and apparent pseudo-first-order rate constants and transformation product(s) for TCE, CFC-113, and CTFE.

The incubations contained 10 μM vitamin B₁₂ and 5 mM titanium(III) citrate as reductant. The standard error was determined using the LINEST function in Microsoft Excel (Office 365 version 16.17, Microsoft Corp., Redmond, WA). No dehalogenation was observed in control incubations without vitamin B₁₂ or the reductant.

Compound	Pseudo-first-order-rate constant (d ⁻¹)	Product(s)
TCE	0.066 ± 0.009	cDCE, VC, ethene
cDCE	0.003 ± 0.001	VC, ethene
VC	-	ethene [†]
CFC-113	0.23 ± 0.01	CTFE, TFE
CTFE	0.10 ± 0.01	TFE, cDFE

[†]Ethene was detected, but VC-to-ethene conversion was very slow and no rate constant was determined.

Co-metabolic reductive dehalogenation by corrinoid-producing bacteria

The homoacetogen *Sporomusa ovata*, a known corrinoid producer, mediated reductive dechlorination and reductive defluorination of CFC-113. Within a 1-week incubation period, 8.7 (± 0.8) μmol of CFC-113 was converted to 5.4 (± 0.9) μmol of CTFE and 1.8 (± 1.0) μmol of TFE, indicating that CFC-113 was sequentially dechlorinated to TFE via CTFE (Figure 54a). In the incubations with 5.2 (± 0.9) μmol of CTFE, 5.1 (± 1.0) μmol of TFE and 0.2 (± 0.0) μmol of cDFE were formed (Figure 54b), indicating that corrinoid produced by *S. ovata* catalyzed not only reductive dechlorination but also reductive defluorination reactions.

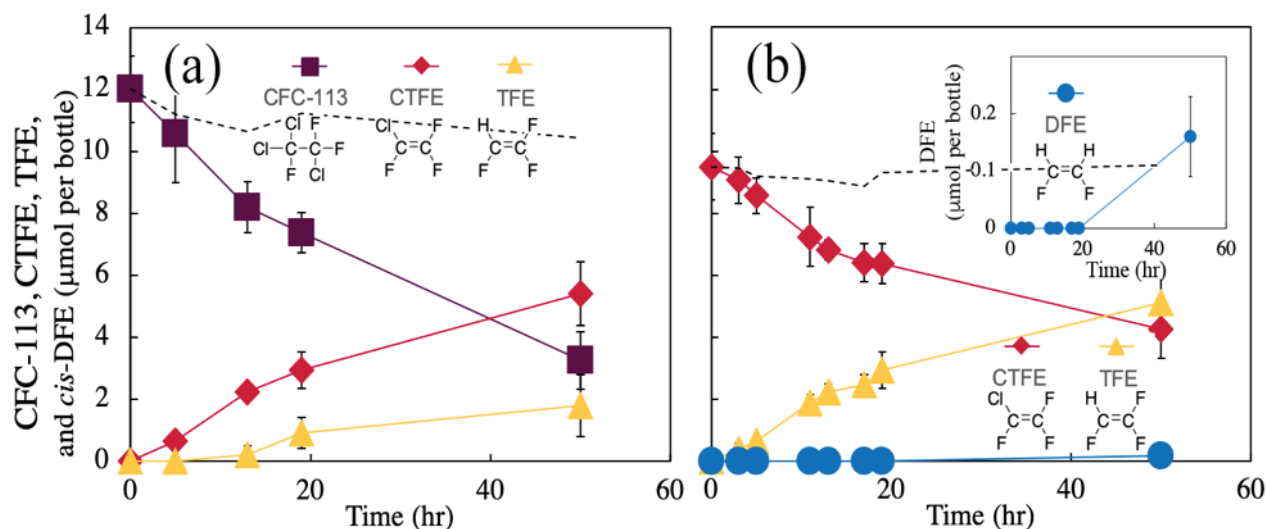


Figure 54. Co-metabolic reductive dehalogenation by the corrinoid-producing homoacetogenic bacterium *Sporomusa ovata* strain H1.

(a) Reductive dechlorination of CFC-113 to CTFE and TFE. (b) Reductive dechlorination of CTFE to TFE and reductive dechlorination of TFE to cDFE. Error bars represent standard deviations of triplicate cultures and are not visible when smaller than the symbols.

Discussion - Effects of CFC-113

Chlorinated ethenes are major risk drivers at many sites, and bioremediation based on the microbial reductive dechlorination process is a viable option (Lendvay et al., 2003; Löffler et al., 2013a; Major et al., 2002). For the reductive dechlorination of chlorinated ethenes, *Dhc* (He et al., 2003b; Löffler et al., 2013a) and *Dhgm* (Yang et al., 2017c) are the only known organisms capable of metabolic reduction of cDCE and VC to ethene, and our findings provide a possible explanation for stalled degradation activity in plumes with CFC-113 as co-contaminant. CFC-113 has been detected as a co-contaminant in TCE-contaminated aquifers, (Höhener et al., 2003; Jackson et al., 1999; Lesage et al., 1990; Parker et al., 2005; Weidhaas et al., 2013) and concentrations of up to 480 μM (90 mg/L) have been reported (Parker et al., 2005). This study demonstrated that even much lower concentrations of CFC 113 (i.e., 7.6 μM or 1.4 mg/L) resulted in severe inhibition of *Dhc* reductive dechlorination activity (i.e., cDCE dechlorination to VC and ethene) and incomplete TCE degradation (i.e., cDCE stalls) (Figure 47b). These findings suggest that *in situ* bioremediation treatment of chlorinated ethenes can be compromised when CFC-113 exists as a co-contaminant. It should be noted that the reductive dechlorination of chlorinated ethenes to environmentally benign ethene is not limited to *Dhc*, and a member of the *Dhgm* genus,

'*Candidatus Dehalogenimonas etheniformans*', has been demonstrated to use VC as a respiratory electron acceptor (Yang et al., 2017c). CFC-113 also inhibited reductive dechlorination of 1,2-D by *Dhgm lykanthroporepellens* strain BL-DC-9 (Figure 50), suggesting that CFC-113 also negatively affects dehalogenation activity of *Dhgm*. The observation that higher concentrations of CFC-113 also impacted TCE reductive dechlorination suggested that bacteria responsible for PCE/TCE reductive dechlorination to cDCE are also susceptible to inhibition by CFC-113.

The recovery of *Dhc* activity following prolonged incubation (up to 42 days) under unfavorable conditions (e.g., low pH, starvation) has been demonstrated; (Lee et al., 2006; Yang et al., 2017b) however, *Dhc* activity was not recovered after a 17-day exposure period to CFC-113. Although the inhibition mechanism remains to be elucidated, the observation that *Dhc* dechlorination activity was not recovered after removing CFC-113 (Figure 47f) suggests that CFC-113 irreversibly inactivated the *Dhc* RDases. Chlorinated ethanes have been reported to reversibly inhibit TCE dechlorination in *Dhc*-containing consortia, and VC dechlorination was more inhibited than TCE and cDCE dechlorination by 1,1,1-trichloroethane (1,1,1-TCA), and 1,1-dichloroethane was less inhibitory than 1,1,1-TCA (Chan et al., 2011). These observations suggest that the mode of inhibition exerted by CFC-113 and chlorinated ethanes on *Dhc* cells and/or RDases is different. Inhibitory responses of *Dhc* have also been reported for exposure to per- and polyfluoroalkyl substances (PFASs), albeit at lower degree (Harding-Marjanovic et al., 2016; Weathers et al., 2016). The inhibition by PFASs was concentration-dependent, but ethene was formed even in the presence of 110 mg/L of perfluorinated sulfonates (Yin et al., 2019) or 22-66 mg/L of total perfluoroalkyl acids (Weathers et al., 2016).

A number of studies reported biological dechlorination of CFC-113 (Lesage et al., 1990) (Balsiger et al., 2005; Deipser et al., 1997; Lesage et al., 1992). In methanogenic landfill leachate, for example, CFC-113 was transformed to 1,2-dichloro-1,1,2-trifluoroethane (HCFC-123a), which was further reductively dechlorinated to 1-chloro-1,2,2-trifluoroethane (HCFC-133) and 1-chloro-1,1,2-trifluoroethane (HCFC-133b) (Figure 46) (Deipser et al., 1997; Lesage et al., 1992; Lesage et al., 1990). Similar reductive transformation was observed in sewage sludge and aquifer sediment microcosms, and CFC-113 was dechlorinated to HCFC-123a and CTFE to TFE as end-products (Figure 46) (Balsiger et al., 2005). Although transformation of CFC-113 has been documented, (Deipser et al., 1997; Lesage et al., 1992; Lesage et al., 1990) no specific microorganisms have so far been identified associated with this activity, and it remains unclear whether the observed dehalogenation reactions were fortuitous (i.e., co-metabolic or indirect biological) or due to metabolic processes (e.g., organohalide respiration). In this study, similar reductive dechlorination patterns of CFC-113 were observed in the microcosm experiments with sediment samples collected from three geographically distinct locations. Despite repeated efforts, the activity could not be sustained in transfer cultures, thus limiting detailed studies of the microorganisms and processes involved in CFC-113 transformation.

Iron-bearing minerals have been widely studied in the context of halogenated compound degradation (Butler et al., 1999; Ferrey et al., 2004; Jeong et al., 2007; Lee et al., 2002a; Lee et al., 2002b; O'Loughlin et al., 2003), and the control experiments demonstrated that the mineral phases used in this study had activity toward TCE. However, none of these reactive mineral phases catalyzed the transformation of CFC-113, suggesting that abiotic degradation, at least with the iron-bearing minerals tested, does not affect the fate and longevity of CFC-113 in environmental systems. This is a relevant finding as it suggests that a limited number of processes contribute to CFC-113 degradation under *in situ* conditions.

An interesting observation was reductive dechlorination of CFC-113 followed by reductive

defluorination to form cDFE by the biomimetic reaction with reduced vitamin B₁₂. This reaction sequence was observed in *in vitro* incubations and in *in vivo* incubations with a corrinoid-producing, axenic bacterial culture, the homoacetogen *S. ovata*. These findings suggest that natural attenuation of CFC-113 can occur in environments with low redox potential where bacteria (e.g., homoacetogens) and possibly archaeae (e.g., methanogens) are abundant and produce corrinoids that can mediate the cleavage of carbon-chlorine and carbon-fluorine bonds. Such conditions can be achieved by the addition of fermentable substrates to increase hydrogen flux and stimulate reductive dechlorination activity, and biostimulation can potentially increase the abundance of corrinoid-producing bacteria and archaea, which can enhance co-metabolic transformation of CFC-113. The dechlorination rate of CFC-113 by vitamin B₁₂ was higher compared to the rates observed for chlorinated ethenes and CTFE (Im et al., 2019) suggesting that reduced cobamides may react preferentially with CFC-113. It should also be noted that the CFC-113 degradation intermediates, CTFE, TFE, and cDFE, did not inhibit TCE dechlorination by *Dhc*, indicating that the initial reductive transformation step can overcome cDCE stalls.

When designing a remediation strategy, an important consideration is the toxicity of transformation products. Lethal concentration 50 (LC₅₀) of CFC-113 transformation products, i.e., CTFE and TFE, are higher than that of the parent compound (Im et al., 2019) suggesting that the degradation process could potentially increase the combined toxicity at sites with CFC-113 as the major contaminant. However, since chlorinated ethenes are risk drivers at many contaminated sites, (Löffler et al., 2013a) the reductive transformation of CFC-113 to alleviate the inhibition of chlorinated solvent degradation could be a sound strategy to meet remediation goals.

In summary, this study demonstrated an inhibitory effect of CFC-113 on bacterial reductive dechlorination of chlorinated ethenes and 1,2-D, and performed a comprehensive investigation of potential attenuation mechanisms for CFC-113. CFC-113 can severely compromise *in situ* bioremediation of chlorinated ethenes and 1,2-D, suggesting that at sites where CFC-113 is a co-contaminant, conventional bioaugmentation and/or biostimulation strategies to treat chlorinated solvent plumes may not achieve cleanup goals. Removal of CFC-113 with biomimetic strategies utilizing reduced corrinoid, (Habeck et al., 1995; Sorel et al., 2001) or *in situ* stimulation of corrinoid-producing bacteria and archaea may be required before successful bioremediation of chlorinated ethenes can be implemented.

Inhibition of reductive dechlorination by N₂O

The development of a data mining approach for generating predictive understanding of ethene formation (i.e., detoxification) at sites impacted with chlorinated ethenes (Task 6) revealed that the presence of nitrate and/or nitrite were key determinants for detoxification. While nitrate and nitrite are competing electron acceptors and therefore may limit the electron flow towards reductive dechlorination, the strong correlation between nitrate/nitrite and stalled reductive dechlorination was unexpected. To shed light on this prediction of the machine learning-based data mining approach, a series of growth and kinetic experiments were conducted with axenic and mixed organohalide-respiring culture.

N₂O affects reductive dechlorination performance in *Geobacter lovleyi* strain SZ cultures

In the absence of N₂O, *Geo* strain SZ cultures completely dechlorinated 38.1 ± 3.1 μmol of PCE to stoichiometric amounts of cDCE over a 5-day incubation period, and an average PCE-to-cDCE dechlorination rate of 155.6 ± 27.2 $\mu\text{mol Cl}^- \text{L}^{-1} \text{d}^{-1}$ was measured (Figure 55A). Cultures amended with 9.5 μM or higher concentrations of N₂O exhibited decreased dechlorination rates and

incomplete PCE-to-cDCE dechlorination. In the presence of 9.5 μM N_2O , *Geo* strain SZ cultures dechlorinated the initial amount of PCE ($38.0 \pm 3.9 \mu\text{mol}$) at a dechlorination rate of $90.0 \pm 21.6 \mu\text{mol Cl}^- \text{L}^{-1} \text{d}^{-1}$, leading to an extended time period of at least 7 days to achieve complete consumption of PCE. Although PCE was completely consumed in cultures that received 9.5 μM N_2O , small amounts of TCE ($7.6 \pm 0.3 \mu\text{mol}$) remained, even after an extended incubation period of 180 days. With the addition of 19.1 μM N_2O , the average PCE dechlorination rate in *Geo* strain SZ cultures decreased to $64.2 \pm 4.6 \mu\text{mol Cl}^- \text{L}^{-1} \text{d}^{-1}$, and no more than $78 \pm 3.4\%$ of the initial amount of PCE ($38.5 \pm 1.1 \mu\text{mol}$) was dechlorinated. Following an extended incubation period of 180 days, PCE ($9.9 \pm 0.2 \mu\text{mol}$), TCE ($6.6 \pm 1.6 \mu\text{mol}$) and cDCE ($25.0 \pm 3.2 \mu\text{mol}$) were measured in strain SZ cultures that had received an initial dose of $38.5 \pm 1.1 \mu\text{mol}$ of PCE and 19.1 μM N_2O . A further decline in dechlorination rate to $12.0 \pm 2.1 \mu\text{mol Cl}^- \text{L}^{-1} \text{d}^{-1}$ was observed in cultures that received 57.3 μM N_2O , and $63.4 \pm 4.5\%$ of the initial amount of PCE ($38.3 \pm 3.7 \mu\text{mol}$) remained in culture bottles at the termination of the experiments (Figure 55A). In all culture bottles with observed inhibition of dechlorination activity, electron donor (i.e., 5 mM acetate) was not limiting electron acceptor reduction. Furthermore, consistent with the absence of N_2O reductase (*nos*) operons in the genome of *Geo* strain SZ, (Wagner et al., 2012) the amended N_2O remained constant throughout the experiment.

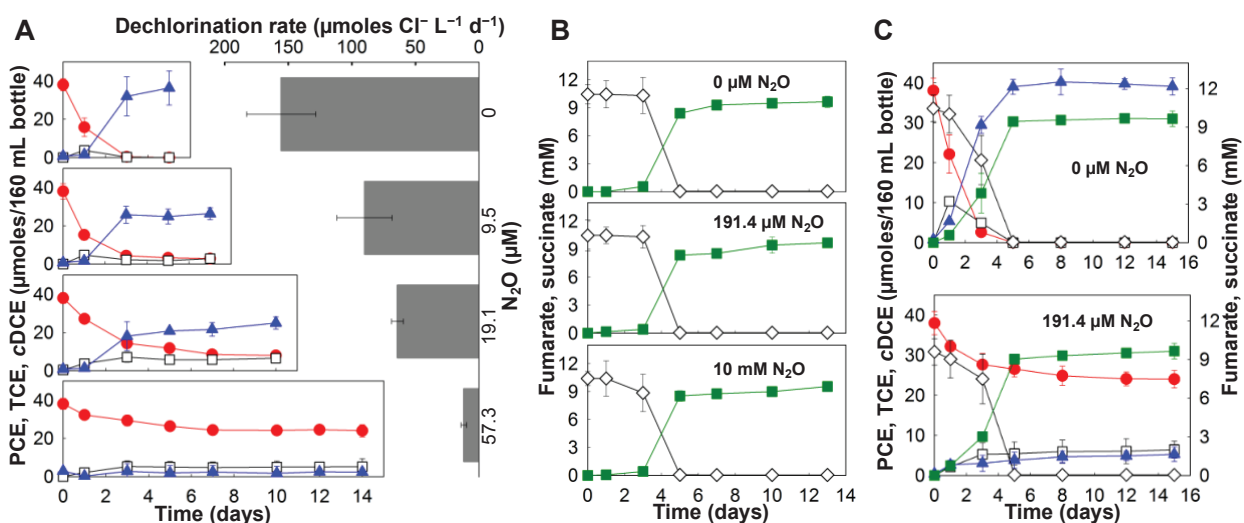


Figure 55. Effect of N_2O on the consumption of PCE in *Geobacter lovleyi* strain SZ cultures. Effect of N_2O on the consumption of PCE (A), fumarate (B), or both PCE and fumarate (C) as electron acceptors in cultures of the corrinoid-prototrophic bacterium *Geo* strain SZ. (A) PCE-to-cDCE reductive dechlorination rates and extents in *Geo* strain SZ cultures without N_2O (top panel) and in the presence of 9.5, 19.1 and 57.3 μM N_2O . (B) Fumarate-to-succinate reduction in *Geo* strain SZ cultures without N_2O and in the presence of 191.4 μM and 10 mM N_2O . (C) PCE-to-cDCE reductive dechlorination and fumarate-to-succinate reduction in *Geo* strain SZ cultures in the absence of N_2O (top panel) and in the presence of 191.4 μM N_2O (bottom panel). Solid red circles, PCE; open squares, TCE; solid blue triangles, cDCE; open diamonds, fumarate; and solid green squares, succinate. Error bars represent the standard deviations of triplicate cultures.

In addition to catalyzing PCE-to-cDCE dechlorination, *Geo* strain SZ also derives energy from acetate oxidation coupled to fumarate to succinate reduction (Sung et al., 2006a). In contrast to organohalide respiration catalyzed by corrinoid-dependent RDases, fumarate respiration via fumarate reductase does not involve a corrinoid-dependent enzyme system (Iverson et al., 1999). Therefore, investigating the impact of N₂O on fumarate reduction by *Geo* strain SZ cultures served as a control experiment to illustrate the selective effect of N₂O on the PCE RDase in *Geo* strain SZ. As shown in

Figure 55B, the presence of 191.4 μ M and 10 mM N₂O did not impact fumarate to succinate reduction rates and extents in *Geo* strain SZ cultures. In the absence of N₂O, *Geo* strain SZ reduced PCE and fumarate concomitantly (Figure 55C, top panel); however, in cultures amended with 191.4 μ M N₂O, only fumarate was reduced to succinate and no PCE reductive dechlorination occurred (Figure 55C, bottom panel).

N₂O affects cDCE and VC reductive dechlorination performance in *Dhc* strain BAV1 cultures

Without N₂O, *Dhc* strain BAV1 dechlorinated cDCE ($97.2 \pm 3.1 \mu\text{mol}$) to stoichiometric amounts of ethene ($102.3 \pm 6.1 \mu\text{mol}$) within a 14-day incubation period at an average cDCE-to-ethene dechlorination rate of $146.1 \pm 21.6 \mu\text{mol Cl}^- \text{L}^{-1} \text{d}^{-1}$ (Figure 56A). In contrast, cultures amended with 9.5 μ M or higher N₂O concentrations all exhibited incomplete transformation of cDCE and VC, and required longer incubation periods (up to 28 days) before stable dechlorination product patterns were observed. Cultures that received 9.5 μ M N₂O showed a significantly lower dechlorination rate of $66.8 \pm 21.6 \mu\text{mol Cl}^- \text{L}^{-1} \text{d}^{-1}$, and the initial amount of cDCE ($97.2 \pm 1.2 \mu\text{mol}$) was dechlorinated to a mixture of VC ($74.1 \pm 1.2 \mu\text{mol}$) and ethene ($23.1 \pm 0.7 \mu\text{mol}$). Cultures that received 29.0 μ M N₂O dechlorinated only about half ($50.7 \pm 3.3\%$) of the initial amount of cDCE ($96.3 \pm 1.1 \mu\text{mol}$) to predominantly VC ($48.8 \pm 3.2 \mu\text{mol}$) at a rate of $24.4 \pm 1.1 \mu\text{mol Cl}^- \text{L}^{-1} \text{d}^{-1}$ and only small amounts of ethene ($4.7 \pm 0.2\%$) were produced. At a higher N₂O concentration of 57.3 μ M, strain BAV1 dechlorinated cDCE to VC at a dechlorination rate of $18.9 \pm 0.7 \mu\text{mol Cl}^- \text{L}^{-1} \text{d}^{-1}$, no ethene was formed, and about one third ($26.3 \pm 0.4 \mu\text{mol}$) of the initial amount of cDCE remained. Extended incubation periods of up to 6 months did not result in further dechlorination in all tested strain BAV1 culture vessels with N₂O. Notably, in all N₂O-amended *Dhc* strain BAV1 cultures, the VC-to-ethene dechlorination step occurred at such low rates resulting in VC, rather than ethene formation as the major product.

To further investigate the impact of N₂O on the reductive dechlorination of VC, *Dhc* strain BAV1 cultures amended with VC as electron acceptor received N₂O at concentrations of 2.9, 5.7, and 19.1 μ M. Cultures without N₂O completely dechlorinated the initial amount of $41.2 \pm 0.4 \mu\text{mol}$ of VC to ethene within 11 days at an average VC dechlorination rate of $37.2 \pm 2.7 \mu\text{mol Cl}^- \text{L}^{-1} \text{d}^{-1}$ (Figure 56B). N₂O had a profound impact on VC dechlorination in *Dhc* strain BAV1 cultures, and the VC dechlorination rates decreased to 18.3 ± 2.1 and $9.8 \pm 0.4 \mu\text{mol Cl}^- \text{L}^{-1} \text{d}^{-1}$ in the presence of 2.9 and 5.7 μ M N₂O, respectively. Compared to the complete VC to ethene conversion in control incubations without N₂O, the amount of VC dechlorinated to ethene was diminished by $37.0 \pm 1.3\%$ and $76.2 \pm 4.1\%$, respectively, in cultures amended with 2.9 and 5.7 μ M N₂O. The most pronounced inhibition was observed in *Dhc* strain BAV1 cultures that received 19.1 μ M of N₂O (Figure 56B), and only negligible amounts ($< 1.6 \pm 0.6 \mu\text{mol}$) of ethene were formed even after extended incubation periods of 180 days, indicating that the VC to ethene step was particularly susceptible to N₂O inhibition.

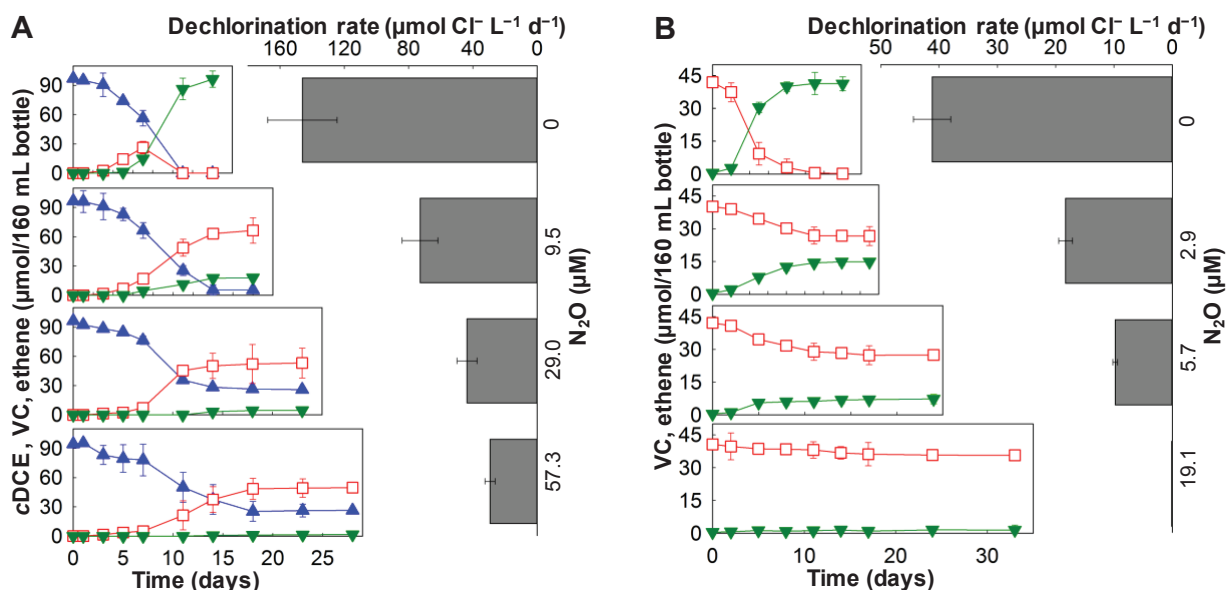


Figure 56. Effect of N_2O on reductive dechlorination of cDCE (A) and VC (B) in corrinoid auxotrophic *Dhc* strain BAV1. (A) cDCE reductive dechlorination rates and extents in *Dhc* strain BAV1 without N_2O (top panel) and in the presence of 9.5, 29.0 and 57.3 μM N_2O . (B) VC reductive dechlorination rates and extents in *Dhc* strain BAV1 without N_2O (top panel) and in the presence of 2.9, 5.7 and 19.1 μM N_2O . Solid blue triangles, cDCE; open red squares, VC; inverted, solid green triangles, ethene. Error bars represent the standard deviations of triplicate cultures.

Quantification of N_2O inhibition in whole cell suspension dechlorination assays

To further investigate the inhibitory effects of N_2O on reductive dechlorination, whole cell suspension assays were performed. Plots of dechlorination rates versus initial substrate concentrations with increasing N_2O concentrations are presented in Figure 57 and Figure 58. In all cases, the maximum dechlorination rates decreased with the addition of increasing N_2O concentrations. In *Geo* strain SZ cell suspensions without N_2O addition, a maximum PCE-to-cDCE dechlorination rate ($V_{max,PCE}$) of 76.3 ± 2.6 nmol Cl^- released min^{-1} mg protein $^{-1}$ was calculated using non-linear regression in the Michaelis-Menten model (Figure 57A and Table 33). With the addition of 10 and 60 μM N_2O , $V_{max,PCE}$ in *Geo* strain SZ cell suspensions declined to 61.3 ± 4.1 and 30.9 ± 4.2 nmol Cl^- released min^{-1} mg protein $^{-1}$, respectively. Even more pronounced inhibitory effects of N_2O were observed for cDCE and VC dechlorination in *Dhc* strain BAV1 assays. In the presence of 10 and 60 μM N_2O , the maximum cDCE-to-VC dechlorination rate decreased from a $V_{max,cDCE}$ of 119.4 ± 2.1 nmol Cl^- released min^{-1} mg protein $^{-1}$ in assays without N_2O to 81.1 ± 5.1 and 31.2 ± 2.4 nmol Cl^- released min^{-1} mg protein $^{-1}$, respectively (Figure 58A and Table 33). The strongest inhibitory effect of N_2O was observed in whole cell suspension assays of strain BAV1 with VC as electron acceptor. Compared to the maximum VC-to-ethene dechlorination rate $V_{max,VC}$ of 123.3 ± 2.2 nmol Cl^- released min^{-1} mg protein $^{-1}$ without N_2O , the addition of 15 and 50 μM N_2O reduced $V_{max,VC}$ to 78.9 ± 2.1 and 19.9 ± 1.3 nmol Cl^- released min^{-1} mg protein $^{-1}$, respectively (Figure 58C and Table 33). No dechlorination was detected in control incubations, confirming that suspended cells, rather than any abiotic reactions, were responsible for the observed dechlorination activity.

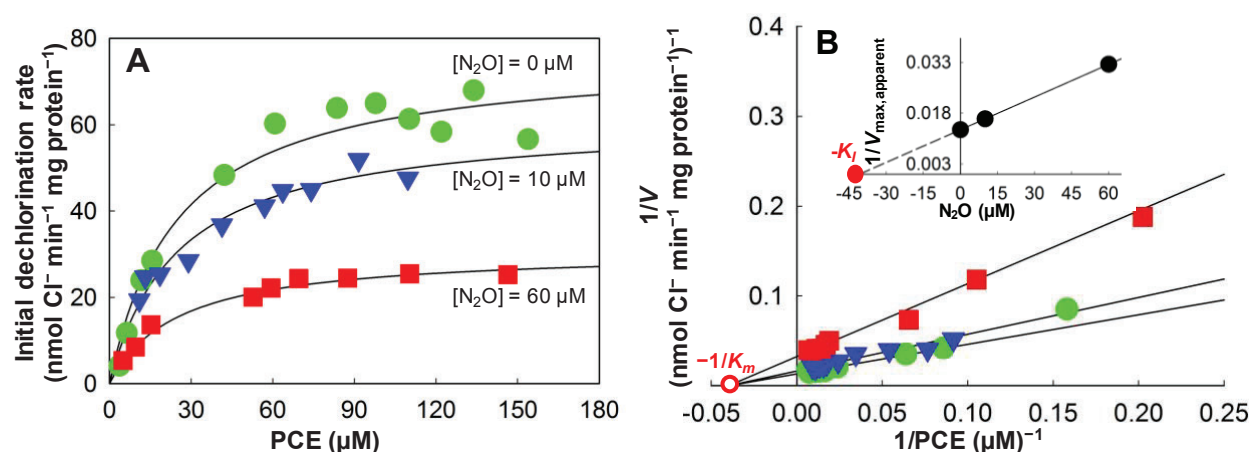


Figure 57. Kinetics of PCE-to-cDCE reductive dechlorination in cell suspensions of *Geo* strain SZ in the presence of increasing concentrations of N₂O.

Kinetics of PCE-to-cDCE reductive dechlorination in cell suspensions of *Geo* strain SZ in the presence of increasing concentrations of N₂O. (A) Michaelis-Menten plot of initial PCE-to-cDCE dechlorination rates versus PCE concentrations without and in the presence of 10 and 60 μM N₂O. (B) Lineweaver-Burk plot with inserted Dixon plot illustrating N₂O inhibition on PCE-to-cDCE reductive dechlorination. Solid lines represent the best fit to each data set based on nonlinear regression using the noncompetitive inhibition model. Solid green circles represent rate data measured in the absence of N₂O; solid blue triangles show rate data measured in the presence of 10 μM N₂O; and solid red squares show rate data measured in the presence of 60 μM N₂O. The solid and open red circles depict the graphical determination of -K_i and -1/K_m, respectively.

Kinetic parameters reveal pronounced N₂O inhibition

The experimental data generated in *Geo* strain SZ cell suspension assays fit the Michaelis-Menten model and the corresponding Lineweaver-Burk plot ($R^2 > 0.95$) (Figure 57). A maximum PCE dechlorination rate $V_{\max, \text{PCE}}$ of 76.3 ± 2.6 nmol Cl⁻ released min⁻¹ mg protein⁻¹ and a half-velocity constant $K_{m, \text{PCE}}$ of 25.1 ± 2.9 μM characterized PCE-to-cDCE dechlorination kinetics for strain SZ in the absence of N₂O (Figure 57B; Table 33). Without N₂O and in the presence of 10 and 60 μM N₂O, the PCE-to-cDCE dechlorination data fit the competitive, uncompetitive and noncompetitive inhibition models ($R^2 > 0.90$); however, with the noncompetitive inhibition model exhibited the highest R^2 and the lowest AICc and Sy.x. values (Figure 57 and Table A-46). Using the noncompetitive inhibition model, an inhibitory constant, K_i , of N₂O for PCE dechlorination in *Geo* strain SZ whole cell suspensions of 40.8 ± 3.8 μM was determined (inserted Dixon plot, Figure 57B; Table 33).

Table 33. Kinetic ($V_{\max}$, K_m) and inhibition (K_I) parameters for PCE, cDCE and VC reductive dechlorination in cell suspensions of *Geo* strain SZ and *Dhc* strain BAV1 in response to increasing N_2O concentrations.^a

e ⁻ Acceptor	Culture	N ₂ O (μ M)	$V_{\max}$ (nmol Cl ⁻ min ⁻¹ mg protein ⁻¹)	K_m (μ M)	K_I (μ M)
PCE	Strain SZ	0	76.3 ($\pm$ 2.6)	25.1 ($\pm$ 2.9)	40.8 ($\pm$ 3.8)
		10	61.3 ($\pm$ 4.1)		
		60	30.9 ($\pm$ 4.2)		
cDCE	Strain BAV1	0	119.4 ($\pm$ 4.1)	19.9 ($\pm$ 2.5)	21.2 ($\pm$ 3.5)
		10	81.1 ($\pm$ 5.1)		
		60	31.2 ($\pm$ 2.4)		
VC	Strain BAV1	0	123.3 ($\pm$ 3.2)	18.9 ($\pm$ 1.3)	9.6 ($\pm$ 0.4)
		15	78.9 ($\pm$ 2.1)		
		50	19.9 ($\pm$ 1.3)		

^a The best fit data were achieved with the Michaelis-Menten noncompetitive inhibition model. Error values represent 95% confidence intervals.

The experimental data generated in *Dhc* strain BAV1 whole cell suspension assays also fit the Michaelis-Menten model simulations and the corresponding Lineweaver-Burk plots ($R^2 > 0.90$) (Figure 58). In the presence of increasing N_2O concentrations, both cDCE-to-VC and VC-to-ethene reductive dechlorination assays showed the best fit to the noncompetitive inhibition model based on the highest R^2 and the lowest AICc and Sy.x. values (Figure 58 and Table A-46). While *Dhc* strain BAV1 assays produced comparable $V_{\max, \text{cDCE}}$ and $V_{\max, \text{VC}}$ values of 119.4 ± 2.1 and 123.3 ± 2.2 nmol Cl⁻ released min⁻¹ mg protein⁻¹, respectively, the K_I of N_2O for cDCE dechlorination (21.2 ± 3.5 μ M) was approximately 2-fold greater than for VC dechlorination (9.6 ± 0.4 μ M) (inserted Dixon plots, Figure 58B and Figure 58D; Table 33), consistent with the greater inhibitory effect of N_2O observed for the VC dechlorination step. To investigate if the differences in susceptibility of the cDCE and VC dechlorination steps to N_2O resulted from different affinities of the BvcA RDase for cDCE and VC, the K_m values for these two electron acceptors were compared. Based on the *Dhc* strain BAV1 assays, similar $K_{m, \text{cDCE}}$ (19.9 ± 2.5 μ M) and $K_{m, \text{VC}}$ (18.9 ± 1.3 μ M) values were determined (Figure 58B and Figure 58D; Table 33), indicating that the substrate affinity of the BvcA RDase does not explain the more potent inhibition of the VC-to-ethene dechlorination step by N_2O .

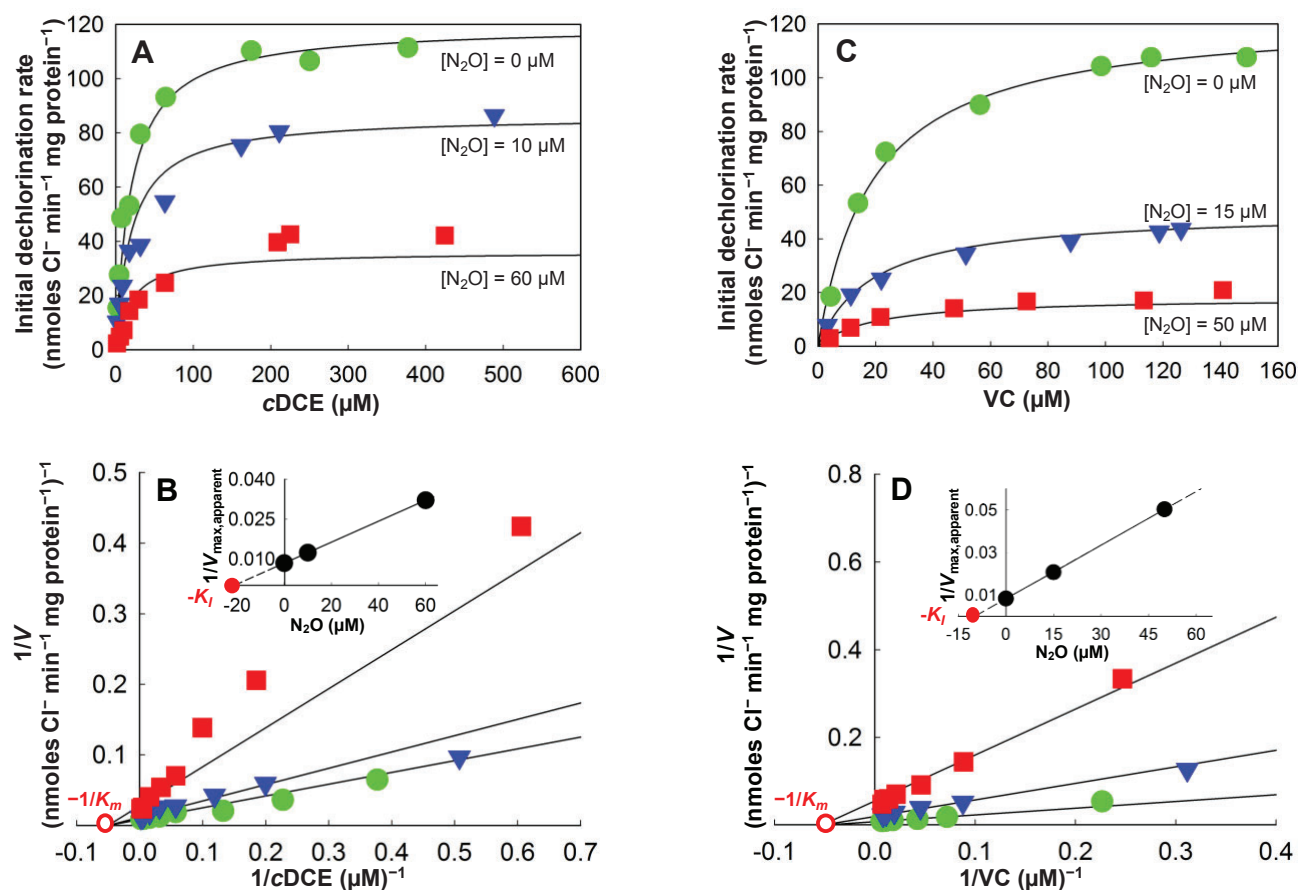


Figure 58. Kinetics of cDCE-to-VC and VC-to-ethene reductive dechlorination in cell suspensions of *Dhc* strain BAV1 in the presence of increasing concentrations of N_2O . (A) Michaelis-Menten plot of initial cDCE-to-VC dechlorination in cell suspension of *Dhc* strain BAV1 without and in the presence of 10 and 60 μ M N_2O . (B) Lineweaver-Burk plot with inserted Dixon plot illustrating N_2O inhibition of cDCE-to-VC reductive dechlorination. (C) Michaelis-Menten plot of initial VC-to-ethene dechlorination in cell suspension of *Dhc* strain BAV1 without and in the presence of 15 and 50 μ M N_2O . (D) Lineweaver-Burk plot with inserted Dixon plot illustrating N_2O inhibition of VC-to-ethene reductive dechlorination. Solid lines represent the best fit to each data set based on nonlinear regression using the noncompetitive inhibition model. Solid green circles represent rate data measured in the absence of N_2O ; solid blue triangles and solid red squares show dechlorination rate data measured in the presence of N_2O (panels A and B, cDCE; panels C and D, VC). The solid and open red circles shown in panels B and D depict the graphical determination of $-K_i$ and $-1/K_m$, respectively.

Discussion - Inhibition of reductive dechlorination by N₂O

Effects of N₂O on corrinoid-dependent processes

Toxic effects of N₂O were first observed in the mid 1950s after two patients died following prolonged N₂O inhalation (Lassen et al., 1956). This incident triggered studies to elucidate the mechanism underlying N₂O toxicity, and detailed investigations revealed that N₂O reacts with cobalt-containing corrinoids (e.g., cobalamin) when the coordinated cobalt atom is in the reduced Co(I) state (Banks et al., 1968; Blackburn et al., 1977). Due to the vital roles of enzyme systems with corrinoid prosthetic groups, (Matthews, 2009; Yan et al., 2018) the N₂O-mediated oxidation of the Co(I) supernucleophile interferes with key metabolic functions in all domains of life (Drummond et al., 1994a; Drummond et al., 1994b). For example, N₂O inhibits the corrinoid-dependent methionine synthase (MetH) required for the biosynthesis of the essential amino acid methionine (Royston et al., 1988). When used as inhalation anesthetic for human patients, N₂O can lead to a malfunction of MetH, resulting in elevated levels of homocysteine in plasma (i.e., hyperhomocysteinemia) (Singer et al., 2008). Patients generally recover from N₂O exposure after 3 – 4 days, (Royston et al., 1988) presumably through de novo MetH synthesis or replenishment of Co(I) corrinoid (Weimann, 2003).

The lowest reported N₂O concentration that affected MetH activity in animals and humans was around 4.1 mM (~1,000 ppm); (Weimann, 2003) however, much lower N₂O concentrations inhibited members of the Bacteria and Archaea, presumably due to the more diverse roles of corrinoid-dependent enzyme systems in their metabolisms (Matthews, 2009). For example, in the denitrifying bacterium *Paracoccus denitrificans* strain PD1222, 0.1 mM N₂O not only repressed MetH but also modulated the expression of anabolic genes under the control of vitamin B₁₂ riboswitches (Sullivan et al., 2013). To compensate for the loss of MetH function, organisms such as *P. denitrificans* and *Escherichia coli* activate a corrinoid-independent methionine synthase, MetE (Matthews, 2009; Sullivan et al., 2013). *Geo* strain SZ possesses both the *metE* and *metH* genes, (Wagner et al., 2012) what may explain the observation that up to 10 mM N₂O did not inhibit the bacterium's growth with fumarate in defined minimal medium. In contrast, N₂O at micromolar concentrations inhibited the growth of *Geo* strain SZ cultures when PCE served as the sole electron acceptor. These observations corroborate that the inhibitory effect of N₂O to organisms varies markedly based on whether corrinoid-dependent enzyme systems are essential for key metabolic steps, including respiratory energy conservation.

OHRB as a model to study the effects of N₂O inhibition

A key feature of OHRB is the involvement of corrinoid-dependent RDases in electron transfer to the chlorinated organohalogen electron acceptor (Adrian et al., 2016; Bommer et al., 2014; Payne et al., 2015; Wang et al., 2018). Functional and structural analyses demonstrated that RDases represent a distinct subfamily of corrinoid-dependent enzymes and a Co(I) supernucleophile is crucial for RDases to initiate the cleavage of carbon-chlorine bonds (Bommer et al., 2014; Fincker et al., 2017; Payne et al., 2015). Since corrinoid-dependent RDases are essential in the energy metabolism of OHRB, N₂O inhibition on Co(I) corrinoid-dependent RDases can be readily observed by quantitative measurement of dechlorination activity and growth when alternate electron acceptors are absent, or the energy metabolism of the OHRB is restricted to chlorinated electron acceptors, as is the case for *Dhc* (Löffler et al., 2013b). This effect was convincingly demonstrated in both *Geo* strain SZ and *Dhc* strain BAV1 cultures amended with chlorinated electron acceptors and N₂O. Thus, OHRB are excellent model organisms to assess the inhibitory effects of N₂O on enzymes involving the Co(I) supernucleophile in catalysis. Consistent with this

assumption, experimental results showed that N₂O at micromolar concentrations exhibited strong inhibitory effects on reductive dechlorination performance in both *Geo* strain SZ and *Dhc* strain BAV1 cultures. Theoretically, a shortage of methionine caused by N₂O inhibition on MetH could also have affected dechlorination activity; however, growth of *Geo* strain SZ with fumarate was not impaired at much higher N₂O concentrations of up to 10 mM, indicating that N₂O inhibition of RDases diminished dechlorination performance.

The PCE-to-*c*DCE dechlorinating *Geo* strain SZ tolerated at least 3-fold higher N₂O concentrations than *Dhc* strain BAV1 before cessation of dechlorination activity was observed, which possibly relates to strain SZ's ability for *de novo* cobamide biosynthesis (Wagner et al., 2012). Active cobamide biosynthesis may allow strain SZ to maintain some level of catalytically active PCE RDase. In contrast, the characterized obligate organohalide-respiring *Dhc* strains (Löffler et al., 2013b; Seshadri et al., 2005) and *Dehalogenimonas* spp., including the VC-respiring *Candidatus* Dehalogenimonas etheniformans, (Yang et al., 2017c) cannot *de novo* synthesize corrinoid, although exceptions may exist (Brisson et al., 2012). All characterized *c*DCE and VC dechlorinators strictly require exogenous corrinoid, which renders these bacteria more susceptible to N₂O inhibition, and low micromolar concentrations of N₂O (i.e., 3-10 µM) repressed the growth of the corrinoid-auxotroph *Dhc* strain BAV1. Unlike corrinoid-auxotrophic Dehalococcoidia, the majority of PCE-to-TCE and TCE-to-*c*DCE-dechlorinating OHRB are corrinoid prototrophs (Schubert et al., 2018) what may enable these organisms to tolerate higher N₂O concentrations. The difference in the ability for *de novo* corrinoid biosynthesis is one possible explanation why PCE and TCE dechlorination to *c*DCE is generally achieved at sites with nitrate, (Lee et al., 2016; Verce et al., 2015) but ethene is not produced (Amaral et al., 2011; Dugat-Bony et al., 2012; Schmidt et al., 2008; Tillotson et al., 2017).

Corrinoid-dependent enzyme systems fulfill essential metabolic functions for organisms in all branches of life, but only a subset of the bacteria and archaea have the machinery for *de novo* corrinoid biosynthesis (Matthews, 2009; Shelton et al., 2019; Zhang et al., 2009). Therefore, N₂O effects on microbial processes that hinge on the activity of corrinoid-dependent enzyme systems may expand beyond organohalide respiration.

Elevated groundwater N₂O and kinetic parameters

Based the current day atmospheric N₂O concentration of 330 ppb, the theoretical equilibrium concentration of N₂O in groundwater should be around 7 nM, assuming no mass transfer limitations; however, much higher groundwater N₂O concentrations were reported indicating other sources exist (Jurado et al., 2017; Reay et al., 2012). For example, the increased usage of synthetic nitrogen fertilizer for agricultural production causes substantial nitrate run-off and elevated N₂O concentrations in groundwater (Jurado et al., 2017; Thomson et al., 2012). Indeed, correlations between fertilizer application and associated nitrate run-off with elevated groundwater N₂O concentrations have been established (Reay et al., 2012; Thomson et al., 2012). Nitrate is not conservative and processes including denitrification, (Thomson et al., 2012; Zumft, 1997) respiratory ammonification (i.e., dissimilatory nitrate reduction to ammonium, DNRA) (Yoon et al., 2015) and ensuing nitrification, (Daims et al., 2016) as well as chemodenitrification (Onley et al., 2018) contribute to the formation of N₂O. Such biogeochemical processes are likely responsible for elevated N₂O concentrations and up to 140 µM N₂O were observed in watersheds impacted by agricultural activities (Jurado et al., 2017). Thus, the N₂O concentrations measured in many groundwater aquifers exceed the theoretical equilibrium with atmospheric N₂O by up to 5 orders in magnitude, and intensified agriculture will exacerbate this issue.

A K_I value indicates the inhibitor (i.e., N_2O) concentration, at which the maximum reaction rate V_{max} (i.e., the reductive dechlorination rates of chlorinated ethenes) is reduced by 50%. The determined K_I values for N_2O on reductive dechlorination of PCE, cDCE and VC are in the range of 40, 20 and 10 μM , respectively, well below reported N_2O concentrations encountered in many groundwater aquifers, (Jurado et al., 2017) particularly at sites impacted by agricultural run-off (Jurado et al., 2017). Consequently, N_2O inhibition could be a major cause for incomplete reductive dechlorination and cDCE and VC stalls observed at field sites (Amaral et al., 2011; Dugat-Bony et al., 2012; Schmidt et al., 2008; Tillotson et al., 2017; Verce et al., 2015). Of note, a 50% $V_{max,cDCE}$ and $V_{max,VC}$ inhibition occurred in strain BAV1 at 2- and 4-fold lower N_2O concentrations, respectively, compared to the same level of inhibition of $V_{max,PCE}$ in strain SZ. The higher K_I values for N_2O determined for *Geo* strain SZ compared to strain BAV1 may be related to the ability of strain SZ for *de novo* corrinoid biosynthesis (see above), or to mechanistic differences in the dechlorination steps catalyzed by the PceA versus the BvcA RDases. Similar K_m values for cDCE and VC were determined in strain BAV1 indicating the organism exhibits similar affinities for cDCE and VC; however, the 2-fold higher K_I value for the cDCE versus the VC dechlorination step cannot be easily explained considering the same BvcA RDase is involved in both dechlorination steps, (Schubert et al., 2018; Tang et al., 2013a) and detailed mechanistic studies are warranted.

Predicting the fate of chlorinated ethenes at bioremediation sites relies on accurate estimates of the intrinsic kinetic parameters of OHRB; (Huang et al., 2009) however, kinetic constants determinations using various dechlorinating cultures at different cell densities reported highly variable V_{max} and K_m values (or K_s values when Monod kinetics were applied) (Baelum et al., 2014; Berggren et al., 2013; Mayer-Blackwell et al., 2016). Likely explanations for these discrepancies are that different, potentially competing types of dechlorinators with distinct RDases and present in varied abundances contributed to cDCE reductive dechlorination (Berggren et al., 2013; Buttet et al., 2018; Chan et al., 2011). The current study accomplished kinetic measurements in axenic cultures under defined conditions and over short incubation periods (< 6h, no growth occurred), which facilitates the determination of intrinsic kinetic parameters (Huang et al., 2009). The Michaelis-Menten model simulations predicted the behaviors of *Geo* strain SZ and *Dhc* strain BAV1 ($R^2 > 0.90$) and both organisms fit the noncompetitive inhibition model ($R^2 > 0.96$) with micromolar levels of N_2O as the inhibitor. These findings imply N_2O as a noncompetitive inhibitor that oxidizes the Co(I) corrinoid cofactor of RDases, thereby decreasing reductive dechlorination rates.

Implications for *in situ* bioremediation

Electron donor (i.e., hydrogen) limitations (Fennell et al., 1997), nutrient availability (e.g., fixed nitrogen) (Lendvay et al., 2003), unfavorable redox potential (Nelson et al., 2002; Schmidt et al., 2008), low pH conditions (Yang et al., 2017b), or toxic and/or inhibitory effects of co-contaminants (e.g., sulfide, chloroform, 1,1,1-trichloroethane) (Berggren et al., 2013; Chan et al., 2011) can impact the microbial reductive dechlorination process. The findings of the current study indicate that decreased reductive dechlorination performance can be the result of N_2O inhibition. A common strategy to improve *in situ* degradation of chlorinated ethenes involves the injection of nutrients (i.e., biostimulation), typically fermentable substrates aimed at increasing the flux of hydrogen (Löffler et al., 2006; McCarty, 2010). The formulations can include fertilizer (nitrate, ammonium, urea, phosphorus) to proactively address possible nutrient limitations (Lendvay et al., 2003; Löffler et al., 2006; Schaefer et al., 2010; Steffan et al., 2016). Biogeochemical transformations of fixed nitrogen will generate N_2O (Daims et al., 2016; Onley et al., 2018; Yoon

et al., 2015), and exert inhibitory effects on microbial reductive dechlorination, which can result in undesirable *c*DCE or VC stalls. Thus, practitioners should carefully evaluate the need for fixed nitrogen additions to avoid possible N₂O inhibition.

The inhibitory constants, K_i , for N₂O inhibition of PCE, *c*DCE and VC dechlorination were within the N₂O concentration ranges observed in groundwater aquifers (i.e., up to 143 μ M) (Jurado et al., 2017), suggesting that N₂O should be of concern at contaminated sites where practitioners seek to rely on microbial reductive dechlorination as a remedial strategy. Considering the relevance of the microbial reductive dechlorination process for achieving cleanup goals and the commonality of elevated N₂O concentrations in aquifers, groundwater monitoring regimes should include nitrate/nitrite and N₂O measurements, so that potential inhibition and *c*DCE and VC stalls can be explained and predicted.

Impact of fixed nitrogen (NH₄⁺) availability on *Dehalococcoides mccartyi* reductive dechlorination activity

Biostimulation with fermentable organic substrates is standard treatment to increase the *in situ* reductive dechlorination activity. This approach increases the flux of hydrogen, the key electron donor for *Dhc*, and generates acetate *Dhc* can use as a source of carbon. Another key macronutrient required for microbial growth and activity is nitrogen; however, the value of biostimulation with fixed nitrogen (e.g., ammonium) is uncertain. Laboratory and field-scale experiments were performed to explore the effects of nitrogen limitations and biostimulation with fixed nitrogen on *Dhc* growth and reductive dechlorination activity.

Effects of NH₄⁺ availability and *Dhc* dechlorination activity and growth

Cultures capable of reductive dechlorination of chlorinated ethenes to ethene were obtained from microcosms established with Third Creek (TC) sediment. In addition, a PCE-to-ethene-dechlorinating PW4 enrichment culture harboring *Dhc* was established with groundwater collected from site in Australia impacted with chlorinated ethenes. Growth experiments with culture PW4 demonstrated cDCE-to-ethene dechlorination regardless of NH₄⁺ amendment. A 5-fold increase in dechlorination rates (i.e., 3.82 ± 0.48 vs 20.62 ± 1.58 $\mu\text{M Cl}^- \text{d}^{-1}$) was observed when NH₄⁺ was added (Figure 59A,B). Accordingly, the time required for complete cDCE to ethene dechlorination was substantially longer in incubations without NH₄⁺ (i.e., 29.0 ± 2.4 vs 143 ± 3.5 days). *Dhc* 16S rRNA gene copy numbers (i.e., *Dhc* cell numbers) increased 200-fold from $(1.7 \pm 0.5) \times 10^6$ (cells introduced with the inoculum) to $(1.8 \pm 0.9) \times 10^8/\text{mL}$ after 29 days of incubations with NH₄⁺, whereas the *Dhc* cell numbers increased less than 25-fold to $(4.1 \pm 0.8) \times 10^7/\text{mL}$ after 143 days without NH₄⁺ amendment. Complete cDCE to ethene dechlorination also occurred in PCE-to-ethene-dechlorinating TC cultures regardless of NH₄⁺ amendment (Figure 59D,E), but without NH₄⁺, a 2-fold reduction in dechlorination rates extended the time required to achieve complete dechlorination (i.e., 88 ± 5 vs 160 ± 7 days). In incubations with NH₄⁺, the abundance of *Dhc* 16S rRNA genes increased from $(1.93 \pm 0.15) \times 10^6$ (cells introduced with the inoculum) to $(9.6 \pm 0.8) \times 10^7$ over an 88-day incubation period required to achieve complete cDCE dechlorination to ethene. In incubations without NH₄⁺, *Dhc* cell numbers of $(2.0 \pm 0.2) \times 10^7 \text{ mL}^{-1}$ were measured after 160 days when dechlorination to ethene was complete. In PW4 and TC incubation vessels without NH₄⁺ and argon headspace (no N₂ present), no more than 17% of the initial amount of cDCE was reduced to VC and no ethene was produced. The observed dechlorination was attributed to carry over of fixed N or possible intrusion of N₂ gas through the stopper. Slight increases in *Dhc* cell numbers were observed, but titers declined at the end of the incubation period (Figure 59C-F). Complete cDCE-to-ethene dechlorination occurred in pure cultures of *Dhc* strain BAV1 or strain GT in approximately 40 days in the medium supplemented with NH₄⁺. In contrast, no growth or reductive dechlorination occurred without NH₄⁺ consistent with the absence of *nifD* and *nifK* (i.e., incomplete *nif* operons) in *Dhc* strains affiliated with the Pinellas group (Kube et al., 2005).

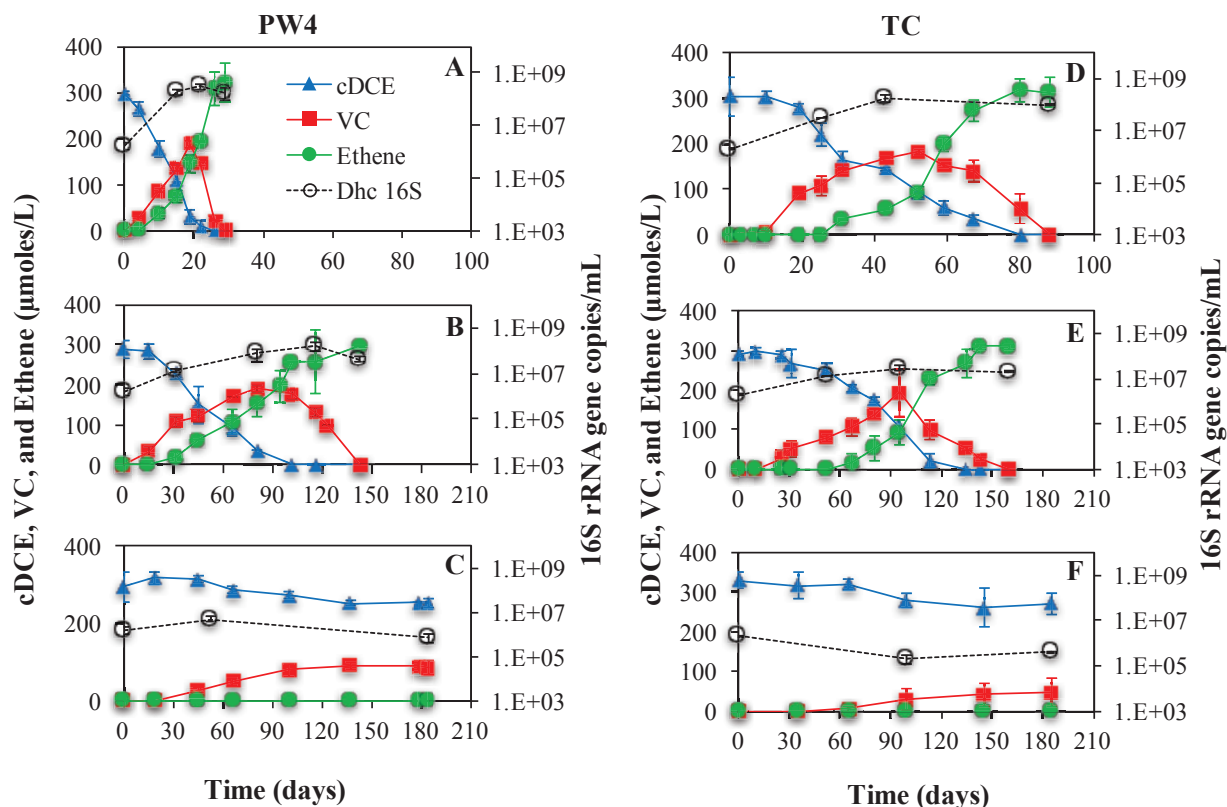


Figure 59. cDCE dechlorination and *Dhc* cell growth in enrichment cultures PW4 and TC. (A) PW4 cultures with NH_4^+ , (B) PW4 cultures without NH_4^+ , and (C) PW4 cultures with argon headspace (without NH_4^+ and N_2). (D) TC cultures with NH_4^+ , (E) TC cultures without NH_4^+ , and (F) TC cultures with argon headspace (without NH_4^+ and N_2). The data represent the average of triplicate measurements each obtained from three independent experiments. Error bars represent standard deviations (not shown if they are smaller than the symbols).

Effect of NH_4^+ availability on biomarker gene abundances

The *Dhc* cells detected in groundwater from well PW4 belonged to the Cornell group (Figure 60A, 1st column), and *Dhc* of the Pinellas/Victoria groups were either present below the qPCR detection limit of 10^2 16S rRNA gene copies/L or absent. In PW4 cultures grown with 0.3 mM cDCE without NH_4^+ , the final number of total *Dhc* cells of $4.1 \pm 0.8E+07$ equaled the number of Cornell-type *Dhc* (Figure 60A, 3rd column). In the presence of NH_4^+ , PW4 cultures produced $1.8 \pm 0.9E+08$ *Dhc* cells following cDCE dechlorination to ethene but only about 50% ($9.8 \pm 5.3E+07$ copies/mL) were Cornell-type *Dhc*. Apparently non-Cornell-type *Dhc* strains, presumably lacking the ability to fix N_2 , responded to the supplementation with NH_4^+ . In the TC cultures, *Dhc* Cornell 16S rRNA genes were not detected (Figure 60B).

The RDase genes *tceA* and *vcrA* were detected in PW4 cultures regardless of NH_4^+ amendment. In PW4 incubations with NH_4^+ , *tceA* and *vcrA* gene copy numbers had increased about 95- and 77-fold, respectively (Figure 60A, 2nd column), when cDCE had been completely dechlorinated to ethene, while without NH_4^+ , lower increases of 37-fold (*tceA*) and 28-fold (*vcrA*) were observed (Figure 60A, 3rd column). The total abundances of *vcrA* genes ($8.3 \pm 0.1E+07$ and $3.1 \pm 0.1E+07$ /mL with and without NH_4^+ , respectively) was within 76-78% of the total Cornell-type *Dhc* cell

numbers ($1.1 \pm 0.5 \times 10^8$ and $4.1 \pm 0.3 \times 10^7$ /mL with and without NH_4^+ , respectively) indicating that at least one Cornell strain harbors *vcrA*. In TC cultures with NH_4^+ , *vcrA* and *tceA* gene copies increased 60- and 50-fold, respectively (Figure 60B, 2nd column), whereas substantially lower increases were observed in cultures without NH_4^+ (Figure 60B, 3rd column). The *bvcA* RDase gene was only present in TC cultures and a 50-fold increase to $4.86 \pm 0.11 \times 10^6$ *bvcA* genes/mL was observed in incubations with NH_4^+ (Figure 60B, 2nd column). In contrast, the increase was only 3-fold in incubations without NH_4^+ (Figure 60B, 3rd column), indicating that NH_4^+ limitation impacted *Dhc* strains carrying *bvcA* more than other *Dhc* strains.

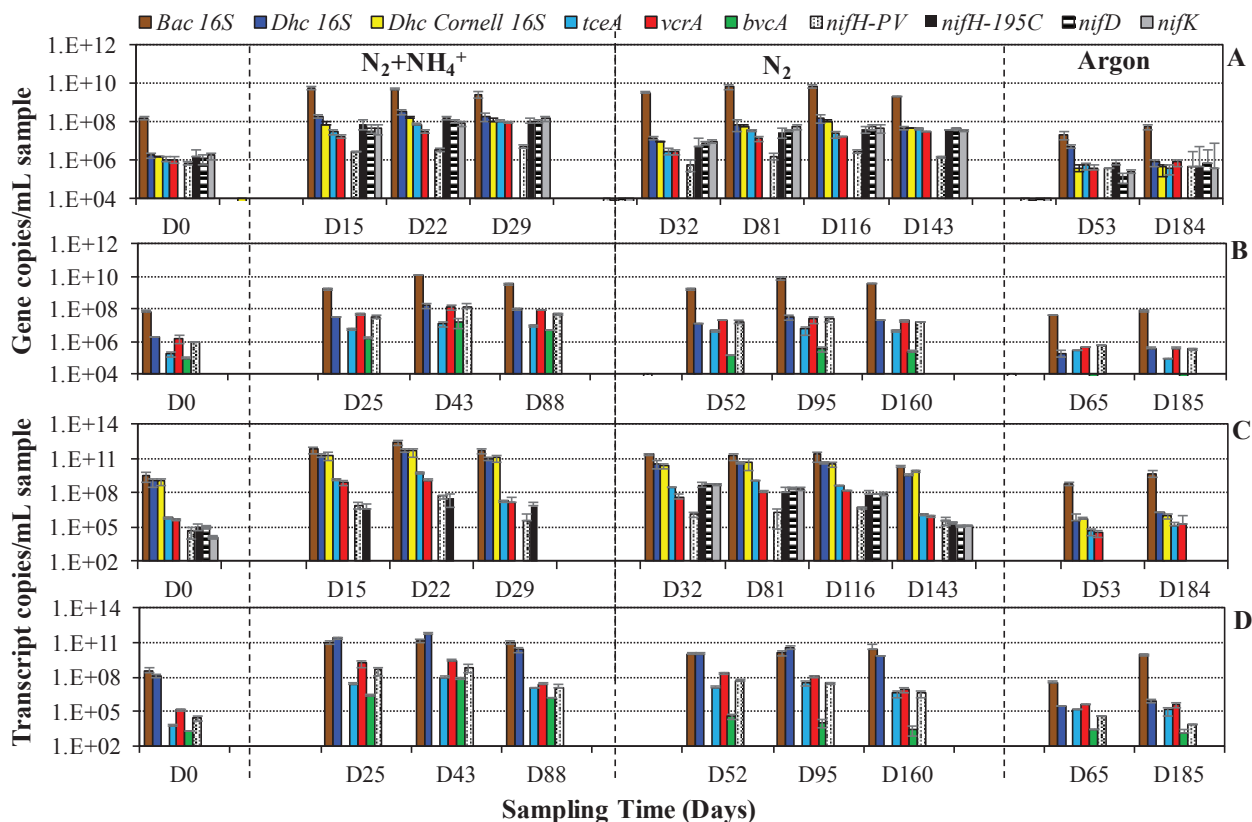


Figure 60. Gene and transcript abundance changes in PW4 and TC enrichment cultures with and without NH_4^+ over the course of cDCE dechlorination.

Panels A and C show PW4 cultures and panels B and D show TC cultures. 1st column: day 0, 2nd column: incubations with NH_4^+ , 3rd column: incubations without NH_4^+ , 4th column: incubations with argon headspace (without NH_4^+ and N_2). The data represent the average of triplicate measurements each obtained from three independent experiments. Error bars represent standard deviations (not shown if they are smaller than the symbols).

The Cornell-type *nif* genes *nifD*, *nifK*, and *nifH* (*nifH-195C*) were solely detected in PW4 cultures, whereas the Pinellas/Victoria-type *Dhc nifH* gene (*nifH-PV*) was present both in PW4 and TC cultures regardless of NH_4^+ amendments (Figure 60A-B). When PW4 cultures were incubated with NH_4^+ , the copy number of Cornell-type *nifD*, *nifK*, and *nifH-195C*, and Pinellas/Victoria-type *nifH-PV* genes increased by 87-, 81-, 70-, and 8-fold, respectively, with copies ranging between $5.19 \pm 0.87 \times 10^6$ and $1.34 \pm 0.36 \times 10^8$ /mL. In contrast, in incubations without NH_4^+ , the overall

increase of those genes was about 34-, 22-, 22-, and 2-fold, respectively, with copies ranging between $1.37 \pm 0.13 \times 10^6$ and $4.09 \pm 0.44 \times 10^7$ /mL. The copy numbers of *nifH-PV* genes in PW4 cultures were 10-15-fold lower than that of Cornell-type *nif* genes indicating the dominance of Cornell-type *Dhc* cells over Pinellas/Victoria-type *Dhc* cells, which possibly reflects the relative abundance of these *Dhc* types in fixed N-limited PW4 groundwater.

The detection *vcrA*, *tceA* and *bvcA* in both PW4 and TC cultures suggests the involvement of multiple *Dhc* strains in reductive dechlorination, because these genes have not been found on a single *Dhc* genome. In contrast, the *tceA* gene has been found on Cornell group genomes (i.e., strain ANAS1 and strain 195) and on Pinellas group genomes (i.e., strain KB1/VC and strain FL2). Only the *vcrA* RDase gene has been detected in genomes of *Dhc* strains belonging to all three groups (i.e., Cornell, Pinellas, and Victoria), whereas *bvcA* appears restricted to Pinellas group genomes (Krajmalnik-Brown et al., 2004; Saiyari et al., 2018).

Consistent with the absence of Cornell-type *Dhc* 16S rRNA genes in TC cultures (Figure 60B), Cornell-type *Dhc* *nifD* and *nifK* genes were not detected. In TC cultures, the numbers of *nifH-PV* genes increased to from $1.01 \pm 0.18 \times 10^6$ to $5.03 \pm 0.44 \times 10^7$ and to $1.64 \pm 0.44 \times 10^7$ copies/mL in the presence and in the absence of NH_4^+ , respectively. Overall, similar increasing trends were observed in the total *Dhc* 16S rRNA and the RDase gene abundances in PW4 and TC cultures regardless of NH_4^+ amendment during cDCE-to-ethene dechlorination.

Effects of NH_4^+ availability on biomarker gene expression

In PW4 cultures, the *tceA* gene was the most highly transcribed RDase. The expression level increased to $5.26 \pm 0.4 \times 10^9$ transcripts/mL at day 22 with NH_4^+ amendment, before declining to $1.74 \pm 0.27 \times 10^7$ transcripts/mL at day 29, when all chlorinated ethenes had been dechlorinated to ethene (Figure 61C, 2nd column). Transcript-to-gene ratios (TGRs) for *tceA* were 0.6 at day 0 (0% cDCE consumed), 50 at day 15 (about 75% of cDCE consumed), 75 at day 22 (about 90% of cDCE consumed), and 0.2 at day 29 (all chlorinated ethenes dechlorinated to ethene) (Figure 61A). A similar trend was observed in PW4 cultures without NH_4^+ with TGRs for *tceA* ranging between 0.03 and 114 over the 29-day incubation period (with lower TGRs observed when complete dechlorination had occurred, and higher TGRs observed during cDCE dechlorination). *vcrA* transcripts followed a similar decreasing trend as dechlorination progressed with TGRs ranging between 0.4 and 41 in cultures with NH_4^+ and between 0.02 and 17 in cultures without NH_4^+ (Figure 61A). In TC cultures, the *vcrA* gene was the most highly transcribed RDase (Figure 61D, 3rd column) with TGRs ranging between 0.3 and 34 (with NH_4^+) and 0.4 and 10 (without NH_4^+). TGRs were lower when the chlorinated electron acceptors had been consumed (i.e., dechlorination to ethene was complete) and higher TGRs were observed during active dechlorination (Figure 61B). Similar trends were observed for the expression patterns of the *tceA* and *bvcA* RDase genes.

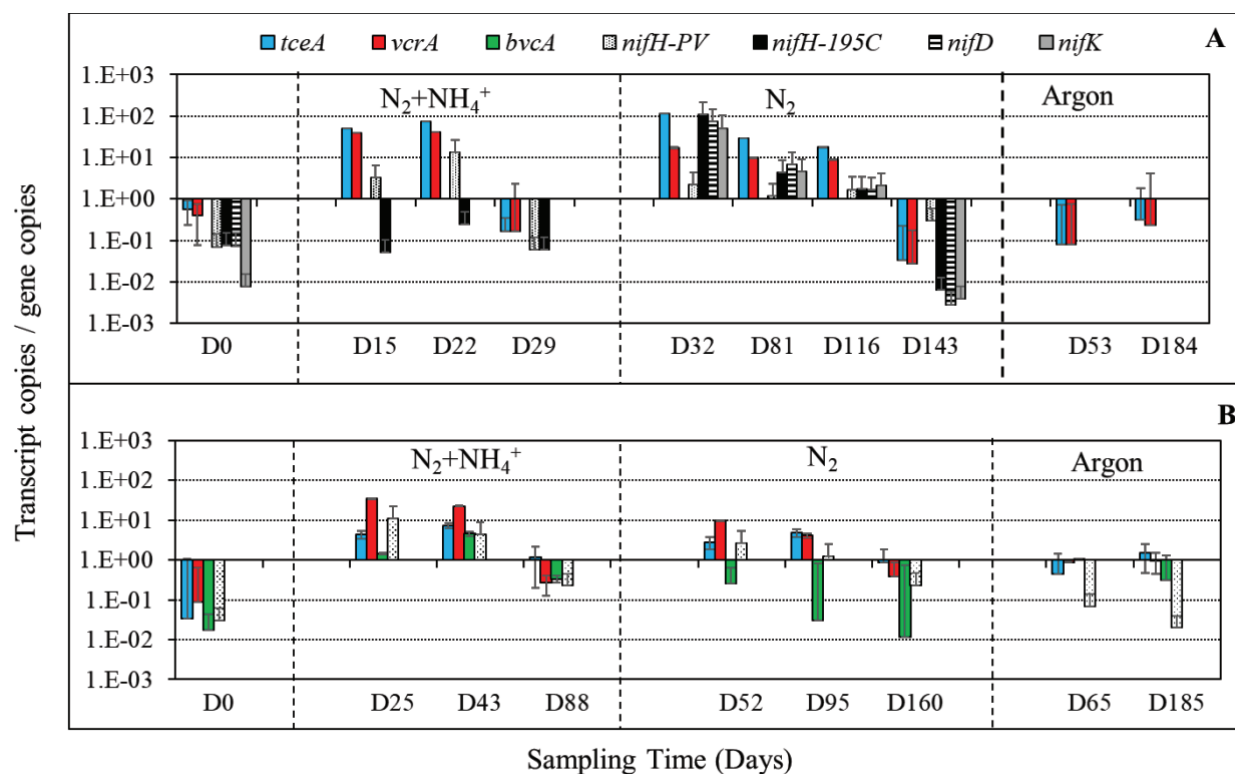


Figure 61. Changes in the expression ratios of *Dhc* RDase genes, the Cornell-type *Dhc nif* genes, and the *Dhc* Pinellas-type *nif* genes in PW4 and TC cultures.

Changes in the expression ratios (i.e., transcript abundances normalized to the respective gene copies) during cDCE-to-ethene dechlorination of the *Dhc* RDase genes *vcrA*, *iceA*, and *bvcA*, the Cornell-type *Dhc nif* genes *nifK*, *nifD*, and *nifH-195C*, and the *Dhc* Pinellas-type *nif* gene *nifH-PV* in (A) PW4 and (B) TC culture incubations: 1st column: day 0 (D0), 2nd column: with NH_4^+ , 3rd column: without NH_4^+ , and 4th column: without N (i.e., no NH_4^+ and N_2 since argon replaced N_2 in the headspace). (D: Days).

The Cornell-type *nifD* and *nifK* transcripts were only detected in PW4 cultures without NH_4^+ (Figure 61C, 3rd column), highlighting the role of these genes in N_2 fixation. As dechlorination proceeded, similar to the RDase genes, the Cornell-type *nif* genes in PW4 cultures without NH_4^+ followed a decreasing trend with TGRs ranging between 51-108 with 20% of cDCE consumed, declining to 4-7 with 90% of cDCE consumed, and declining further to 0.003-0.006 when complete dechlorination to ethene had been achieved (Figure 61A). In TC cultures, transcripts of the *nifH-PV* gene also declined with TGRs ranging between 0.2 and 11 (with NH_4^+) and 0.2 and 3 (without NH_4^+) with the lowest ratio observed at the completion of cDCE-to-ethene dechlorination. Similar to *nifH-195C* gene expression patterns in PW4 cultures, *nifH-PV* transcripts were measured in TC cultures regardless of NH_4^+ amendment, suggesting that the expression of these genes was not regulated by NH_4^+ . Taken together, all monitored *Dhc* genes were expressed at higher levels in the presence of NH_4^+ . About 1 to 2-orders of magnitude differences in transcriptional levels were observed with an initially high increase followed by a decline during the course of electron acceptor (i.e., cDCE and VC) consumption in both PW4 and TC cultures (Figure 61C, D).

Effects of NH_4^+ on the microbial community

The 16S rRNA gene amplicon sequencing data revealed community differences associated with PW4 groundwater collected within a contaminated aquifer in Australia and TC river sediment collected in Knoxville, TN where infiltrating groundwater is contaminated with chlorinated solvents (Şimşir et al., 2017). The analysis of PW4 and TC enrichment cultures indicated that NH_4^+ amendment impacted the microbial community composition during cDCE dechlorination. In PW4 well groundwater, *Actinobacteria* (42%), *Proteobacteria* (22%), *Firmicutes* (9%), and *Bacteroidetes* (8%) were the most abundant bacterial phyla (Table 34), whereas the phylum *Chloroflexi* (1%) was less abundant. Over the course of repeated transfers of the PW4 enrichment culture to fresh medium with cDCE provided as electron acceptor, *Actinobacteria* diminished while the relative abundances of *Firmicutes* and *Chloroflexi* increased regardless of NH_4^+ amendment. At the genus level, in PW4 culture incubations without NH_4^+ , *Sulfurospirillum* and *Acetobacterium* substantially increased in abundance when compared to their levels in PW4 groundwater (i.e., increases from 4 to 46% and from 1 to 42%, respectively), suggesting that members of these genera contributed to N_2 fixation. Sequences affiliated with *Clostridium* (37%) and unclassified *Bacteroidales* (28%) were most abundant in cultures amended with NH_4^+ (day 29), whereas their abundances decreased without NH_4^+ . Consistent with the qPCR results (Figure 60A), the abundance of *Dhc* increased regardless of NH_4^+ amendment (Table 34), albeit higher *Dhc* cell numbers were attained with NH_4^+ (i.e., $1.8 \pm 0.9 \times 10^8$ vs $4.1 \pm 0.8 \times 10^7$ copies/mL).

The TC sediment community was dominated by *Proteobacteria* (44%) and *Bacteroidetes* (26%), while *Chloroflexi* and *Firmicutes* were less abundant (<1%) (Table 34). Over the course of repeated transfers of the TC enrichment culture to fresh medium with cDCE provided as electron acceptor, the relative abundance of *Proteobacteria* declined, whereas *Bacteroidetes* and *Firmicutes* (i.e., *Proteiniclasticum*) increased substantially regardless of NH_4^+ amendment (Table 34). Similar to PW4 culture incubations, the abundance of *Dhc* increased regardless of NH_4^+ amendment (Table 34) albeit the increase was more pronounced in cultures with NH_4^+ (6.7% vs 2.3% of sequences), which was consistent with the qPCR results (Figure 60B).

Table 34. Microbial community composition and relative abundance (%) based on 16S rRNA gene amplicon sequencing in PW4 and TC enrichment cultures.

Microbial community composition and relative abundance (%) based on 16S rRNA gene amplicon sequencing. Shown are the most abundant phyla and taxa detected in well PW4 groundwater used to establish PW4 enrichment cultures, in TC sediment used to establish TC enrichment cultures, and in PW4 and TC enrichment cultures with or without NH_4^+ .

Phyla	Taxon ¹	PW4 culture			TC culture		
		GW	with NH_4^+ Day 29	without NH_4^+ Day 143	Sediment	with NH_4^+ Day 88	without NH_4^+ Day 160
Crenarchaeota ²	f <i>Cenarchaeaceae</i>	<0.1	-	-	2.1	-	-
	<i>Methanocorpusculum</i>	7.6	1.3	0.2	<0.1	<0.1	<0.1
	<i>Candidatus</i>	<0.1	-	-	7.1	<0.1	<0.1
Euryarchaeota ²	<i>Methanoregula</i>	<0.1	-	-	<0.1	1.6	0.8
	<i>Methanospirillum</i>	<0.1	-	-	3.5	<0.1	<0.1
	<i>Methanosaeta</i>	<0.1	-	-	<0.1	<0.1	<0.1
Actinobacteria	f <i>Coriobacteriaceae</i>	40.7	<0.1	<0.1	<0.1	<0.1	<0.1
	o <i>Bacteroidales</i>	7.6	28.0	6.1	20.6	40.7	32.4
	f <i>Cytophagaceae</i>	<0.1	<0.1	-	2.8	<0.1	<0.1
Bacteroidetes	o <i>Saprospirales</i>	<0.1	-	-	2.6	<0.1	<0.1
	f <i>Ignavibacteriaceae</i>	-	-	-	4.3	<0.1	<0.1
	<i>Dehalococcoides</i>	1.0	4.6	1.5	<0.1	6.7	2.3
Deferribacteres	f <i>Deferribacteraceae</i>	2.8	<0.1	-	-	<0.1	<0.1
Firmicutes	<i>Bacillus</i>	1.8	<0.1	<0.1	<0.1	<0.1	-
	<i>Clostridium</i>	1.8	36.9	<0.1	<0.1	1.1	0.1
	<i>Proteiniclasticum</i>	<0.1	<0.1	<0.1	<0.1	14.7	33.6
	f <i>Clostridiaceae</i>	<0.1	-	-	<0.1	6.2	0.6
	f <i>EtOH8</i>	-	-	<0.1	<0.1	1.9	0.1
	<i>Acetobacterium</i>	0.2	1.9	41.7	0.1	1.4	2.6
	<i>Dehalobacter</i>	1.0	3.7	0.9	0.1	0.1	0.1
	<i>Syntrophobotulus</i>	-	<0.1	<0.1	<0.1	2.5	0.1
	<i>Syntrophomonas</i>	<0.1	<0.1	<0.1	<0.1	0.4	6.5
	<i>Anaeromusa</i>	<0.1	<0.1	-	<0.1	4.9	2.3
	<i>Acidaminobacter</i>	0.6	1.8	<0.1	<0.1	0.5	0.3
	<i>PSB-M-3</i>	<0.1	<0.1	-	0.1	2.2	5.9
	o <i>Nitrospirales</i>	<0.1	<0.1	<0.1	5.4	<0.1	<0.1
	f <i>Rhodobacteraceae</i>	12.0	5.2	0.1	<0.1	<0.1	<0.1
	f <i>Comamonadaceae</i>	<0.1	<0.1	<0.1	15.1	<0.1	<0.1
Proteobacteria	<i>Desulfomicrobium</i>	<0.1	1.6	0.1	<0.1	<0.1	<0.1
	<i>Desulfovibrio</i>	4.5	<0.1	<0.1	<0.1	<0.1	<0.1
	<i>Geobacter</i>	<0.1	<0.1	<0.1	4.6	1.4	0.5
	<i>Anaeromyxobacter</i>	<0.1	-	-	2.3	-	<0.1
	<i>Syntrophus</i>	<0.1	2.2	0.1	5.0	3.5	2.8
	<i>Sulfurospirillum</i>	3.5	7.0	45.7	<0.1	0.2	0.6
	o <i>Alteromonadales</i>	1.2	<0.1	<0.1	4.7	<0.1	-
	<i>Crenothrix</i>	<0.1	<0.1	-	2.9	<0.1	<0.1
	o <i>PL-11B10</i>	<0.1	-	-	<0.1	0.4	1.5
Spirochaetes	f <i>Sphaerochaetaceae</i>	<0.1	0.2	1.8	0.3	0.1	1.6
Synergistetes	<i>vadinCA02</i>	1.3	1.9	0.7	<0.1	0.2	0.2
Tenericutes	o <i>RF39</i>	4.0	-	-	<0.1	<0.1	-
Verrucomicrobia	f <i>auto67 4W</i>	<0.1	-	-	3.2	<0.1	<0.1
Unassigned	Unassigned ³	2.7	-	-	<0.1	<0.1	<0.1

¹OTUs not assigned to any genus classified either at family (f) or order (o) level. ²Archaea. ³Sequences that could not be assigned to any of these taxonomic ranks. “-”: Not detected.

Relative sequence abundance (%): 0.1  0.2  2.7  45.7

Detection of biomarker proteins in the PW4 enrichment cultures and source groundwater

TceA (matching *Dhc* strain 195) and VcrA (matching *Dhc* strain VS) were detected in relative abundances corresponding to their transcription levels (Figure 60C, D). The *tceA* gene was transcribed 1.2- to 8-fold higher than the *vcrA* gene, whereas the relative spectral counts of VcrA peptides (NSAF of 280-436 and 62-386 with or without NH_4^+ , respectively) were 2- to 4-fold higher compared to the counts of the TceA peptides (Table 35). In addition to TceA and VcrA, peptides of seven other *Dhc*-type RDase proteins were detected in PW4 samples, albeit at lower abundances, while peptides of non-*Dhc* RDases were not detected in PW4 enrichment cultures.

The proteomic analysis detected peptides of the nitrogenase proteins NifD, NifK, and NifH and three homologs of the nitrogen regulatory protein P-II matching those of *Dhc* strain 195 only in PW4 cultures without NH_4^+ (Table 35). Relative abundances of nitrogenase peptides were higher at the beginning of cDCE dechlorination (day 32) compared to those near completion (day 143) (Table 35), and thus corresponded to their respective transcript levels (Figure 60C, D). Similarly, peptides of the NH_4^+ transporter protein AmtB were solely detected in PW4 cultures without NH_4^+ . Peptides matching *Dhc* type glutamine and glutamate synthase were expressed in PW4 cultures regardless of NH_4^+ amendment (Table 35). Despite the detection of *nifH* transcripts in cultures with NH_4^+ , peptides of other nitrogenase proteins were not detected, indicating that *nifH* transcripts do not serve as biomarkers for N_2 fixation and that NH_4^+ regulated the expression of these proteins. Peptides of methylglyoxal synthase (MGS) and the transcriptional regulator MraZ matching those of *Dhc* strain 195 were detected only in the incubations without NH_4^+ (Table 35). MGS synthesizes methylglyoxal, a toxic electrophile, that can inhibit growth (Tötemeyer et al., 1998), while MraZ synthesizes a conserved transcription factor implicated in the regulation of cell division, (Eraso et al., 2014) suggesting these proteins have regulatory functions in Cornell-type *Dhc* strains. The proteomics analysis also detected peptides of non-*Dhc* nitrogenases in cultures without NH_4^+ , but not in cultures with NH_4^+ .

Table 35. Relative peptide abundance of selected *Dhc* type proteins analyzed through global proteomics in PW4 enrichment cultures. Relative peptide abundance of selected *Dhc* type proteins analyzed through global proteomics in PW4 groundwater (used to establish PW4 enrichment cultures) and in PW4 culture incubations with or without NH_4^+ during cDCE-to-ethene dechlorination. Spectral counts of identified peptides were normalized to obtain the normalized spectral abundance factor (NSAF). For better visualization, the NSAF values were multiplied by a constant number (100,000).

Description	GW	with NH_4^+			without NH_4^+			
		D15	D22	D29	D32	D82	D116	D143
Nitrogen regulatory protein P-II ^a	0	0	0	0	1320	602	102	380
Nitrogen regulatory protein P-II ^a	0	0	0	0	478	444	74	257
Nitrogen regulatory protein P-II ^a	0	0	0	0	112	16	2	12
Nitrogenase reductase (nitrogenase iron protein), NifH ^a	3	0	0	0	357	115	20	43
Nitrogenase molybdenum-iron protein alpha chain, NifD ^a	0	0	0	0	68	26	5	17
Nitrogenase molybdenum-iron protein beta chain, NifK ^a					21	12	3	0
Ammonium transporter, AmtB ^a	0	0	0	0	24	28	7	142
Molybdenum ABC transporter, periplasmic molybdate-binding protein ^a	0	0	0	0	54	61	10	40
Glutamine synthetase, type I ^a	228	81	56	39	320	435	355	424
Glutamine synthetase, type I ^b	0	0	0	0	103	222	245	136
Glutamine synthetase, glna gene product ^b	0	49	95	141	4	0	0	0
Glutamate synthase-like protein protein, alpha subunit-like protein ^c	161	7	15	11	19	16	1	9
Glutamate synthase, alpha subunit ^b	2	5	11	18	39	35	5	40
Glutamate synthase-like protein, gltb-like fragment ^b	0	0	12	24	54	42	30	53
Glutamate synthase (NADPH), homotetrameric ^b	0	0	0	11	0	3	0	4
Glutamate synthase (NADPH), homotetrameric ^c	37	23	10	0	11	0	1	7
Trichloroethene reductive dehalogenase ^a	180	144	148	171	63	165	26	37
Vinyl chloride reductive dehalogenase ^c	334	280	346	436	285	386	62	156
Reductive dehalogenase ^d	335	280	342	431	269	386	63	220
Reductive dehalogenase ^a	96	12	31	37	21	31	4	34
Reductive dehalogenase ^a	2	0	0	0	0	24	5	18
Reductive dehalogenase homologous protein RdhA8 ^c	0	0	0	0	0	4	0	0
Reductive dehalogenase homologous protein RdhA9 ^c	0	0	0	0	0	3	0	0
Reductive dehalogenase ^b	0	5	0	0	13	0	2	0
Reductive dehalogenase ^b	0	0	0	0	0	3	0	0
MraZ ^a	0	0	0	0	0	16	7	41
MraZ ^c	0	0	0	0	0	0	0	9
Methylglyoxal synthase (MGS) ^a	0	0	0	0	0	20	7	0

D: Day. *Dhc*: *Dehalococcoides mccartyi*. Peptide of the protein matches related protein of ^a *Dhc* strain 195, ^b all *Dhc* strains, ^c *Dhc* strain VS, ^d *Dhc* strain GT, and ^e CBDB1.

Effects of NH₄⁺ amendment on *Dhc* activity and biomarker proteins in a contaminated groundwater aquifer

Monitoring of well PW4 groundwater demonstrated stalled dechlorination with VC as the dominant chlorinated ethene and a decline of *Dhc* 16S rRNA, *vcrA*, and *tceA* genes over time (Baldwin et al., 2017). Proteomic analysis of groundwater samples revealed the expression of the *Dhc* nitrogenase proteins NifH and NifD (Table 36), suggesting that *Dhc* were experiencing fixed N limitation.

Table 36. Relative peptide abundances of selected proteins analyzed through global proteomics in groundwater from well PW4. Well PW4 is located within a chlorinated solvent plume. The data in the table represent the normalized spectral counts of identified peptides. For better visualization, the normalized spectral abundance factor (NSAF) values were multiplied by a constant factor (100,000).

Groundwater harvested (date) 		Periodic addition of Aquasol fertilizer containing urea 		
Protein		Nov. 2013	Jan. 2014	Oct. 2014
		NSAF values		
Nitrogenase iron protein, NifH ^a		14.4	30.1	10.3
Nitrogenase molybdenum-iron protein alpha chain, NifD ^a		0.0	2.6	0.0
Glutamine synthetase, type I ^a		3.3	8.1	0.0
Glutamate synthase, alpha subunit-like protein ^b		0.0	3.5	0.0
Nitrogen fixation protein, NifX ^c		0.0	39.2	0.0
Vinyl chloride reductive dehalogenase, VcrA ^b		0.8	0.0	3.80
Trichloroethene reductive dehalogenase, TceA ^a		0.0	0.9	2.0
Tetrachloroethene dehalogenase ^d		0.0	1.3	9.1
Tetrachloroethene reductive dehalogenase ^e		0.0	0.0	10.8
Formate dehydrogenase, alpha subunit ^a		0.0	0.4	0.0
[Ni/Fe] hydrogenase, grp. 1, large subunit, putative ^a		0.0	0.7	0.0
Chaperonin GroEL ^a		35.0	5.3	4.7
Chaperonin GroES ^a		11.90	0.0	0.0

Peptides match protein of *Dehalococcoides mccartyi* ^astrain 195 and ^bstrain VS, ^c*Anaeromyxobacter* sp., ^d*Dehalobacter restrictus* strain DSM 9455, and ^e*Dehalobacter* sp. strain UNSWDHB.

Total Kjeldahl N (TKN) and NH_4^+ -N measurements (Table 37) supported this conclusion.

Table 37. Nitrogen measurements in groundwater collected from well PW4.

Sampling Date	Nitrogen (mg/L)	
	TKN	NH_4^+ -N
Nov-2013	<0.05	0.11
Jan-14	1.1	0.3
Feb-14	0.11	0.25
Apr-14	<0.05	0.18
Jul-14	4.4	0.46
Sep-14	1.3	0.54

Therefore, soluble Aquasol fertilizer containing urea was added to wells up-gradient of PW4. In response, elevated TKN and NH_4^+ -N values (Table 37), enhanced dechlorination activity, and higher abundances of *Dhc* 16S rRNA, *vcrA*, and *tceA* genes were observed. Consistent with the qPCR results, proteomic analysis demonstrated an increase of TceA and VcrA peptides (NSAF values of 0.8-0.9 before versus 2-3.8 after Aquasol fertilizer addition) as well as of non-*Dhc* RDase peptides such as tetrachloroethene reductive dehalogenase, PceA, of *Dehalobacter* sp. UNSWDHB and PceA of *Dehalobacter restrictus* DSM 9455, but a decrease of *Dhc* nitrogenase proteins NifH and NifD (NSAF values of 30.1 and 2.6 versus 10.3 and 0, respectively) after fertilizer addition (Table 36).

Discussion - Impact of fixed nitrogen availability on *Dhc* reductive dechlorination activity

Effects of NH_4^+ on *Dhc* growth and reductive dechlorination performance

groundwater aquifers are often oligotrophic (i.e., nutrient-poor), (Ghiorse et al., 1988; Löffler et al., 2006; Skubal et al., 2001) and fixed N limitation can negatively impact reductive dechlorination activity. NH_4^+ limitation impacted *Dhc* growth and reductive dechlorination activity and resulted in lower reductive dechlorination rates, longer time frames to complete dechlorination, and lower *Dhc* biomarker gene abundances in PW4 and TC enrichment cultures. The availability of NH_4^+ also affected the relative abundance of *Dhc* strains belonging to the Cornell versus the Pinellas/Victoria groups in PW4 cultures, consistent with gene content and physiology attributed to Cornell versus Pinellas/Victoria group *Dhc* (Cheng et al., 2009; Lee et al., 2011a; Lee et al., 2012; Lee et al., 2009; Seshadri et al., 2005; West et al., 2008). Cornell-type *Dhc* have an advantage over Pinellas/Victoria-type *Dhc* under NH_4^+ -limiting conditions due to their N_2 -fixing ability. N_2 fixation is an energetically demanding process, diverts electron flow and reduces energy gain, and thus impacts dechlorination activity, as was observed experimentally in PW4 cultures dominated by Cornell-type *Dhc*. Pinellas/Victoria-type *Dhc* lack the ability to fix N_2 but TC culture incubations without NH_4^+ also achieved complete cDCE-to-ethene dechlorination (Figure 60B), presumably due to the activity of diazotrophs present in the culture. This is a relevant observation indicating that non- N_2 fixing *Dhc* strains depend on community diazotrophs for supplying fixed N under N-limiting conditions.

Although the addition of fixed N (e.g., NH_4^+ , Aquasol fertilizer) improved *Dhc* activity and reductive dechlorination performance, elevated NH_4^+ -N concentrations (i.e., ≥ 0.5 g NH_4^+ /L) can

decrease dechlorination rates, particularly the conversion of the crucial VC-to-ethene dechlorination step (Delgado et al., 2016). Furthermore, fixed N turnover in groundwater aquifers results in the formation of nitrous oxide (N₂O), (Jurado et al., 2017; Reay et al., 2012) which is a potent inhibitor of *Dhc* reductive dechlorination activity and a possible cause for cDCE or VC stalls (Yin et al., 2019). Therefore, the decision of fixed N addition requires careful evaluation and should be based on the presence of *Dhc* types (i.e., N₂-fixing Cornell-type *Dhc* versus non-N₂-fixing Pinellas/Victoria-type *Dhc*), the time frames to reach desirable remediation endpoints, and the ability to implement a rigorous monitoring regime to alert about potential dechlorination stalls due to elevated N₂O concentrations (Yin et al., 2019).

Effects of NH₄⁺ on microbial community composition

Without fixed N additions to PW4 and TC cultures, bacterial groups characterized as diazotrophs increased in abundance. For example, species of *Sulfurospirillum*, *Acetobacterium* and *Spirochaetes* possess complete *nif* operons, (Lilburn et al., 2001) suggesting N₂ fixation capabilities. Furthermore, *Sulfurospirillum*, *Acetobacterium*, *Clostridium*, and *Spirochaetes* are often found in groundwater contaminated with chlorinated ethenes (Adamson et al., 2003; Atashgahi et al., 2017; Pérez-de-Mora et al., 2014) and have been implicated in supplying *Dhc* with essential nutrients such as H₂, (John et al., 2009; Kruse et al., 2018) acetate, (Kruse et al., 2018) and corrinoid (He et al., 2007; Macbeth et al., 2004). *Proteiniclasticum*, a major population in the TC culture without NH₄⁺ reportedly digests proteins (Zhang et al., 2010) and releases nitrogenous metabolites (e.g., from necromass) that can fulfill the nutritional N requirement of *Dhc*. Since diazotrophs are common members of groundwater aquifer microbiomes, N₂ fixation will occur under fixed N-limiting conditions and nitrogenous metabolites will be generated and become available to *Dhc*. While this “do-nothing” approach may not result in the highest possible dechlorination rates, it saves the operational costs for fertilizer biostimulation and avoids the likelihood of stalled reductive dechlorination due to elevated N₂O concentrations as explained above. None of the 16S rRNA gene amplicon sequences derived from the enrichment cultures affiliated with the genus *Dehalogenimonas*. Members of this genus have been implicated in reductive dechlorination of chlorinated ethenes, and *Dehalogenimonas* sp. WBC-2 dechlorinates *trans*-1,2-dichloroethene to VC and ‘*Candidatus Dehalogenimonas etheniformans*’ strain GP dechlorinates trichloroethene (TCE) to ethene (Manchester et al., 2012; Yang et al., 2017c). A 16S rRNA-based survey demonstrated the broad distribution of *Dehalogenimonas* spp. in groundwater aquifers impacted with chlorinated ethenes, suggesting that members of this group contribute to reductive dechlorination and detoxification (Clark et al., 2018). Based on the gene content of the five *Dehalogenimonas* genomes spp. (NCBI:txids 1217799, 943347, 1536648, 1839801, 552811), only *Dehalogenimonas* sp. strain WBC-2 (NCBI:txid943347) has a complete *nif* operon and can potentially fix N₂.

Effects of NH₄⁺ on biomarker gene and transcript abundances

Dhc share highly similar 16S rRNA gene sequences but not all *Dhc* strains have the ability to dechlorinate cDCE and VC to ethene (Lee et al., 2008b). Accordingly, specific metabolic functions cannot be inferred from *Dhc* 16S rRNA gene-targeted approaches, and the presence of *Dhc* does not guarantee that VC is efficiently dechlorinated to ethene. RDase genes (i.e., *tceA*, *bvcA*, and *vcrA*) that encode RDase proteins with assigned functions can diagnose and demonstrate *Dhc* growth and specific dechlorination activities. Hence, efforts to monitor the responses of *Dhc* populations to treatment and evaluate bioremediation performance should include 16S rRNA gene and RDase gene analyses (Ritalahti et al., 2006). In PW4 cultures, *tceA*

was consistently more abundant than *vcrA* regardless of NH_4^+ amendment, while the *bvcA* gene was not detected. In contrast, in TC cultures, the abundance of the *vcrA* gene was highest followed by *tceA* and *bvcA* gene abundances regardless of NH_4^+ amendment. Despite the presence of the *bvcA* gene in TC cultures, this gene was only upregulated in incubations with NH_4^+ . The results indicate that the effect of NH_4^+ limitation was much more pronounced at the transcriptional level than at gene level. *Dhc* strains harboring *bvcA*, a gene that encodes BvcA responsible for cDCE and VC reductive dechlorination, were most sensitive to NH_4^+ availability (Figure 59), suggesting that NH_4^+ limitation impacts *Dhc* strains lacking complete *nif* operons differently.

RDase gene transcripts declined as chlorinated electron acceptors (i.e., cDCE, VC) were depleted regardless of NH_4^+ amendments (Figure 60C-D). The TGRs were within the ranges reported in prior studies performed with cDCE-degrading *Dhc* enrichment cultures (i.e., TGRs between 10 and 100), (Lee et al., 2006; Rahm et al., 2006; Rahm et al., 2008b) suggesting a direct link between TGRs and electron acceptor availability. A similar declining trend in TGR values was observed in the transcript numbers of *nif* genes as dechlorination progressed. Interestingly, *nifH*-PV and *nifH*-195C transcripts were detected in PW4 cultures with NH_4^+ , but *nifK* and *nifD* transcripts were only detected in cultures without added NH_4^+ , suggesting that *nifK* and *nifD*, but not *nifH* transcripts, are indicators of *Dhc* nitrogenase activity. These observations are consistent with the grouping of the Pinellas/Victoria *nifH* with cluster IV *nifH* sequences (i.e., homologs of diverse *nifH* gene sequences) not involved in N_2 fixation (Ju et al., 2007; Raymond et al., 2004).

Detection of biomarker proteins during NH_4^+ limitation

Monitoring merely the presence/absence of organism- and process-specific biomarker genes is generally not sufficient to imply microbial activity (Lee et al., 2008b) and expression data can provide more direct evidence of activity. A factor complicating transcript analysis is the inherent instability and fast decay of RNA (Heavner et al., 2018). The analysis of proteins in groundwater has become feasible and this measurement can provide additional evidence about the microbial process of interest (Heavner et al., 2019; Solis et al., 2019). Of note, DNA-based tools would not have uncovered that fixed N limitations limited reductive dechlorination rates in the area surrounding well PW4, highlighting the value of untargeted proteomics analysis.

The detection of several *Dhc* proteins implicated in N_2 fixation, N metabolism and the control of growth (i.e., MGS and *MraZ*) in incubations without NH_4^+ suggests their potential role as biomarker proteins for *Dhc* activity. Nitrogenase proteins were only identified in PW4 cultures without NH_4^+ and in groundwater samples of well PW4 prior to fertilizer additions, indicating that *Dhc* *NifK* and *NifD* can serve as activity biomarkers for N_2 fixation. The observation that *NifH* was expressed in *Dhc* cultures with NH_4^+ indicated that this protein is not a useful biomarker for N_2 fixation, which is consistent with the results of the transcript analysis.

The main bacterial pathway for NH_4^+ incorporation into amino acids, including *Dhc*, is through glutamine synthetase (GS) encoded by *glnA* and glutamate:2-oxoglutarate aminotransferase (GOGAT) encoded by *gltA*, also known as the GS-GOGAT cycle (Lee et al., 2012; Leigh et al., 2007). The detection of the peptides of *Dhc*-type GS and glutamate synthase (Table 35) suggests that non- N_2 fixing *Dhc* strains acquired NH_4^+ , possibly derived from necromass. All three homologs of the N regulatory protein P-II control the formation of GS and were detected in PW4 cultures without NH_4^+ (Huergo et al., 2012; Leigh et al., 2007). Two of three genes that encode the N regulatory protein P-II are located within the *nif* operon and their expression reportedly coupled to the *nif* genes (Lee et al., 2012). The third P-II gene is co-located with GS-GOGAT pathway genes (*glnA* and *gltA*) and adjacent to an NH_4^+ transporter gene (*amtB*). The NH_4^+ transporter protein AmtB is required for growth under NH_4^+ -limiting conditions, (Wang et al., 2010) and was

only detected in PW4 cultures without NH_4^+ . These findings suggest that the N regulatory protein P-II and AmtB can also serve as potential biomarkers for NH_4^+ or fixed N limitation.

Implications for enhanced *in situ* bioremediation

Enhanced anaerobic bioremediation through biostimulation with or without bioaugmentation has achieved extensive reductions in the concentrations of chlorinated ethenes at many chlorinated solvent-impacted sites (Schaefer et al., 2010; Tillotson et al., 2017). To sustain *in situ* bioremediation, the nutritional requirements of *Dhc* must be met and the buildup of inhibitory chemicals has to be avoided. (Carol et al., 2013) Biostimulation generally involves the addition of fermentable substrates with the goal of increasing the flux of hydrogen. The results from this study illustrate that NH_4^+ limitation impairs *Dhc* reductive dechlorination activity, and biostimulation with fixed N can overcome this bottleneck. However, possible negative feedback on *Dhc* reductive dechlorination activity must be considered, including inhibition by elevated NH_4^+ following fertilizer addition or by N_2O resulting from increased N turnover (Delgado et al., 2016; Yin et al., 2019). Without fixed N biostimulation, native diazotrophs will fix N_2 under N-limiting conditions and provide fixed N to organisms lacking this ability, including *Dhc* of the Pinellas and Victoria groups. While this “do nothing” approach will not result in the highest possible reductive dechlorination rates, it does not require fertilizer additions and prevents unintended negative consequences, including N_2O emissions to the atmosphere (i.e., N_2O is a strong greenhouse gas with ozone destruction potential) and inhibition of *Dhc* dechlorination activity leading to cDCE and VC stalls.

Dhc reductive dechlorination activity with different cobamides

Dhc and *Dhgm* isolates lack the ability for *de novo* synthesis of the corrin ring system but the RDase enzyme systems strictly require corrinoid to perform reductive dechlorination. To perform reductive dechlorination, corrinoid auxotrophs must acquire complete cobamides or precursor molecules from the environment. Only subsets of the bacteria and the archaea have the complete set of genes for *de novo* biosynthesis of cobamides, but the majority of organisms, including mammals, require corrinoid for essential enzymatic functions (Shelton et al., 2019). In groundwater aquifers, members of the microbial community generate corrinoid, which corrinoid auxotrophs, including *Dhc* and *Dhgm*, can acquire and repurpose for the assembly of functional RDases. Detailed laboratory experiments were conducted to better understand the corrinoid requirement of *Dhc* and to elucidate the impact of geochemistry of the corrinoid pool.

The lower base affects dechlorination rates

The cobamides DMB-Cba, 5-MeBza-Cba, 5-OMeBza-Cba and Bza-Cba supported complete dechlorination of cDCE to ethene in *Dhc* strain BAV1 cultures. The highest cDCE-to-ethene dechlorination rates of 107.0 ± 12.0 and 74.3 ± 1.0 $\mu\text{M Cl}^-$ released day^{-1} were measured in the presence of DMB-Cba and 5-MeBza-Cba, respectively (Figure 62A), and the initial amount of $72.2 (\pm 1.4)$ μmol cDCE was completely dechlorinated to ethene in 12-18 days. Longer time periods of 38 and 86 days were required to achieve complete cDCE and VC reductive dechlorination to ethene in strain BAV1 cultures amended with 5-OMeBza-Cba or Bza-Cba due to lower cDCE dechlorination rates of $33.2 (\pm 2.6)$ and $16.8 (\pm 1.1)$ $\mu\text{M Cl}^-$ released day^{-1} , respectively (Figure 62A). Strain GT cultures amended with DMB-Cba and 5-MeBza-Cba dechlorinated cDCE to ethene at rates of $67.4 (\pm 1.4)$ and $26.7 (\pm 1.9)$ $\mu\text{M Cl}^-$ released day^{-1} , respectively (Figure 62B). Similar to strain BAV1 cultures, cDCE dechlorination rates decreased in strain GT cultures amended with 5-OMeBza-Cba or Bza-Cba; however, the VC-to-ethene dechlorination step occurred at such low rates that VC, rather than ethene, was formed as dechlorination end product (Figure 62B). In strain GT cultures amended with 5-OMeBza-Cba or Bza-Cba, no more than 23% (17.5 μmol) of the total VC produced from cDCE dechlorination was further dechlorinated to ethene over a 77-day incubation period (Figure 62B).

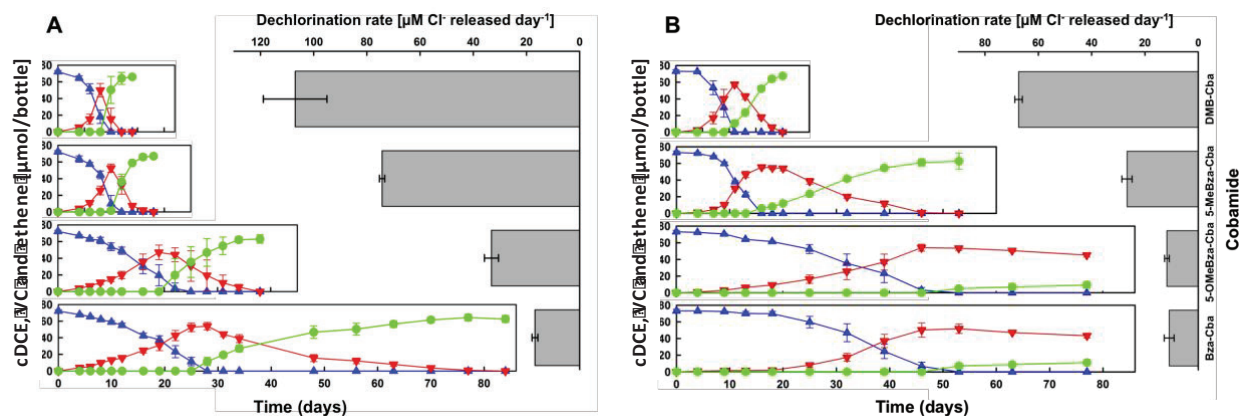


Figure 62. Reductive dechlorination of cDCE in cultures of *Dhc* strain BAV1 harboring the BvcA RDase and strain GT harboring the VcrA RDase in the presence of different cobamides.

Triplicate cultures of strain BAV1 (A) and strain GT (B) were amended with cobamides carrying different benzimidazole derivatives as lower bases at initial concentrations of 36.9 nM. Blue triangles, cDCE; red inverted triangles, VC; green circles, ethene. Error bars represent the standard deviations of triplicate cultures.

The lower base affects *Dhc* growth yields

Enumeration of *Dhc* cell numbers after cDCE-to-ethene dechlorination was complete (i.e., in all BAV1 cultures and DMB-Cba- or 5-MeBza-Cba-amended GT cultures) or ceased (i.e., in 5-OMeBza- or Bza-Cba-amended GT cultures) revealed that strain BAV1 cultures reached similar growth yields of $1.38 (\pm 0.09)$ to $1.57 (\pm 0.03) \times 10^8$ cells per $\mu\text{mol Cl}^-$ released with all cobamides tested (Table 38). For *Dhc* strain GT, the highest final cell densities of $1.29 (\pm 0.22) \times 10^8$ and $1.30 (\pm 0.41) \times 10^8$ cells/mL were measured in cultures amended with DMB-Cba and 5-MeBza-Cba, respectively, and these results were consistent with the growth yield expected from complete cDCE-to-ethene dechlorination (Table 38). With 5-OMeBza-Cba or Bza-Cba, strain GT cultures reached cell yields of $6.95 (\pm 1.04)$ and $7.72 (\pm 0.87) \times 10^7$ cells per $\mu\text{mol Cl}^-$ released, respectively, which were on average 31% and 23% lower compared to growth yields of $0.92 (\pm 0.15)$ and $1.10 (\pm 0.24) \times 10^8$ cells per $\mu\text{mol Cl}^-$ released measured in DMB-Cba or 5-MeBza-Cba amended cultures, respectively. The lower growth yields suggested that the VC-to-ethene reductive dechlorination step was uncoupled from growth in strain GT cultures amended with 5-OMeBza-Cba or Bza-Cba.

Table 38. Growth of *Dhc* pure cultures amended with different cobamides.

<i>Dhc</i> strain	Cobamide ^a	DMB ^b	<i>Dhc</i> cell density (16S rRNA gene copies/mL)		Growth yield (cells [$\mu\text{mol Cl}^-$] ⁻¹) ^c
			Initial	Final	
BAV1 (cDCE)	DMB-Cba	-	$4.26 \pm 0.87 \times 10^6$	$1.82 \pm 0.25 \times 10^8$	$1.38 \pm 0.09 \times 10^8$
	5-MeBza-Cba	-	$4.26 \pm 0.87 \times 10^6$	$2.09 \pm 0.51 \times 10^8$	$1.54 \pm 0.39 \times 10^8$
	5-OMeBza-Cba	-	$4.26 \pm 0.87 \times 10^6$	$1.85 \pm 0.39 \times 10^8$	$1.42 \pm 0.20 \times 10^8$
	Bza-Cba	-	$4.26 \pm 0.87 \times 10^6$	$2.07 \pm 0.10 \times 10^8$	$1.57 \pm 0.03 \times 10^8$
GT (cDCE)	DMB-Cba	-	$5.22 \pm 0.54 \times 10^6$	$1.29 \pm 0.22 \times 10^8$	$0.92 \pm 0.15 \times 10^8$
	5-MeBza-Cba	-	$5.22 \pm 0.54 \times 10^6$	$1.30 \pm 0.41 \times 10^8$	$1.10 \pm 0.24 \times 10^8$
	5-OMeBza-Cba	-	$5.22 \pm 0.54 \times 10^6$	$4.97 \pm 0.99 \times 10^7$	$6.95 \pm 1.04 \times 10^7$
	Bza-Cba	-	$5.22 \pm 0.54 \times 10^6$	$5.72 \pm 0.38 \times 10^7$	$7.72 \pm 0.87 \times 10^7$
GT (VC)	DMB-Cba	-	$2.61 \pm 0.27 \times 10^6$	$0.96 \pm 0.06 \times 10^8$	$1.17 \pm 0.15 \times 10^8$
	5-MeBza-Cba	-	$2.61 \pm 0.27 \times 10^6$	$1.10 \pm 0.10 \times 10^8$	$1.39 \pm 0.20 \times 10^8$
	5-OMeBza-Cba	-	$2.61 \pm 0.27 \times 10^6$	$2.08 \pm 0.01 \times 10^6$	- ^d
	5-OMeBza-Cba	+	$2.61 \pm 0.27 \times 10^6$	$1.21 \pm 0.34 \times 10^8$	$1.45 \pm 0.33 \times 10^8$
	Bza-Cba	-	$2.61 \pm 0.27 \times 10^6$	$2.37 \pm 0.00 \times 10^6$	- ^d
	Bza-Cba	+	$2.61 \pm 0.27 \times 10^6$	$1.22 \pm 0.08 \times 10^8$	$1.48 \pm 0.11 \times 10^8$

^a Cobamide concentration was 36.9 nM

^b DMB was supplied at 10 μM

^c Growth yield was estimated from the final cell density after complete or stalled cDCE or VC dechlorination

^d No growth occurred

Impact of the lower base on dechlorination extent

VC stall and lower growth yields suggested that 5-OMeBza-Cba and Bza-Cba were not fully functional in strain GT, and VC was not used as a growth substrate. The key feature distinguishing *Dhc* strain GT from strain BAV1 is the VC RDase: strain GT uses VcrA whereas strain BAV1 uses BvcA for cDCE and VC reductive dechlorination (Krajmalnik-Brown et al., 2004; Müller et al., 2004; Parthasarathy et al., 2015; Sung et al., 2006b; Tang et al., 2013a). To further investigate the impact of the lower base on the catalytic activity of the VcrA RDase, growth experiments in medium amended with VC as electron acceptor were performed. Consistent with the observation in cDCE-dechlorinating cultures, only DMB-Cba and 5-MeBza-Cba sustained VC dechlorination in strain GT cultures, and rates of $40.0 (\pm 5.1)$ and $16.5 (\pm 2.8) \mu\text{M VC day}^{-1}$, respectively, were observed. Ethene production was negligible ($<0.8\%$ of the total VC added) in strain GT cultures amended with 5-OMeBza-Cba or Bza-Cba (Figure 63).

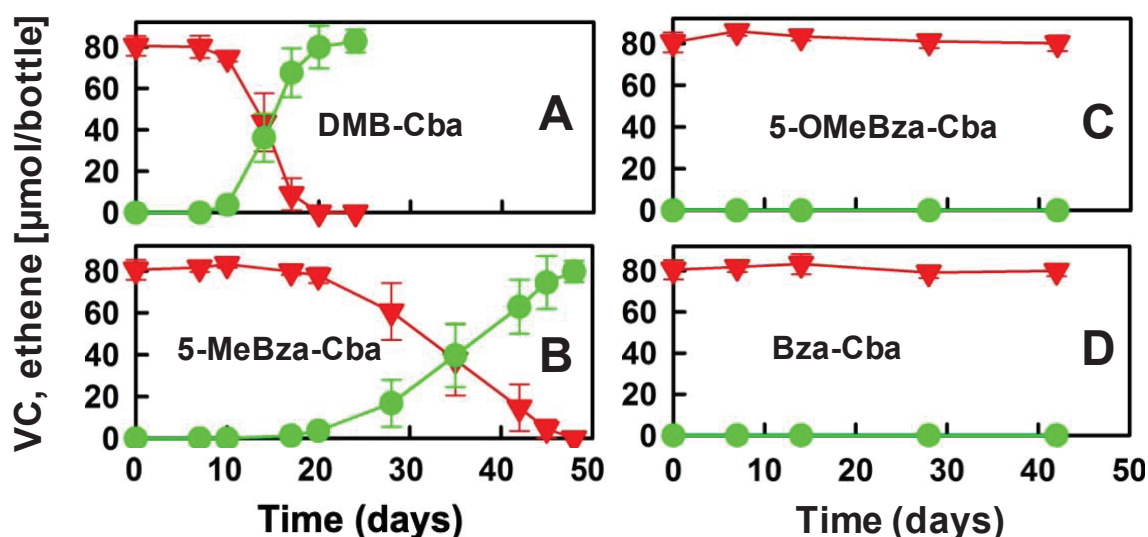


Figure 63. Reductive dechlorination of VC in *Dhc* strain GT cultures. Reductive dechlorination of VC in *Dhc* strain GT cultures amended with an initial concentration of 36.9 nM (A) DMB-Cba, (B) 5-MeBza-Cba, (C) 5-OMeBza-Cba or (D) Bza-Cba. Red inverted triangles, VC; green circles, ethene. Error bars represent the standard deviations of triplicate cultures.

In contrast, strain BAV1 cultures that received 5-OMeBza- or Bza-Cba completely dechlorinated VC to ethene. Enumeration of *Dhc* cells with qPCR confirmed the inability of 5-OMeBza-Cba and Bza-Cba to support growth of strain GT with VC as an electron acceptor. In vessels with initial cell densities of $2.61 (\pm 0.27) \times 10^6$ cells/mL (i.e., cells introduced with the inoculum), strain GT cell numbers decreased to $2.08 (\pm 0.01) \times 10^6$ and $2.37 (\pm 0.09) \times 10^6$ cells/mL with 5-OMeBza-Cba or Bza-Cba provided as corrinoid cofactor (Table 38). In contrast, strain GT cell numbers increased to $0.96 (\pm 0.06) \times 10^8$ and $1.10 (\pm 0.10) \times 10^8$ cells/mL in cultures amended with DMB-Cba and 5-MeBza-Cba, respectively, corresponding to growth yields of $1.17 (\pm 0.15) \times 10^8$ and $1.39 (\pm 0.20) \times 10^8$ cells per $\mu\text{mol VC}$ dechlorinated (Table 38).

We next tested if the addition of DMB could rescue the VC-to-ethene dechlorination phenotype in strain GT cultures that had received 5-OMeBza-Cba or Bza-Cba. Three weeks following DMB addition to inactive cultures, all VC was dechlorinated to ethene, whereas no ethene formation

occurred in cultures without DMB amendment (Figure 64A). To verify if the restoration of VC dechlorination activity following DMB addition was due to corrinoid remodeling, intracellular corrinoids from strain GT cells were extracted and purified. DMB-Cba was only detected in corrinoid extracts of cells collected from vessels amended with DMB, and DMB-Cba accounted for nearly half of the total intracellular cobamides in strain GT cells grown in medium with 5-OMeBza-Cba or Bza-Cba (Figure 64B). Cell enumeration with qPCR demonstrated that additional growth occurred following restoration of VcrA activity. Cultures that initially received 5-OMeBza-Cba or Bza-Cba and were then amended with DMB to restore the VC dechlorinating phenotype produced $1.21 (\pm 0.34)E+08$ and $1.22 (\pm 0.08) E+8$ cells/mL culture, respectively (Table 38). The growth yields in 5-OMeBza-Cba and Bza-Cba cultures following DMB addition were $1.45 (\pm 0.33)E+08$ and $1.48 (\pm 0.11)E+08$ cells per μmol VC dechlorinated, respectively, which are comparable to those observed in strain GT cultures amended with DMB-Cba ($1.17 (\pm 0.15)E+08$ cells per μmol VC dechlorinated) (Figure 64C).

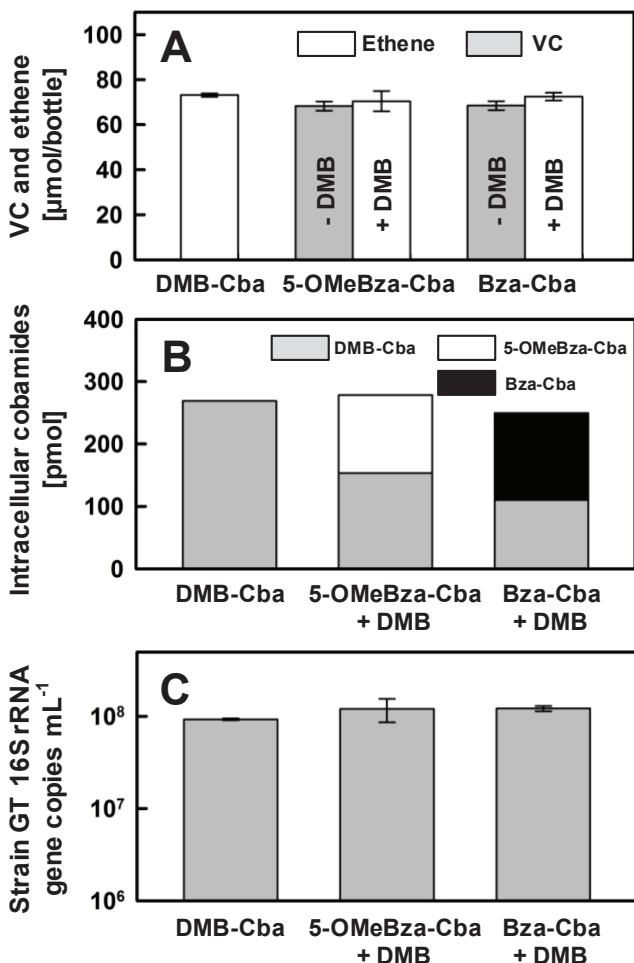


Figure 64. Restoration of the VC dechlorination phenotype by DMB addition to inactive *Dhc* strain GT cultures. **(A)** Comparison of VC-to-ethene dechlorination activity in 5-OMeBza-Cba- or Bza-Cba- (36.9 nM each) amended strain GT cultures in the presence or absence of $10 \mu\text{M}$ DMB. **(B)** Quantification of intracellular cobamides extracted from strain GT biomass grown with 5-OMeBza-Cba or Bza-Cba in the presence of DMB and collected from 100 ml cell suspensions. **(C)** Comparison of the final cell densities in in 5-OMeBza-Cba- or Bza-Cba-amended strain GT cultures with or without DMB (see Table 38).

Cobamide transport in *Dhc*

To explore if differential transport of cobamides with distinct lower bases across the *Dhc* surface layer and/or cytoplasmic membrane could explain the observations, cDCE-dechlorinating *Dhc* cultures were supplied with equimolar mixtures of DMB-Cba, 5-OMeBza-Cba, and Bza-Cba. Following cDCE conversion to ethene in strain BAV1 and strain GT cultures, the molar concentration ratios of DMB-Cba:5-OMeBza-Cba:Bza-Cba were 1.06:1.00:1.00 and 1.00:1.00:1.02 in strain BAV1 and strain GT culture supernatants, respectively, and therefore not significantly different than the initial conditions. Further, statistically identical concentrations of DMB-Cba (24.4 ($\pm$ 1.2) / 24.1 ($\pm$ 1.9) nM), 5-OMeOBza-Cba (23.1 ($\pm$ 1.1) / 24.0 ($\pm$ 1.0) nM), and Bza-Cba (23.2 ($\pm$ 1.3) / 24.6 ($\pm$ 1.5) nM) were recovered from strain BAV1 / strain GT culture supernatants (Figure 65). These findings indicate that surface layer and membrane transport did not discriminate between the three benzimidazole-type cobamides. Intracellular cobamides were also analyzed, and DMB-Cba, 5-OMeBza-Cba, and Bza-Cba were present in similar amounts ranging from 2E+03 to 5E+03 molecules per cell in strain BAV1 and strain GT cultures.

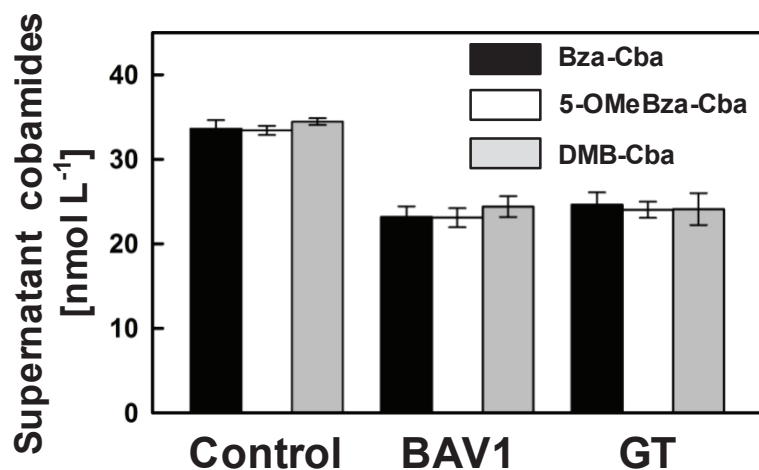


Figure 65. Quantification of supernatant-associated cobamides recovered from cDCE-dechlorinating *Dhc* strain BAV1 and strain GT cultures. The cultures were supplied with an equimolar mixture of DMB-Cba, 5-OMeBza-Cba and Bza-Cba (36.9 nM each).

Discussion - *Dhc* reductive dechlorination activity with different cobamides

Corrinoids in complete form (i.e., cobamides) are essential enzyme cofactors exclusively synthesized by a subset of the *Archaea* and *Bacteria*, even though these molecules play critical roles for the majority of organisms in all domains of life (Banerjee, 1999; Gruber et al., 2011; Martens et al., 2002; Miles et al., 2011; Parks et al., 2013; Schneider et al., 1987). Naturally occurring corrinoids carry a variety of lower bases, and as many as 16 benzimidazole, nucleobase, and phenol derivatives have been identified (Allen et al., 2008; Stupperich et al., 1990). Microorganisms such as *Escherichia coli* (ATCC 11105) and *Lactobacillus leichmannii* (ATCC 4797) are capable of utilizing natural corrinoids with at least 11 different lower bases to fulfill their nutritional requirements (Burkholder, 1951; Schneider et al., 1987; Thompson et al., 1950). The human gut anaerobe *Bacteroides thetaiotaomicron* grows with indistinguishable doubling times with DMB-Cba, 5-MeBza-Cba, 5-OMeBza-Cba, Bza-Cba, pseudo-B₁₂ (i.e., Cba with adenine as the lower base) or 2-methyladeninyl-Cba as corrinoid cofactor (Degnan et al., 2014). *Sporomusa ovata* and *Sporomusa* sp. strain KB-1, which natively synthesize phenolic type cobamides (e.g. Phe-Cba and *p*-Cre-Cba), were also capable of utilizing cobamides with benzimidazole type lower

bases (i.e. DMB-Cba, 5-MeBza-Cba, 5-OMeBza-Cba, Bza-Cba) during growth with H₂/CO₂, betaine or fructose as substrate (Mok et al., 2013; Yan et al., 2013). Some inhibitory effects were observed in *Sporomusa ovata* cultures amended with 5-MeBza when methanol or 3,4-dimethoxybenzoate served as growth substrates, suggesting that the structure of the lower base affected cobamide function and essential metabolic pathways (Mok et al., 2013; Yan et al., 2013). Reduced versatility in terms of the lower base utilization was observed with the corrinoid-auxotrophic *Dhc* strains, which exhibited a clear preference for cobamides with DMB or 5-MeBza lower bases to perform organohalide respiration with cDCE and VC as electron acceptors.

The PCE-to-cDCE RDase PceA of *Sulfurospirillum multivorans* and the *ortho*-dibromophenol RDase NpRdhA of *Nitratireductor pacificus* strain pht-B shed first light on the structure of RDase enzyme systems and provided details on interactive corrinoid-RDase binding (Bommer et al., 2014; Payne et al., 2015). In both RDases, the corrinoid cofactor (norpseudo-B₁₂ for PceA and DMB-Cba for NpRdhA) is involved in the catalytic cleavage of carbon-chlorine bonds. The lower bases adenine in norpseudo-B₁₂ or DMB in DMB-Cba are uncoordinated and in the base-off conformation to anchor the cofactors deeply inside the RDase scaffold, suggesting that the lower base is not directly involved in catalysis (PDB ID = 4UR0, 4RAS). Interestingly, kinetic studies on non-RDase cobamide-dependent enzymes suggested varying affinities of the apoprotein to cobamides with different lower bases (Barker et al., 1960; Lengyel et al., 1960) (Tanioka et al., 2010). For example, both the mammalian (sheep) kidney and bacterial (*Propionibacterium shermanii*) methylmalonyl-CoA mutase have high affinity to DMB-Cba (K_m values of 0.021 and 0.024 μ M, respectively), whereas the K_m values for Bza-Cba were about an order in magnitude greater (Lengyel et al., 1960). In contrast, the glutamate mutase of *Clostridium tetanomorphum* preferred Bza-Cba over DMB-Cba with K_m values of 0.24 and 18 μ M, respectively (Barker et al., 1960). Preference for a cobamide with a specific lower base was also observed with the methionine synthase of *Spirulina platensis* strain NIES-39, which bound pseudo-B₁₂ with a K_m of 0.07 μ M, and a much higher K_m of 16.0 μ M was determined for DMB-Cba (Tanioka et al., 2010). These observations suggest that the affinity of the enzyme to cobamides with different lower bases affects the assembly of the functional holoenzyme, which can explain the observed lower base effects on reductive dechlorination rates and extents.

Differential transport of cobamides into the cell can affect RDase maturation and is another possible explanation for the observed decrease in dechlorination rates. However, the experimental data indicated that DMB-, 5-OMeBza- and Bza-Cba were equally transported across the surface layer/cytoplasmic membrane in *Dhc* strains BAV1 and GT, suggesting that differential cobamide uptake cannot explain the observed decrease in dechlorination performance. *Dhc* strains lack a peptidoglycan cell wall, and a *btuB* homolog for controlling cobamide translocation across the outer membrane into the periplasmic space is absent in the sequenced *Dhc* genomes (Chimento et al., 2003; Löffler et al., 2013b; Yi et al., 2012). Experimental evidence obtained with the human gut anaerobe *Bacteroides thetaiotaomicron* corroborated that BtuB, rather than the ATP-binding cassette corrinoid transporter system BtuFCD is the necessary component for selective cobamide transport (Degnan et al., 2014).

An unexpected finding was that the activity of the VC RDases BvcA and VcrA was differentially influenced by cobamides with distinct lower bases. Apparently, lower bases can exert post-translational control over corrinoid-dependent enzyme systems, in this example RDases. An observation supporting this hypothesis was made in the PCE-to-cDCE dechlorinator *Sulfurospirillum multivorans*. Following DMB addition, DMB replaced the native adenine lower base to generate nor-B₁₂, which inhibited PceA maturation and export, and impacted PCE

dechlorination and growth of *S. multivorans* (Keller et al., 2014). This finding further indicated that the lower base affects the binding between the cobamide and the apo-form of the RDase, and the incorporation of a cobamide with an unfavorable lower base impacts RDase maturation. In *Dhc* strains BAV1 and GT, inefficient maturation and impaired export of BvcA and VcrA due to incorporation of a corrinoid cofactor carrying unfavorable 5-OMeBza or Bza lower bases may explain the decreased dechlorination rates, or even the complete loss of VC dechlorination ability in strain GT.

The pure culture studies with cDCE/VC-dechlorinating *Dhc* strains revealed that methyl substitutions of the benzimidazole backbone at the 5 and 6 positions affect *Dhc* strains harboring *vcrA* (strain GT) or *bvcA* (strain BAV1) differently. Apparently, the growth-supporting benzimidazole type corrinoids are functionally not equivalent in *Dhc* strains with distinct RDases. In strain BAV1 (BvcA) and strain GT (VcrA), reductive dechlorination rates decreased in the order DMB-Cba > 5-MeBza-Cba > Bza-Cba, and a similar trend was observed in *Dhc* strain 195 (Yi et al., 2012). DMB-Cba supported the highest TCE dechlorination rates in strain 195 cultures, but no growth occurred with Bza-Cba. Further, *Dhc* strains are unable to grow with 5-OHBza-Cba in axenic culture or during co-cultivation with a 5-OHBza-Cba-producing methanogen, indicating that substitution of the hydrophobic methyl group with a hydroxyl group in the 5 position and removal of the methyl group in 6 position resulted in a non-functional lower base (Yan et al., 2013; Yi et al., 2012). Corrinoid cofactor-protein interactions are governed by hydrogen bonding, as revealed by the crystal structures of PceA and NpRdhA (Bommer et al., 2014; Payne et al., 2015). The findings presented here suggest that the methyl group substitutions in the 5 and 6 positions of the benzimidazole ring have relevant roles for VcrA and BvcA activity, and are possibly involved in stabilizing the mature RDase complex. Such methyl group-protein hydrophobic interactions provide a plausible explanation for the preference of the *Dhc* RDases BvcA (strain BAV1), VcrA (strain GT), and TceA (strain 195) for a corrinoid with DMB as the lower base.

The effects of the lower base on dechlorination rates and end points have implications for bioremediation practice. A common contaminated site management practice is to supply electron donor when dechlorination rates and extent following initial bioremediation treatment (i.e., biostimulation alone or combined with bioaugmentation) decrease (Löffler et al., 2006). The assumption is that hydrogen, the required electron donor for *Dhc*, is limiting and additions of fermentable carbon substrates increase hydrogen flux. Although repeated electron donor additions can sustain reductive dechlorination activity, evidence that hydrogen flux was actually limiting *Dhc* activity is difficult to ascertain. A carefully executed long-term study under conditions of sustained low hydrogen flux indicated that vitamin B₁₂ additions, not hydrogen, limited *Dhc* reductive dechlorination activity and VC to ethene conversion (Fennell et al., 1997). In contaminated aquifers, organohalide-respiring *Chloroflexi* depend on corrinoid scavenging to acquire this essential cofactor from the environment. Depending on the site biogeochemical conditions and the type of substrate(s) used for biostimulation (i.e., electron donors), microorganisms that produce the “wrong” lower base(s) may dominate. In such scenarios, continued biostimulation and bioaugmentation will not sustain *Dhc* activity and lead to efficient contaminant detoxification. Knowledge of the exact lower base requirements of the keystone corrinoid-auxotrophic dechlorinators, as well as the biogeochemical conditions that favor the synthesis of required growth factors by indigenous microbes, will enable strategies to overcome nutritional limitations of organohalide-respiring *Chloroflexi* and promote faster detoxification rates.

Task 6: Database mining

Available biogeochemical datasets from sites impacted with chlorinated ethenes were collected to explore if the integrated analysis of existing information can be used to develop a predictive understanding of ethene formation (i.e., detoxification). To accomplish this goal, a machine learning-based data mining approach using the classification and regression tree (CART) algorithm was applied. The two major goals were (i) to determine relevant parameters that affect *in situ* reductive dechlorination potential, and (ii) to demonstrate the value of a data mining approach for generating predictive models that identify the most promising remediation strategy at a specific site.

Preselection of Geochemical Parameters Associated with Reductive Dechlorination Potential

Correlations between variables were assessed using the Spearman's rank correlation coefficient as shown in Table 39. CH₄, ORP, NO₃⁻, NO₂⁻, Fe²⁺, SO₄²⁻, and TOC showed highly significant ($p < 0.001$) correlations with the 3-month-ahead DP class. *Dhc* 16S rRNA gene copy numbers showed significant ($p < 0.01$) correlations, and weaker correlations ($p < 0.05$) were observed with Cl⁻ concentrations with the 3-month-ahead DP class. Since the lower number DP class reflects high dechlorination potential (i.e., Class 1 > Class 2 > Class 3 in terms of the 3-month-ahead dechlorination potential), *Dhc* 16S rRNA gene copy numbers, CH₄ and Fe²⁺ concentrations, and TOC amounts showed negative correlations with the DP class, whereas ORP and NO₃⁻, NO₂⁻, and SO₄²⁻ concentrations had positive correlations with the DP class. For example, a higher *Dhc* 16S rRNA gene abundance was a strong indicator for dechlorination potential, and, therefore, correlated with DP Class 1. Likewise, high NO₃⁻ concentrations, which showed negative correlation with the DP class, served as an indicator of low dechlorination potential (i.e., DP Class 3). Unexpectedly, pH and DO, which had been reported to be critical parameters for reductive dechlorination activity, (Amos et al., 2008; Lacroix et al., 2014; Robinson et al., 2009; Taş et al., 2010) showed no significant correlations with dechlorination potential. As a result, eight parameters associated with reductive dechlorination activity including *Dhc* 16S rRNA gene copies, CH₄, Fe²⁺, NO₃⁻, NO₂⁻, SO₄²⁻, TOC, and ORP, were selected as independent variables to develop the CART model.

Table 39. Spearman's rank correlation coefficients (ρ) between variables.

	(1) DP class (N=187)	(2) <i>Dhc</i> (N=187)	(3) CH ₄ (N=187)	(4) pH (N=186)	(5) DO (N=186)	(6) ORP (N=183)	(7) NO ₃ ⁻ (N=181)	(8) NO ₂ ⁻ (N=144)	(9) Fe ²⁺ (N=157)	(10) SO ₄ ²⁻ (N=187)	(11) TOC (N=187)	(12) Cl ⁻ (N=181)
(1)	1.000	-0.250 ^b	-0.395 ^a	0.027	0.137	0.323 ^a	0.447 ^a	0.348 ^a	-0.297 ^a	0.395 ^a	-0.289 ^a	0.154 ^c
(2)		1.000	0.389 ^a	-0.048	-0.238 ^b	-0.315 ^a	-0.295 ^b	-0.218 ^b	0.337 ^b	-0.184 ^c	0.119	0.269 ^b
(3)			1.000	-0.286 ^{***}	-0.309 ^{***}	-0.375 ^a	-0.445 ^a	-0.175 ^c	0.578 ^a	-0.436 ^a	0.293 ^a	-0.005
(4)				1.000	0.077	-0.128	-0.044	-0.316 ^a	-0.291 ^a	0.039	-0.174 ^c	-0.266 ^a
(5)					1.000	0.187 ^c	0.415 ^a	0.302 ^a	-0.214 ^b	0.089	-0.176 ^c	-0.240 ^b
(6)						1.000	0.339 ^a	0.188 ^c	-0.0289 ^a	0.301 ^a	-0.210 ^b	-0.069
(7)							1.000	.593 ^a	-0.174 ^c	0.215 ^b	-0.039	-0.089
(8)								1.000	0.114	0.253 ^b	0.052	0.108
(9)									1.000	-0.250 ^b	0.160 ^c	0.051
(10)										1.000	-0.371 ^a	0.314 ^a
(11)											1.000	0.190 [*]
(12)												1.000

*** p < 0.001, ** p < 0.01, * p < 0.05

Selection of the Representative Model and Important Parameters for Model Construction

After the 10-fold cross-validation for 100 randomly shuffled datasets, 100 individual classification trees for each partitioning ratio (70:30 and 80:20) were constructed and performance indices (i.e., AUC_{total} and true positive rates) were calculated for the training and test sets (Table 40).

Table 40. Percentage of acceptable trees and performance indices of the decision tree models representing two different data partitioning ratios.

Acceptable trees had an average area under the ROC curve (AUT_{TOT}) above 0.7.

Data partitioning ratio			70:30	80:20
Percentage of acceptable trees (AUC _{TOT} >0.7)	Training sets		97%	98%
	Test sets		69%	72%
AUC _{TOT} of the best CART models for each partitioning ratio	Training set		0.8151	0.8214
	Test set		0.7707	0.7617
True positive rates of the best CART models for each partitioning ratio	Training set	Class 1	71.1%	72.7%
		Class 2	80.9%	81.1%
		Class 3	74.4%	69.4%
		Overall	75.8%	74.7%
	Test set	Class 1	77.8%	58.3%
		Class2	76.2%	73.3%
		Class 3	55.0%	50.0%
		Overall	69.5%	61.0%

The percentage of acceptable trees ($AUT_{TOT}>0.7$) for the 80:20 CART models (98% for the training sets and 72% for the test sets) was slightly higher than the percentage for the 70:30 CART models (97% for the training sets and 69% for the test sets). The best CART models for each partitioning ratio also did not show significant differences in the AUC_{total} for both the training and the test sets. However, the 70:30 model had higher true positive rates than the 80:20 model for the test sets (69.5% and 61.0% for the 70:30 and the 80:20 model, respectively), especially for Class 1 (77.8% and 58.3%) and Class 3 (55.0% and 50.0%), and thus, the best CART model for the partitioning ratio of 70:30 was selected as the representative model (Table 40).

Based on these observations, a relative importance ranking of individual parameters towards dechlorination potential was possible, as shown in Table 41. Overall, the presence of NO_3^- , NO_2^- , and Fe^{2+} took precedence over other parameters. The TOC amount, the CH_4 concentration, and the *Dhc* 16S rRNA gene abundance were also essential parameters to predict reductive dechlorination potential. The CART algorithm excluded ORP as a parameter for constructing the model, indicating that ORP data did not improve model performance.

Table 41. Relative importance of independent variables used for constructing the representative classification tree

Independent Variable	Relative Importance ^a
NO ₃ ⁻	1.000
NO ₂ ⁻	0.9368
TOC	0.8295
Fe ²⁺	0.7029
CH ₄	0.6114
<i>Dhc</i>	0.4085

^a Relative importance scores were determined by the CART algorithm based on the usefulness of the variables over all possible splits.

Splitting Rules of the Representative Model

The representative CART model is shown in

Figure 66. A line with the gray mark in a termination node is the final predicted DP class, and the right column in each node shows the number and percentages of the training cases meeting the splitting criteria.

- (1) The first split of the tree was based on NO₃⁻ concentrations. Among 128 cases in the entire training set, NO₃⁻ concentrations from 102 cases were below 2.3 mg/L and the remaining 26 training cases had NO₃⁻ concentrations exceeding 2.3 mg/L.
- (2) For 102 cases with NO₃⁻ concentrations below 2.3 mg/L, the second splitting criterion was the NO₂⁻ concentration. When NO₃⁻ below 2.3 mg/L and NO₂⁻ not exceeding 0.15 mg/L, active production of ethene could be observed in the majority of wells (88.9%) after 3 months.
- (3) For 26 cases with NO₃⁻ concentrations exceeding 2.3 mg/L, TOC was the second splitting criterion. When NO₃⁻ concentrations exceeded 2.3 mg/L and TOC amounts were higher than 13.0 mg/L, the monitoring well was likely to be categorized as DP Class 2, characteristic of VC stalls.
- (4) With NO₃⁻ concentrations exceeding 2.3 mg/L and TOC levels below 13.0 mg/L, dechlorination of polychlorinated ethenes was slow or did not occur at all (DP Class 3).
- (5) For 84 cases with NO₃⁻ < 2.3 mg/L and NO₂⁻ ≥ 0.15 mg/L, Fe²⁺ concentration was the third splitting criterion. With concentrations of NO₃⁻ below 2.3 mg/L, NO₂⁻ above 0.15 mg/L, and Fe²⁺ exceeding 16.1 mg/L, all the wells showed active ethene formation over the 3-month time window.
- (6) For 78 cases with NO₃⁻ < 2.3 mg/L, NO₂⁻ ≥ 0.15 mg/L, and Fe²⁺ < 16.1 mg/L, the CH₄ concentration served as the fourth splitting criterion. With concentrations of NO₃⁻ below 2.3 mg/L, NO₂⁻ above 0.15 mg/L, Fe²⁺ not exceeding 16.1 mg/L, and CH₄ above 0.3 mg/L, the majority of the training cases belonged to DP Class 2 (67.3%).
- (7) Twenty-nine training cases with CH₄ concentrations below 0.3 mg/L were divided into two subgroups based on the abundance of *Dhc* 16S rRNA genes. When *Dhc* 16S rRNA gene copy

numbers were below $1.11\text{E}+05$ cells/L, PCE/TCE or cDCE dechlorination did not occur, and no ethene was produced over the 3-month time window.

- (8) For 19 cases with $\text{NO}_3^- < 2.3$ mg/L, $\text{NO}_2^- \geq 0.15$ mg/L, $\text{Fe}^{2+} < 16.1$ mg/L, $\text{CH}_4 < 0.3$ mg/L, and *Dhc* 16S rRNA gene copies $\geq 1.11\text{E}+05$ cells/L, the TOC amount was the final splitting criterion. With TOC exceeding 18.1 mg/L, ethene was produced over the 3-month time period, whereas no PCE/TCE dechlorination and cDCE stalls were observed at lower TOC levels.

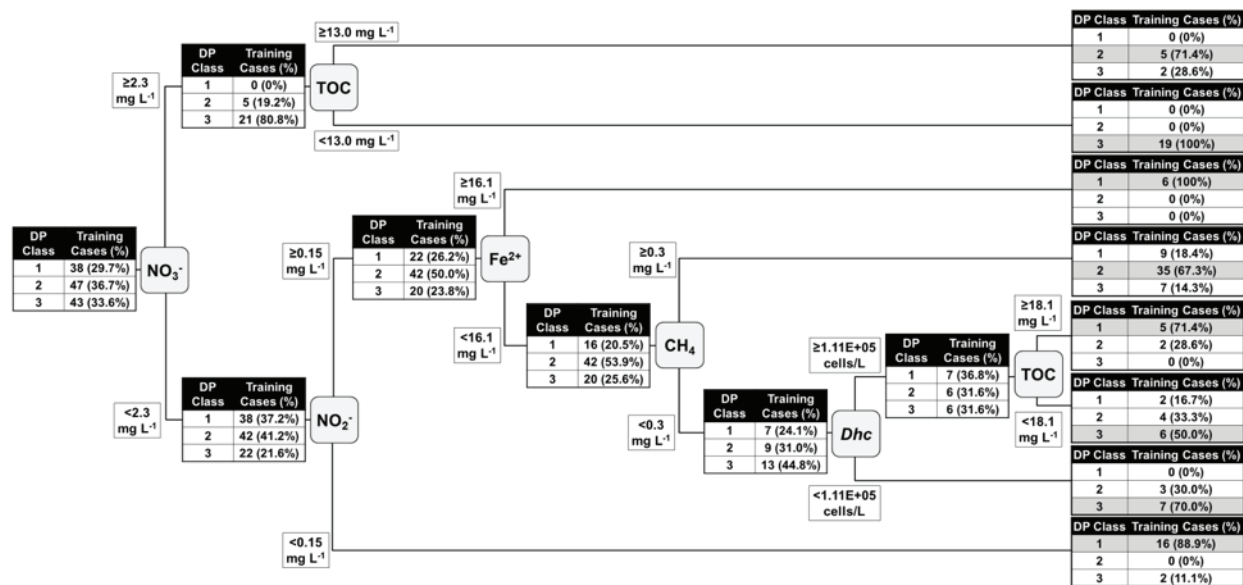


Figure 66. The representative CART model to predict the 3-month-ahead dechlorination potential in the contaminant plume. See text for details.

The lack of reliable tools for deciding on the most promising remediation strategy at contaminated sites presents a major challenge for remediation project managers, and decisions based on weak and unreliable evidence can exacerbate the environmental impact and nullify substantial financial investments. One of the main purposes of the present study was to demonstrate the utility of a data mining model using a machine learning algorithm to predict the detoxification potential at sites contaminated with chlorinated ethenes. The representative CART model was capable of effectively classifying the 3-month-ahead dechlorination potential with 75.8% and 69.5% true positive rates for the training and the test set, respectively. The findings demonstrated that the machine learning-based data mining approach is a promising tool to rank various measurable biogeochemical parameters by relative importance and to build a prediction model using extensive groundwater monitoring data sets for assessing in situ reductive dechlorination potential.

Dhc are strict anaerobes and sensitive to oxygen, and thus, DO affects *Dhc* dechlorinating activity (Adrian et al., 2007a; Amos et al., 2008; Atashgahi et al., 2013; He et al., 2003a). Similarly, inhibitory effects of low pH on dechlorination activity have been observed in the laboratory and under in situ conditions (Lacroix et al., 2014; Robinson et al., 2009; Stroo et al., 2013; Taş et al., 2010; Wiedemeier et al., 1998). Thus, the observation that DO and pH did not show significant correlations with the DP class is counterintuitive and was unexpected (Table 39). However, it

should be noted that small correlation coefficients do not mean that pH and DO are not important parameters for the reductive dechlorination process. Regarding pH, the range of values observed in the majority of wells was conducive for reductive dechlorination activity (95% confidence interval 6.69 to 6.84) (Figure 67a). The lack of data sets from low (pH < 6) and high (pH > 7.5) pH aquifers was a likely reason that the analysis did not reveal correlations between groundwater pH and reductive dechlorination activity. Likewise, most (76%) of the DO values were below 2 mg/L, which indicates oxygen-limited (most likely anoxic) conditions, favoring alternate electron accepting processes (95% confidence interval 1.2 mg/L to 2.2 mg/L) (Figure 67b).

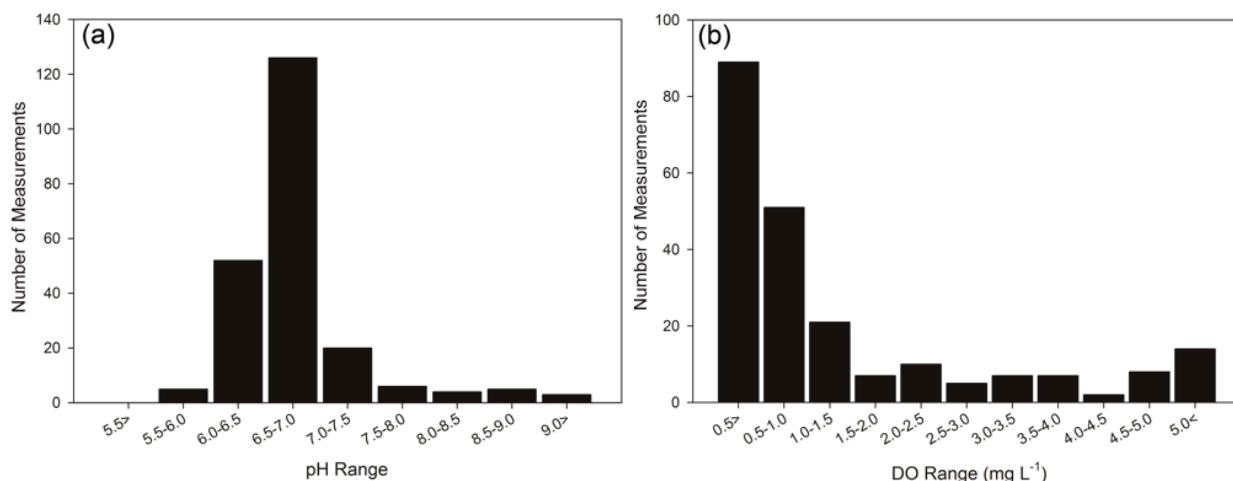


Figure 67. Distribution of pH values and dissolved oxygen concentrations in the groundwater.

Additionally, DO measurements are notoriously unreliable because oxygen contamination and improper calibration of the oxygen-sensitive electrode make the interpretation of low DO concentration measurements tenuous (Wilkin et al., 2001). More reliable indicators of anoxic conditions would be the presence of CH₄ and Fe²⁺, and these measurable parameters should be given more weight for identifying conditions conducive for reductive dechlorination. The conclusions drawn from a study focused on interpreting *Dhc* abundance and observed reductive dechlorination rates support these recommendations (Lu et al., 2006b). Laboratory experiments explored the effects of single parameters on *Dhc* reductive dechlorination performance, and these efforts contributed to the development of guidelines and protocols to estimate *in situ* reductive dechlorination potential (Environmental Restoration Project ER-0518) (Stroo et al., 2013; Wiedemeier et al., 1998).

In situ, *Dhc* experience fluctuating environmental conditions and the analysis of multiple, interrelated (i.e., dependent) parameters is required to improve predictive understanding of *in situ* reductive dechlorination potential. In contrast to previous attempts to correlate geochemical parameters with reductive dechlorination activity, only the data mining approach considers interrelationships and reveals synergistic and antagonistic effects between individual parameters or groups of parameters, which represents a major advance. Extensive groundwater monitoring data have been collected for decades, suggesting that more comprehensive integrative analyses with much larger data sets are theoretically feasible. Unfortunately, existing databases are data-rich-but-information-poor, due to (i) a lack of standardized sampling protocols, that is, inconsistent monitoring schedules and parameter sets, and (ii) poor data curation and availability. These limitations were communicated in the 1980s (Ward et al., 1986) but efforts to rectify these

shortcomings have fallen short and these issues continue to hamper effective metadata analysis (Ronen et al., 2012; Timmerman et al., 2010; Ward et al., 1986) Thus, a concerted effort to build a systematic, comprehensive and open-access database would enable the development of more accurate and robust data mining approaches so that contaminated sites can be categorized and prioritized to ensure that cleanup goals are achieved in the most economical and environmentally benign fashion. The investments for establishing uniform groundwater monitoring regimes and maintaining a curated, open-access database are small compared to the potential cost-savings realized by more targeted remediation technology implementation.

Task 7: Apply new tools to DoD sites impacted with chlorinated solvents

Application of RD-qChip v2 to DoD Sites

Validation of the RD-qChip v2 with groundwater samples from contaminated sites

The RD-qChip v1 was designed for laboratory testing and optimization and was not intended for testing of environmental samples. The goal was to apply the improved RD-qChip v2 for field testing, but several issues delayed these efforts, including the transition to LDFs for standard curve preparation, longer than expected wait time for qChip (i.e., array plate) manufacture, and instrument downtime and repairs. When the RD-qChip v2 array plates finally became available in 2019, we then encountered major difficulties in obtaining samples from field sites. For tool validation, we were interested in samples from sites where baseline characterization (e.g., geochemical data, contaminant concentrations) had been performed, information about (prior) treatment was available, and temporal sample collection was anticipated. Unfortunately, we were not successful obtaining samples in 2018 and in 2019. An opportunity arose in early 2020, and two team members supported groundwater sampling efforts at Site 9 at the Naval Air Station North Island (NASNI) in San Diego. An additional sampling effort occurred at the Lordstown site in Ohio, which is impacted with chlorinated ethenes. These samples were received by the University of Tennessee in early March 2020 and immediately processed. DNA was extracted and prepared for qPCR analysis with the RD-qChip v2 array plates. Unexpectedly, the SARS-CoV-2 pandemic shut down the laboratory and we were unable to analyze the samples. In June 2020, we received permission to access the laboratory facilities that house the QuantStudio PCR system and DNA samples from Site 9 at NASNI, San Diego were run on the RD-qChip v2 array plates. The samples collected at Lordstown site in Ohio yielded very low DNA concentrations. The *Dhc* abundances in the Lordstown DNA samples were determined with the regular *Dhc* 16S rRNA gene-targeted assay in 384-well plate. The results indicated very low *Dhc* abundances with *Dhc* 16S rRNA genes in the detectable range but not quantifiable (DNQ). Therefore, the Lordstown samples were not analyzed on the RD-qChip v2 array plates.

Groundwater samples from Site 9 at NASNI

The Naval Air Station North Island is located in San Diego, CA. The reported primary contaminants of concern at Site 9 include chlorinated ethenes (TCE, cDCE and VC); chlorinated ethanes (1,1,1-TCA and 1,1-DCA); chlorinated methanes (DCM) and 1,4-dioxane. The selected wells and relevant groundwater geochemical parameters are listed in Table 42.

Table 42. Geochemical parameters of groundwater samples collected at NASNI Site 9.

Well ID	GW depth (ft.)	Temp. (°C)	DO (mg/L)	pH	ORP (mV)
S9-IMW-1	8.42	22.4	-0.01	7.30	-209.7
S9-MW-52	19.75	21.6	0.05	6.83	-192.1
S9-MW60-L3	8.60	22.8	0.02	6.53	-99.8
S9-MW60-L5	8.75	23.1	0.46	6.76	-99.4
S9-MW-60-L7	8.22	23.1	0.00	6.99	-321.8
S9-MW-71-L3	9.48	21.9	0.63	7.37	-116.1
S9-MW74L3	15.83	21.2	0.87	7.13	-30.5

[illegible]

Table 43. Information about well locations at Site 9 at NASNL.

Well ID	Location of monitoring well in reference to the source area
S9-IMW-1	Source Area, Layer 3
S9-MW-52	Mid-Plume, Layer 3
S9-MW-60-L3	Source Area, Layer 3
S9-MW-60-L5	Source Area, Layer 3
S9-MW-60-L7	Source Area, Layer 7
S9-MW-71-L3	Shoreline Cluster #2, Layer 3 (in flow path)
S9-MW-74-L3	Mid-Plume, Layer 3 (in flow path)

Groundwater samples from Site 9 at NASNI were collected and passed through Sterivex-GP cartridges *on site* for biomass collection following established procedures (Amos et al., 2008;

Johnson et al., 2005b; Ritalahti et al., 2010a). The Sterivex cartridges were shipped on dry ice using an overnight carrier service to the Löffler lab at the University of Tennessee. Upon arrival, the Sterivex cartridges were immediately transferred and stored at -80°C to preserve biomarker integrity. DNA was isolated from the Sterivex cartridges as described (Amos et al., 2008; Johnson et al., 2005b; Ritalahti et al., 2010a) using the MoBio PowerLyzer PowerSoil Kit (MoBio, Carlsbad, CA) according to the manufacturer's recommendations. DNA concentrations were quantified using the Qubit dsDNA BR Assay (Life Technologies, Grand Island, NY) according to the manufacturer's manual. DNA samples were stored at -80°C until analysis.

For qPCR analysis, each sample was diluted to three different (i.e. 1:10, 1:100 and 1:1,000) dilutions in nuclease-free water to determine if any compounds were present that would interfere with PCR. The *Dhc* 16S rRNA gene assay was chosen to demonstrate the presence of any compound interference (Ritalahti et al., 2006). The *Dhc* 16S rRNA gene assay was performed using the 384-well plate format on the QuantStudio 12K Flex Real-Time PCR instrument. The most dilute sample that gave the best fit within the template DNA standard curve was chosen for further qPCR analysis. For Site 9 samples, the 1:10 dilutions were chosen for analysis using the RD-qChip v2 array plates.

The selected samples collected from seven wells at Site 9 (Table 43) were analyzed in triplicate on the RD-qChip v2 array plates. Triplicate, negative controls consisting of nuclease-free, molecular grade water accompanied each array plate. Quantification of target gene copy numbers was performed by using standard curves generated with LDF mixtures and obtained on the same RD-qChip v2 array plate (see Task 1, Validation of RD-qChip v2).

16S rRNA gene-targeted qPCR analysis of Site 9 samples

In order to validate RD-qChip v2 with environmental samples collected from contaminated sites, seven groundwater samples collected from Site 9 at NASNI (Table 43) were analyzed on RD-qChip v2 array plates. The target 16S rRNA gene assays placed on the RD-qChip v2 are listed in Table 21. For initial regular qPCR analysis, each sample was diluted 1:10, 1:100 and 1:1,000 in nuclease-free water to determine if any compounds were present that would interfere with PCR. The *Dhc* 16S rRNA gene assay was chosen to demonstrate the presence of potential inhibitor interference (Ritalahti et al., 2006). The regular *Dhc* 16S rRNA gene-targeted qPCR assays were performed in the 384-well plate format available on the QuantStudio 12K Flex Real-Time PCR instrument. The least dilute sample that gave the best fit within the template DNA standard curve was determined, and for Site 9 samples, the 1:10 dilutions were chosen for analysis using the RD-qChip v2 array plates. The selected dilution samples were analyzed in triplicate on the RD-qChip v2 array plates. Triplicate, negative controls consisting of nuclease-free, molecular grade water accompanied each array plate. Quantification of target gene copy numbers was performed by using standard curves generated with LDF mixtures and obtained on the same RD-qChip v2 array plate (see Task 1, Validation of RD-qChip v2).

The application of the RD qChip v2 to the total DNA obtained from biomass collected from groundwater obtained from monitoring wells at Site 9 demonstrated the presence of bacteria (assay TBAC_16S) and methanogenic archaea (assay METH_16S). Total bacterial 16S rRNA gene abundances in the samples varied between 1.98E+04 (IMW-1) to 1.22E+06 (MW-71-L3) copies/mL (Figure 69). The abundance of methanogenic archaea in these samples ranged from 6.24E+02 to 5.90E+05 cells/mL. The lowest abundances of 16S rRNA gene sequences of methanogenic archaea were observed for MW-71-L3 and MW-60-L3 with 6.24E+02 and

7.95E+02 gene copies/mL, respectively. The highest abundance was determined for sampling location MW-60-L7 with 5.90E+05 gene copies/mL. Methanogens are commonly reported to co-occur with *Dhc* in dechlorinating enrichment cultures (Men et al., 2012). While co-occurrence may simply be explained by the preference of similar redox environments, some reports hint at specific interactions that benefit *Dhc* (He et al., 2007; Heimann et al., 2006; Wen et al., 2015)

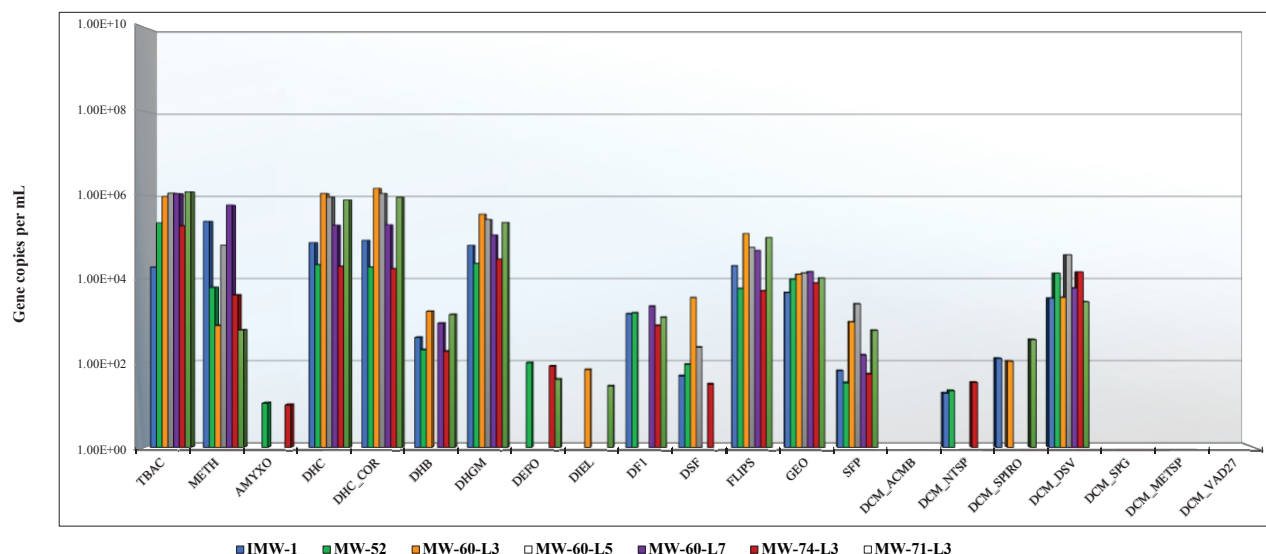


Figure 69. Abundance of 16S rRNA genes in Site 9 groundwater at NASNI.

The analysis was performed with the RD qChip v2 array plate using DNA extracted from biomass collected with Sterivex-GP cartridges from monitoring wells installed at Site 9 at NASNI. For specific information about the assays, see Table 21.

The RD qChip v2 contains assays targeting the 16S rRNA genes of members of the class Dehalococcoidia, specifically *Dhc*, *Dhgm*, and ‘*Ca. Dehalobium chlorocoercia*’. The *Dhc* 16S rRNA gene-targeted assay (DHC_16S) detected *Dhc* in samples collected at all seven well locations. The abundance of *Dhc* cells ranged from 7.51E+04 (IMW-1) to 1.12E+06 (MW-60-L3) cells/mL. It has been reported that *Dhc* abundance should be near or higher than 10^7 *Dhc* cells per liter of groundwater (10^4 *Dhc* cells/ mL of GW) to produce a beneficial rate of $\geq 0.3 \text{ year}^{-1}$ (Imfeld et al., 2008; Lebron, 2011; Lu et al., 2006a; Lu et al., 2006b; Ritalahti et al., 2010b). The *Dhc* abundance in Site 9 groundwater exceeds this 10^7 *Dhc* cells per liter threshold suggesting that the *in situ* dechlorination rates exceed 0.3 year^{-1} . Therefore, *in situ* attenuation of chlorinated ethenes is likely occurring at Site 9 at NASNI, at least in the vicinity of the monitoring wells sampled in this effort (Table 43). Of note, the abundances Cornell group *Dhc* strains, which were obtained with the DHCCOR_16S assay, approximately matched the total *Dhc* abundance obtained with the DHC_16S assay, indicating that the *Dhc* population at Site 9 is dominated by Cornell-type *Dhc*. Among the three *Dhc* subgroups, only Cornell-type *Dhc* strains have the ability to fix dinitrogen, suggesting that the native *Dhc* population can respond to fixed nitrogen limitations (Kaya et al., 2019; Lee et al., 2009).

Also abundant in Site 9 groundwater were 16S rRNA genes of the genus *Dehalogenimonas*. The presence of *Dhgm* was detected in biomass collected from all seven groundwater sampling

locations. The *Dhgm* 16S rRNA gene abundances ranged between 2.40E+04 and 3.58E+05 copies/mL. As is the case for *Dhc*, the known *Dhgm* genomes harbor a single 16S rRNA gene, indicating that the 16S rRNA gene abundances represent that cell numbers. Similar to *Dhc*, members of the genus *Dhgm* are strictly organohalide-respiring bacteria and can only grow when suitable chlorinated electron acceptors are available. *Dehalogenimonas* spp. have been implicated in reductive dechlorination of chlorinated ethenes (Manchester et al., 2012; Molenda et al., 2016; Yang et al., 2017c) and chlorinated alkanes (Key et al., 2017; Moe et al., 2009). The presence of *Dhgm* 16S rRNA genes ranging between 2.40E+04 and 3.58E+05 copies/mL indicate that members of this genus utilize chlorinated compounds as electron acceptors and contribute to contaminant attenuation at Site 9.

The presence of the 16S rRNA gene representing ‘*Ca. Dehalobium chlorocoercia*’ was demonstrated for groundwater collected from five of the seven sampling locations, and 16S rRNA gene abundances ranged between 7.99E+02 to 1.60E+03 copies/mL. Only the samples collected from MW-60-L3 and MW-60-L5 yielded negative results. ‘*Ca. Dehalobium chlorocoercia*’ strain DF-1 is a PCB-respiring bacterium capable of dechlorinating certain PCB congeners (May et al., 2008). In addition, strain DF-1 was shown to dechlorinate chlorinated benzenes with doubly flanked chlorines and PCE/TCE to cDCE and tDCE (Adrian et al., 2016; Miller et al., 2005).

Dehalobacter 16S rRNA genes were present in abundances ranging between 1.94E+02 and 1.73E+03 copies/mL and no *Dhb* 16S rRNA genes were detected in MW-60-L5. *Dehalobacter* spp. are strictly organohalide-respiring bacteria that have been implicated in reductive dechlorination of a variety of chlorinated compounds including chlorinated ethenes (PCE, TCE), chlorinated ethanes (TCA, DCA), CF, chlorinated phenols, and chlorinated benzenes. The RDase for dechlorination of PCE and TCE, PceA, of *Dehalobacter restrictus* strain PER-K23 (PCE→cDCE) has been characterized (Holliger et al., 1998b; Kruse et al., 2013; Maillard et al., 2003). *Dhb* strains with other RDases, including CfrA, TmrA, ThmA (CF→DCM; 1,1,1 TCA→1,1 DCA) (Tang et al., 2013b; Wong et al., 2016; Zhao et al., 2017b), DcrA (1,1 DCA→CA) (Tang et al., 2013b) and DcaA (1,2-DCA→ethene) (Grostern et al., 2009) have been reported. *Dehalobacter* are not uncommon in anoxic environments but occur in low abundances often escaping detection, presumably because chlorinated electron acceptors in natural, uncontaminated environments are scarce. The observed abundances ranging from 1.94E+02 to 1.73E+03 *Dhc* 16S rRNA gene copies/mL at Site 9 suggest that growth of *Dhb* occurred *in situ*, and *Dhb* utilize chlorinated contaminants as electron acceptors. It is likely that members of this genus contribute to contaminant attenuation at Site 9.

Assay DEFO_16S quantified *Dehalobacterium formicoaceticum* 16S rRNA genes at abundances of 1.03E+02, 8.60E+01 and 4.16E+01 per mL in wells MW-52, MW-74-L3 and MW-71-L3, respectively. *Dehalobacterium formicoaceticum* utilizes DCM as carbon and energy source and produce formate and acetate (Chen et al., 2017; Mägli et al., 1996). The 16S rRNA genes of ‘*Ca. Dichloromethanomonas elyunquensis*’, another bacterium capable of anaerobic DCM catabolism yielding acetate, H₂ and CO₂ (Chen et al., 2020; Chen et al., 2018; Kleindienst et al., 2017), were only detected in MW-60-L3 and MW-71-L3 at abundances of 7.08E+01 and 2.88E+01 per mL. These findings indicate that the bacteria capable of degrading DCM are present at Site 9 and are likely contributing to DCM degradation. Of note, based on the current understanding of the physiology of *Dehalobacterium formicoaceticum* and ‘*Ca. Dichloromethanomonas elyunquensis*’, both organisms can only grow with DCM as an energy source, linking their presence with DCM degradation at Site 9.

16S rRNA genes representing the genus *Desulfitobacterium* were detected at abundances ranging between $3.19\text{E}+01$ and $3.68\text{E}+03$ copies/mL in biomass collected from groundwater of wells IMW-1, MW-52, MW-60-L3, MW-60-L5 and MW-74-L3. *Desulfitobacterium* 16S rRNA genes were not detected in wells MW-60-L7 and MW-71-L3. *Desulfitobacterium* species are widely distributed in the environment and several members of this genus have the ability to perform organohalide respirations. For example, *Desulfitobacterium* have been reported to utilize chlorinated ethenes (PCE, TCE) (Bouchard et al., 1996; Reinhold et al., 2012), chlorinated ethanes (TCA, DCA) (Ding et al., 2014; Kunze et al., 2017; Marzorati et al., 2007), chlorinated phenols (TCP, 2,3-DCP, PCP) (Alfán-Guzmán et al., 2017; Boyer et al., 2003), and chloroform (Ding et al., 2014) as electron acceptors. Of note, *Desulfitobacterium* are metabolically versatile and can grow with a variety of electron donors and electron acceptors. Therefore, *in situ* growth of *Desulfitobacterium* spp. cannot be unambiguously linked to reductive dechlorination without more detailed experimentation (e.g., the expression of functional *Desulfitobacterium* biomarkers).

The GEO_16S assay enumerates 16S rRNA gene sequences affiliated with the genus *Geobacter*. *Geobacter* 16S rRNA genes were detected in groundwater collected from all sampling locations and varied in abundance from $4.87\text{E}+03$ to $1.42\text{E}+04$ cells/mL. Similar to *Desulfitobacterium*, *Geobacter* spp. share versatile metabolisms and can use a variety of electron donor/electron acceptor combinations to support growth. *Geobacter lovleyi* can utilize PCE and TCE as electron acceptors for organohalide respiration, and this process co-occurs during ferric iron reduction. Thus, *Geobacter lovleyi* strains may contribute to PCE/TCE reductive dechlorination to cDCE, but additional experimental efforts (e.g., the expression of the PceA RDase of *Geobacter lovleyi*) are needed to determine if *Geobacter* contribute to reductive dechlorination in the Site 9 aquifer.

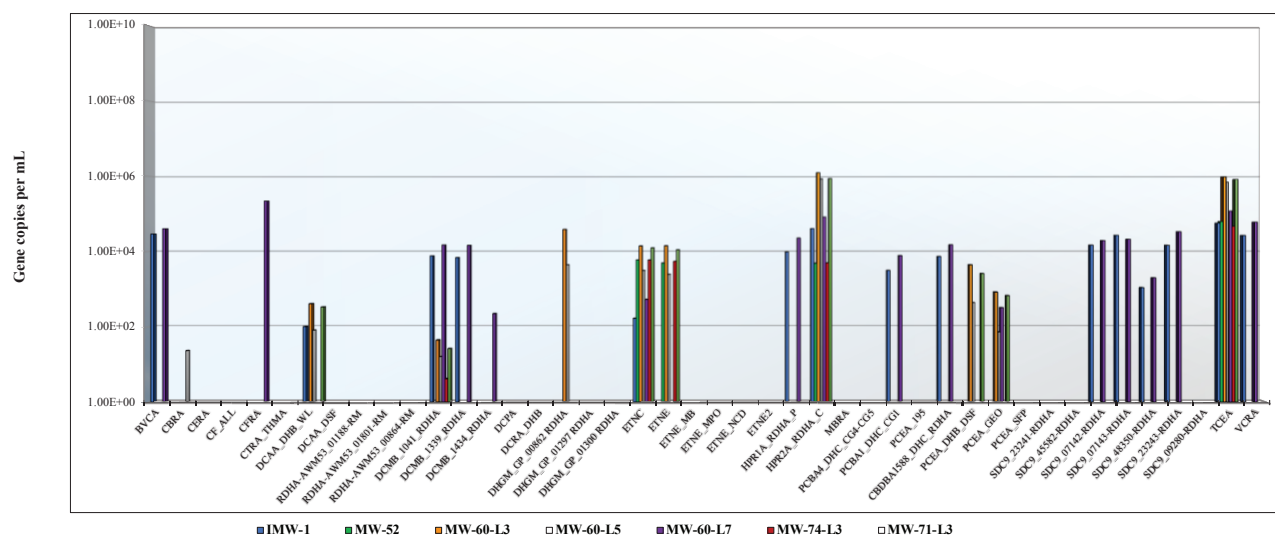
Many *Geobacter* spp. have the ability for *de novo* corrinoid biosynthesis and at least some generate cobamides that support the activity of *Dhc* (Yan et al., 2013; Yan et al., 2012). Therefore, the abundance of *Geobacter* suggests that the microbial community present at Site 9 has the ability to generate cobamides that support *Dhc* activity (and presumably the activity of other corrinoid-auxotrophic Dehalococcoidia) (Yan et al., 2016).

Sulfurospirillum 16S rRNA genes were present at abundances ranging from $3.42\text{E}+01$ to $2.62\text{E}+03$ copies/mL in groundwater from all seven well locations. Some *Sulfurospirillum* species possess a specific *pceA* gene and dechlorinate PCE to TCE or PCE/TCE to cDCE (Butt et al., 2013; Huo et al., 2020; Neumann et al., 1996). Recent efforts suggested that at least some *Sulfurospirillum* spp. perform organohalide respiration under acidic pH conditions (e.g., pH 5.5) (Yang et al., 2017a). *Sulfurospirillum* spp. have versatile metabolisms and can grow with various substrates. Therefore, the presence of *Sulfurospirillum* 16S rRNA genes is not evidence that these organism are actively involved in reductive dechlorination at Site 9, and additional measurement (e.g., expression of the specific PceA RDase) would be needed confirm their role in reductive dechlorination.

Desulfovibrio (assay DCM_DSV_16S) are commonly present in organohalide-respiring enrichment cultures but direct involvement in dechlorination has not been demonstrated. So far, the roles of *Desulfovibrio* for supporting the reductive dechlorination process have remained elusive (May et al., 2008). At Site 9, *Desulfovibrio* 16S rRNA gene abundances ranging between $2.94\text{E}+03$ and $3.85\text{E}+04$ copies/mL were measured (Figure 69). *Desulfovibrio* are known sulfate-reducing and fermenting bacteria. A co-culture study with *Dhc* strain 195 and *Desulfovibrio vulgaris* Hildenborough revealed that *Desulfovibrio* had important function for supplying nutrients to *Dhc*. Specifically *Desulfovibrio* fermented lactate to provide H_2 as electron donor, acetate as

carbon source, and *Desulfovibrio* served as a source of cobalamin and amino acids to support the growth of *Dhc* (Men et al., 2012; Men et al., 2014).

Quantitative analysis of functional genes encoding RDase and VC Oxidases in Site 9 NASNI groundwater



The *tceA* gene was detected in samples from all seven well locations in abundances ranging from 5.71E+04 to 9.62E+05 copies/mL (Table 44). *tceA* occurs as a single copy gene on the known *Dhc* genomes, indicating that the *tceA* gene copy numbers represent *Dhc* cells carrying *tceA*. The data indicate that at least one *Dhc* strain present in Site 9 groundwater carries the *tceA* gene which dechlorinates TCE to VC and ethene. The TceA RDase has been implicated in TCE reductive dechlorination to VC and ethene (Löffler et al., 2013b).

Table 44. Quantitative assessment of *tceA*, *vcrA* and *bvcA* genes in Site 9 groundwater.

Sampling locations (Well ID)	<i>tceA</i> gene copies	<i>vcrA</i> gene copies	<i>bvcA</i> gene copies
S9-IMW-1	5.71E+04	2.69E+04	2.93E+04
S9-MW-52	6.30E+04	Not detected	Not detected
S9-MW-60-L3	9.62E+05	Not detected	Not detected
S9-MW-60-L5	7.00E+05	Not detected	Not detected
S9-MW-60-L7	1.19E+05	6.08E+04	4.04E+04
S9-MW-71-L3	4.69E+04	Not detected	Not detected
S9-MW-74-L3	8.29E+05	Not detected	Not detected

The *vcrA* gene was detected in well location IMW-1 and MW-60-L7 at abundances of 2.69E+04 to 6.08E+04 copies/mL, respectively. *vcrA* was not detected in the remaining five wells included in this sampling campaign (Table 44). The *vcrA* gene occurs as a single copy gene on the genomes of certain *Dhc* strains, and the number of *vcrA* gene copies represent *Dhc* cell numbers. Interestingly, the qPCR analysis also detected the *bvcA* gene in groundwater from sampling locations IMW-1 and MW-60-L7, and *bvcA* gene abundances of 2.93E+04 and 4.04E+04 copies/mL, respectively, were measured. The VcrA RDase is responsible for dechlorination of TCE/cDCE to ethene, and the BvcA RDase was shown to dechlorinate all dichloroethene isomers to ethene (Magnuson et al., 1998; Parthasarathy et al., 2015; Tang et al., 2013a).

The *pceA* gene of *Dhc* strain 195, a member of the Cornell subgroup, was not detected in any of GW samples. The 16S rRNA gene-based analysis indicated that Cornell-type *Dhc* dominated the *Dhc* population in Site 9 groundwater; however, these Cornell-type *Dhc* apparently do not carry the *Dhc*-type *pceA* gene.

Although *Geobacter* 16S rRNA genes were detected in all groundwater samples, the *Geobacter lovleyi*-type *pceA* gene was only detected in wells MW-60-L3, MW-60-L5, MW-60-L7, and MW-71-L3 in abundances of 8.36E+02, 7.27E+01, 3.22E+02, and 6.72E+02 gene copies/mL, respectively. *Geobacter* are common inhabitants of anoxic sediments and subsurface environments, including aquifers, and the occurrence of multiple *Geobacter* spp. at Site 9 is not unexpected. Therefore, it is not surprising that organohalide-respiring *Geobacter* spp. carrying *pceA* represents only a subset of the entire *Geobacter* population.

The *pceA* gene associated with *Dhb* and *Desulfitobacterium* was detected at abundances of 4.42E+03 and 4.37E+02 gene copies/mL in MW-60-L3 and MW-60-L5, respectively. Interestingly, *Dhb* 16S rRNA gene sequences were detected at all well sampling locations (except MW-60-L5) but the *Dhb*/Dsf-type *pceA* gene was only detected at locations MW-60-L3 and MW-60-L5. These findings suggest that *Dhb* with other RDase genes (and likely dechlorination capabilities other than PCE→cDCE) occur in Site 9 groundwater. are strictly organohalide-respiring bacteria and their presence at the abundance levels observed suggests that growth occurred *in situ*.

Dhgm cells were highly abundant in Site 9 groundwater, but *Dhgm* RDase genes with known substrate specificity (e.g., *cerA*, *dcpA*) were not detected in any of the groundwater samples collected from Site 9 wells. *Dhgm* are strictly organohalide-respiring bacteria and their high

abundance is indicative of *in situ* growth. The observations indicate that *Dhgm* strain(s) present in Site 9 groundwater possess different RDase genes with unknown substrate specificity and are most likely involved in contaminant attenuation (Clark et al., 2018).

The *cbrA* RDase gene implicated in reductive dechlorination of chlorinated benzenes (e.g., 1,2,3,4-TeCB → 1,2,4-TCB) was detected in MW-60-L5 at low abundance (2.31E+01 gene copies/mL).

The *cfrA* gene encoding an RDase implicated in CF to DCM and 1,1,1-TCA to 1,1-DCA reductive dechlorination was detected in MW-60-L7 at a high abundance of 2.21 E+05 gene copies/mL. Interestingly, the *cfrA* gene has been reported to occur in *Dhb*; however, the abundance of *Dhb* at this sampling was orders of magnitude lower (i.e., 9.06E+02 cells/mL). This observation may suggest that the *cfrA* gene occurs on non-*Dhb* genomes. The reasons why the *cfrA* gene was only detected in well MW-60-L7 but not the other sampling locations is unclear and additional sampling events should be performed to validate the observations.

The *dcaA* gene (1,2-DCA → ethene) was measured at abundances ranging from 8.09E+01 to 4.09E+02 gene copies/mL in IMW-1, MW-60-L3 and MW-60-L5 groundwater samples.

The HPR2A_RDHA_C assay targeting an RDase gene specific to *Dhc* strain 195 of the Cornell group (locus tag DET1545) was measured in all groundwater samples at abundances ranging from 5.01E+03 to 1.24E+06 gene copies/mL. These results correlate with the observation that 16S rRNA genes of the Cornell-type (assay DHC_COR) represent the dominant *Dhc* population in Site 9 groundwater. Although the substrate specificity of DET1545 (HPR2A rdhA gene, DET1545 for *Dhc* strain 195) is not known, the expression of this gene has been reported in studies of *Dhc* strain 195 grown with PCE or TCE (Fung et al., 2007; Rahm et al., 2008a). The other RDase with unknown substrate specificity (i.e., HPR1a determined with assay HPR1A_RDHA_P) was measured at abundances of 9.73E+03 and 2.29E+04 gene copies/mL in IMW-1 and MW-60-L7. This RDase gene has only been found in members of the Pinellas subgroup of *Dhc*, providing evidence for the presence of Pinellas-type *Dhc* at sampling locations IMW-1 and MW-60-L7. The SDC-9_23243 assay targeting a putative RDase gene of the SDC-9 consortium (DET1080 in *Dhc* strain 195) was measured at abundances of 1.47E+04 and 3.36E+04 in IMW-1 and MW-60-L7 groundwater samples, respectively. Both DET1545 and DET0180 are commonly reported putative RDase genes expressed in *Dhc* strain 195 when grown with PCE or TCE (Fung et al., 2007; Rahm et al., 2008a). These putative RDase genes have also been reported in ANAS1 and ANAS2 *Dhc* strains (Lee et al., 2011a; Lee et al., 2013). The SDC9_45582 and SDC9_09280 assays (both targeting *Desulfitobacterium* RDase genes with unknown substrate specificity and detected in consortium SDC-9) were not detected in Site 9 groundwater samples.

The SDC9_48350 assay designed to target a *Dehalobacter/Desulfitobacterium*-like *pceA* gene in consortium SDC-9 yielded amplicons and 1.10E+03 and 2.01E+03 gene copies/mL in IMW-1 and MW-60-L7 groundwater were measured.

The other RDase genes, SDC9_07142 and SDC9_07143, specific to *Dhc* strains present in consortium SDC-9 (Kucharzyk et al., 2020) were also detected and quantified in high abundances of 1.48E+04 and 2.71E+04 gene copies/mL in IMW-1 and of 1.96E+04 and 2.12E+04 gene copies/mL in MW-60-L7.

Genes involved in the aerobic degradation of VC (i.e., *etnE* and *etnC*) were detected in all groundwater samples in abundances ranging from 1.67E+02 to 1.43E+04 gene copies/mL. This observation confirms prior observations that bacteria capable of aerobic VC oxidation occur in

anoxic aquifers. Aerobic VC oxidation can occur at very low dissolved oxygen concentrations (Gossett, 2010), and should oxygated groundwater infiltrate zones with VC, aerobic VC oxidation can contribute to VC attenuation at Site 9.

The analysis of Site 9 samples demonstrated the utility of the RD-qChip v2 array plate for enumerating gene targets of interest in groundwater collected from field sites. The high-throughput qPCR approach with the ability of enumerating multiple biomarker genes in a parallel format has utility for contaminated site assessment and implementing bioremediation monitoring regimes. Key advantages over conventional 96-well qPCR assays include higher sample throughput, high accuracy and reproducibility, and more comprehensive information about the microbiology involved in contaminant detoxification. To realize the cost saving of the high-throughput qPCR approach using the current instrumentation, the inclusion of 56 or 112 assays should be envisioned, which would allow the parallel processing of 48 or 24 samples, respectively.

Global proteomics analyses of the Mechanicsburg Naval Base and Offutt Airforce Base Groundwater samples

Although the 16S rRNA and RDase gene measurements by qPCR indicated the presence of *Dehalococcoides* (*Dhc*) bacteria in several of the groundwater samples, it does not necessarily prove their participation in active reductive dechlorination. Therefore, to complement the qPCR data collected for *Dhc*, *Dehalogenimonas* (*Dhgm*) and *Dehalobacter* (*Dhb*) bacteria, global proteomics analyses of all Mechanicsburg samples (i.e., M17, M18 and M48) (Table 14) and the six Offutt samples (i.e., 129, 29, 97, 85, 94, 116) (Table 14) were conducted with the same methodology as described in the Methods section, Task 2. Due to the limited peptide recovery of Offutt sample 132 and the lower abundance values of *Dhc* 16S rRNA gene copy numbers reported for the Charleston and Dover samples (most wells below E+04 gene copies/mL), these were not analyzed by the proteomics methodologies described in this report. Based on empirical information, which estimated that for effective dechlorination to occur, *Dhc* gene copies numbers in groundwater should exceed 1×10^7 copies/L (Lu et al., 2006a), samples with higher *Dhc* gene copy numbers were chosen for proteomics analyses.

Based on the qPCR measurements, the abundance of bacterial 16S rRNA genes in the volumes of groundwater processed for proteomics analyses ranked from 2.46E+10 gene copies/mL in groundwater 116 to 7.28E+08 gene copies/mL in groundwater M17 (Figure 71A). Specifically, quantifiable numbers of *Dhc* microorganisms were measured in all samples, *Dhb* bacteria in all samples except for 116 and 94, and *Dhgm* cells only in samples 29, 85, 129 and 94.

The total number of proteins identified from the MS/MS fragmentation spectra collected from tryptic digests of groundwater samples ranged from a maximum of 1,348 proteins for M17 to 190 proteins identified in groundwater sample 116 (Figure 71A). A total of 246 proteins were identified to be in common between the two Mechanicsburg samples M17 and M18; whereas only 2 ATP synthase subunit beta proteins of *Acidipjilium cryptum* strain JF-5 (UniProt ID. A5FZ54) and *Geobacter lovely* strain SZ (B3EA01) were commonly identified between the six Offutt samples (Figure 71B). The global proteomics analysis did not detect any proteins in sample M48, which, according to the qPCR results, had the lowest bacterial cell abundance of 6.49E+05 gene copies/mL. The complete protein identification data are presented in Supplemental Table S3, Excel Document.

The Mechanicsburg samples M17 and M18 showed confirmed presence of *Dhc* bacteria by the

identification of 391 and 24 *Dhc* proteins, respectively (Figure 71C). This information agrees with qPCR data which also tested positive for *Dhc* bacteria. Amongst the *Dhc* proteins uniquely identified in sample M17 were the *BvcA* and *VcrA* RDases, whereas two putative reductive dehalogenases from strains GT (ADC73492) and 195 (Q3Z693) were solely identified in sample M18. Although in both samples quantifiable *vcrA* and *bvcA* gene copy numbers were obtained (i.e., $1.30\text{E}+04$ gene copies/mL and $2.82\text{E}+03$ gene copies/mL respectively) by qPCR, the detection of *VcrA* and *BvcA* proteins in M17, suggests that *Dhc* strains carrying these genes are involved in reductive dechlorination transformations of DCE to ethene in this sample, but not in M18, where strains carrying the genes coding for ADC73492 and Q3Z693 could be participating in dechlorination processes.

In total, 15 *Dhc* proteins were common between samples M17 and M18 and included the housekeeping chaperonin GroEL and the elongation factor Tu (EF-Tu) proteins, but also proteins annotated as “conserved domain proteins” (Figure 71D). The numbers of *Dhb* and *Dhgm lykanthroporepellens* strain BL-DC-9 (*Dhgm* BL-DC-9) protein identifications in samples M17 and M18 were lower in comparison to the number of *Dhc* proteins (Figure 71A); however, like the *Dhc* results, common representatives of *Dhb* proteins in samples M17 and M18 included chaperonin GroEL and EF-Tu, while chaperone ClpB was the unique *Dhgm* BL-DC-9 protein common between these samples (Figure 71D).

In comparison to the Mechanicsburg samples, the average number of proteins identified in the six analyzed Offutt samples was lower (Figure 71A). Except for sample 94, *Dhc* proteins were identified in all Offutt samples analyzed, albeit in smaller numbers than the *Dhb* proteins (Figure 71C). Interestingly, *VcrA* was the only characterized *Dhc* RDase observed in the Offutt samples and was part of the four unique identifications in sample 97, which amongst all the Offutt samples had the the highest *vcrA* gene abundance ($2.95\text{E}+07$ gene copies/mL). Other groups of *Dhc* proteins measured in the Offutt samples included RNA polymerases, beta subunits of ATP synthase, EF-Tus and chaperones DnaK. Although measurements of the *tceA*, *vcrA* and *bvcA* genes by qPCR in each Offutt sample were in the E02-E04 gene copies/mL range (see supplemental SERDP Field Site Analysis Results_qPCR) only *VcrA* was identified in sample 97 by proteomics analyses suggesting that *Dhc* strains containing the *tceA* and *bvcA* genes are not contributing to active dechlorination.

Like *Dhc*, *Dhb* proteins were identified in all Offutt samples (Figure 71C) with a maximum of six overlapping proteins between samples 116 and 94. Some representative examples were pyruvate-flavodoxin oxidoreductases, inosine-monophosphate dehydrogenases, and s-adenosylmethionine synthases. Regarding *Dhgm* BL-DC-9 protein identifications, these were observed in all samples except for sample 116 (Figure 71C), but their numbers ranked from a maximum of two proteins including RNA polymerase subunit beta and chaperone DnaK in sample 129; to a chaperone CplB in sample 94, an EF-Tu in sample 85 and an ATP-synthase subunit alpha in sample 97. Overall, the low number of *Dhgm* associated identifications may be related to the database size and composition used to search the MS/MS spectra results, which only included the proteome of *Dhgm* BL-DC-9 in comparison to six *Dhc* strain proteomes and the proteomes of *Dhb* strains DSM 9455 and UNSWDHB. If higher levels of precision are required, metagenomic analyses of these sites could provide more accurate protein databases that can be used for MS/MS searches and be biologically close to the bacterial populations existing within these environments.

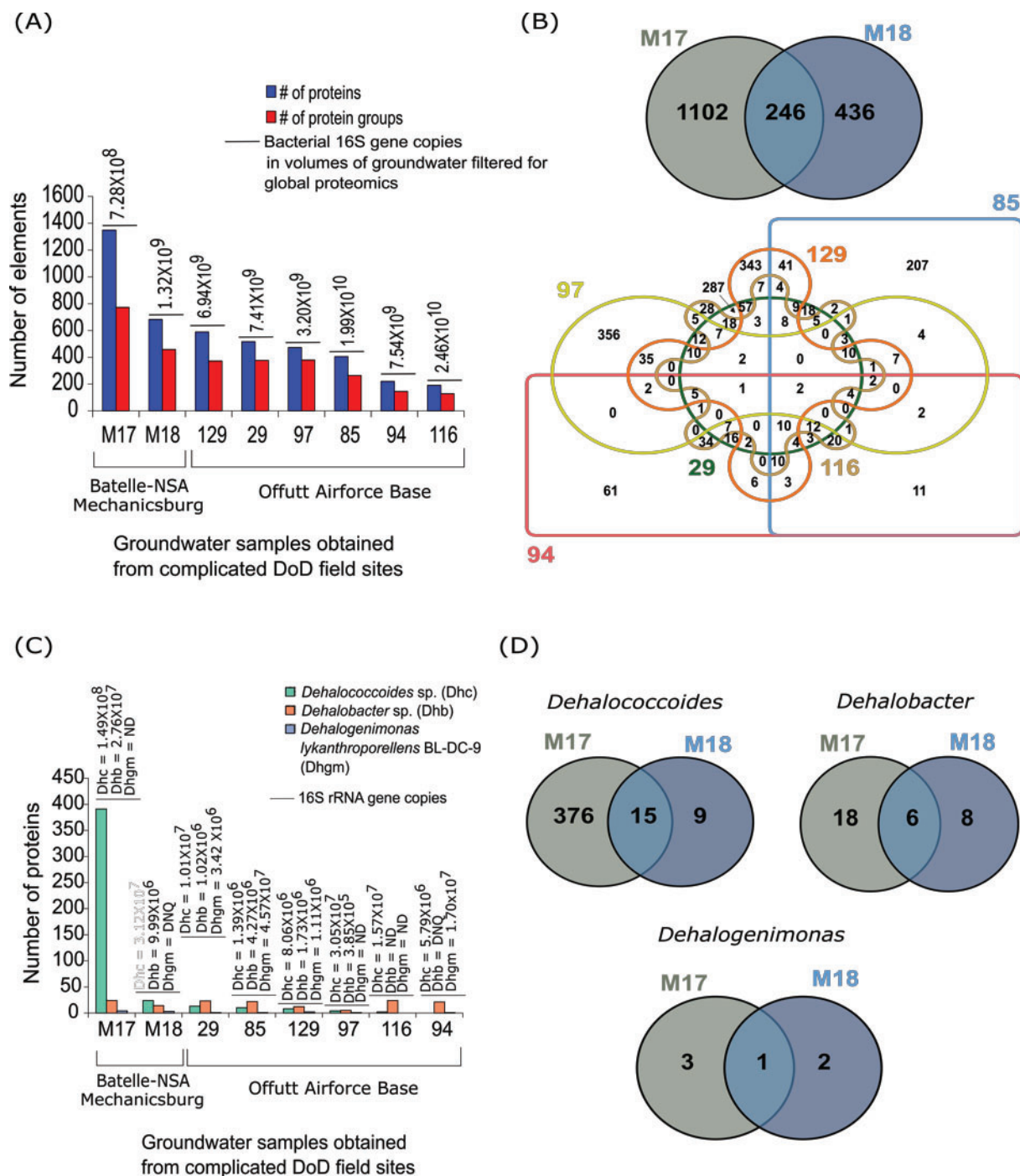


Figure 71. Identification of proteins by global proteomics in groundwater samples. Identification of proteins by global proteomics in groundwater samples extracted from complicated DoD sites. (A) Total number of proteins and protein groups (clustered at > 85% sequence identity). (B) Common and unique total number of proteins between groundwater samples extracted from the same field sites. (C) Total number of proteins identified by microbial species that were considered for 16S rRNA qPCR analysis. (D) Common and unique proteins between groundwater samples extracted from the Mechanicsburg field site and grouped by species. Proteins by species were also grouped for the Offutt samples, but no common identifications were observed amongst the six analyzed samples.

Targeted proteomics analyses of the Mechanicsburg Naval Base and Offutt Airforce Base groundwater samples

Detection of the *Dhc* biomarker proteins (Methods section, Task 2) through targeted proteomics was confirmed only in groundwater sample M17. The expression of chaperonin GroEL homologues was confirmed by the detection of the tryptic peptides IETVAELLPALEK and GNLNILAVK, which are found in the proteomes of multiple *Dhc* strains, including the six most studied *Dhc* isolates (Figure 72). Other biomarkers indicating the presence of *Dhc* and identified in this sample were EF-Tu and S-layer proteins. For EF-Tu, peptides ILDTAEPGDAVGLLLR and NSFPGDEIPIVR were observed. Peptide NSFPGDEIPIVR had a unique match *in-silico* (compared to the entire UniProt database) to the EF-Tu protein sequence of *Dhc* strain 195 and thus suggested the presence of this strain at location M17. Peptide ILDTAEPGDAVGLLLR, which differed by a single threonine residue compared to another targeted peptide (ILDSAEPGDAVGLLLR, see Table of targeted peptides in Task 2), and is found among multiple *Dhc* strains (Supplemental Table S2, Excel Document), demonstrated the utility of the LC-MRM-MS approach to differentiate single amino acid changes in the sequences of the targeted analytes.

The participation of *Dhc* in active reductive dechlorination processes in sample M17 was inferred with the detection of two targeted FdhA peptides and one peptide from the BvcA RDase (Figure 72). While the FdhA (CISM) peptides suggested a broad level of participation in dechlorination processes (Figure 22), the BvcA peptide indicated the involvement in DCE to ethene transformation. The different degrees of proteome specificity provided by these peptides were also helpful to infer the presence of particular *Dhc* strains. For example, one of the FdhA peptides detected was SELEVISSLLSR, which according to the *in-silico* analysis conducted on Task 2, is specific to multiple strains of *Dhc* including strains FL2, GT, BAV1 and CBDB1 but not for others such as strains 195 or VS. This was particularly informative due to the additional detection of the S-layer peptide AGIIDVPATADDATK found in the proteomes of *Dhc* strains FL2 and GT, which corroborated that these strains were present. The occurrence of the BvcA peptide STVAATPVFNSFFR alongside peptides of GroEL, EF-TU, and importantly of FdhA, that can be produced from the tryptic digestion of these proteins in strain BAV1, may point to the participation of BAV1 cells in the dechlorination reactions leading to the transformation of DCE isomers to ethene in sample M17.

Although qPCR detected *Dhc* 16S rRNA genes in the remaining groundwater samples, the absence of peptide identifications by targeted proteomics could be due to two possible reasons: the proteins were not expressed under the prevailing *in situ* conditions, or the expression of the chosen biomarker peptides for the targeted proteins was below the detection limits of the MS measurement. Therefore, the global proteomics data measured initially would provide insight about the presence and abundance of the selected proteins prior to their monitoring by targeted MS measurements.

According to the global proteomics runs, protein biomarkers like GroEL, EF-Tu and S-layer were identified in sample M18, but different tryptic peptides to the ones targeted were detected. Similar cases were observed for the S-layer homologues detected in samples 129 and 116, as well as for the EF-Tu protein detected in sample 85. In the analysis of complicated data derived from environmental samples, it is not uncommon to observe matrix effects, which sometimes are known to cause ion suppression or signal enhancement from peptides generated from the same proteins. In addition, inefficient protein extraction and digestion could have affected peptide detection. Interestingly, the global proteomics analyses did not detect the major housekeeping protein GroEL in all Offutt samples and neither of FdhA, TceA and BvcA, which for the most part agrees with

the targeted proteomics results (Table 45). These observations further suggested that, although *Dhc* are present in these samples, they are not participating in dechlorination processes.

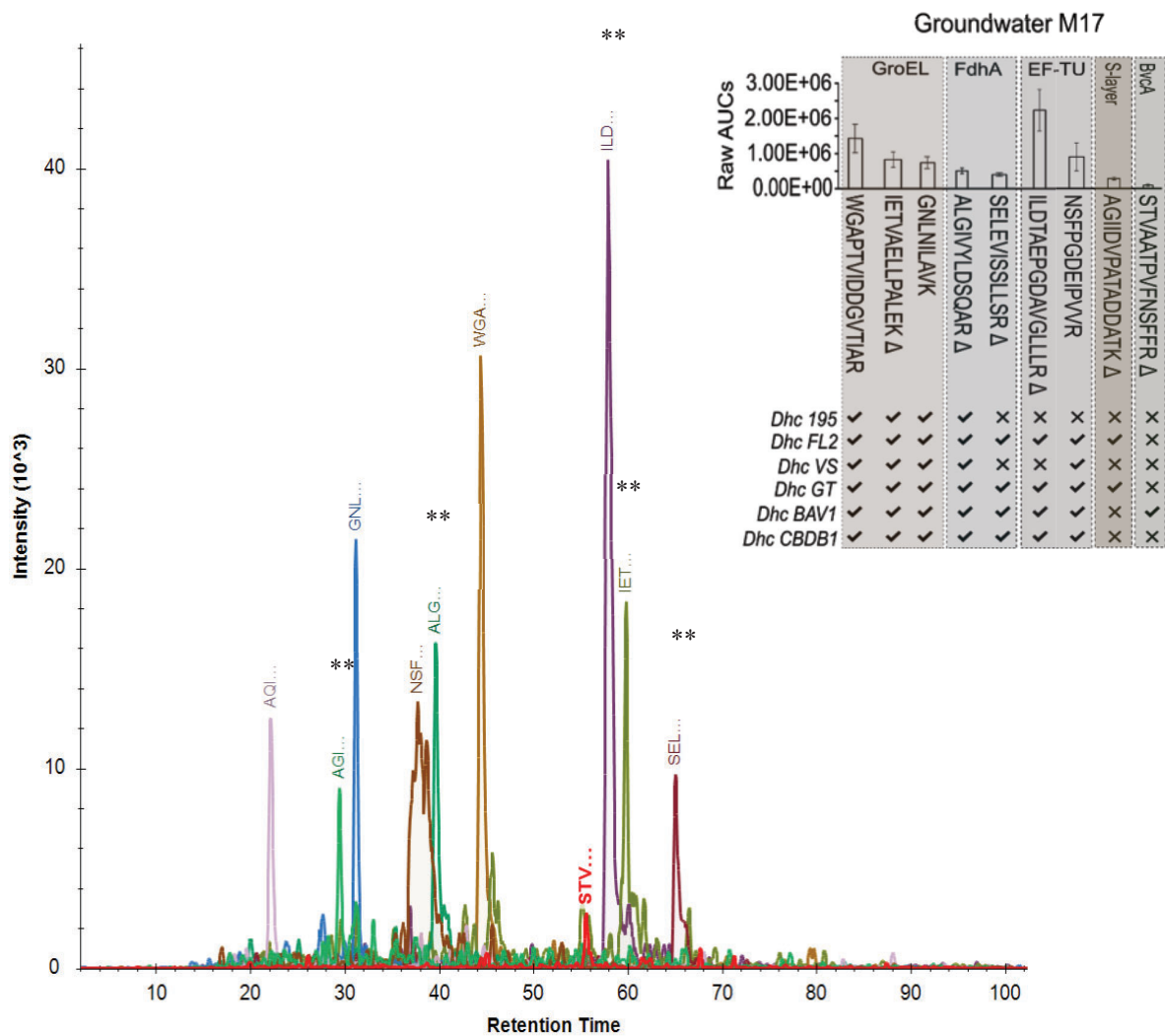


Figure 72. Extracted ion chromatogram (XIC) of a technical replicate of groundwater M17 analyzed by LC-MRM. Signals marked with ** were validated with the use of spiked-in unlabeled peptide standards. Bar charts insert shows the mean raw AUCs calculated. Error bars are the standard error of the mean ($n= 3$ technical replicates).

Table 45. Identification of targeted *Dhc* biomarkers by global proteomics.

Biomarker Protein	Site samples							
	Mechanicsburg samples		Offutt samples					
	M17	M18	97	94	85	29	129	116
GroEL	✓	✓	-	-	-	-	-	-
FdhA	✓	-	-	-	-	-	-	-
TceA	-	-	-	-	-	-	-	-
BvcA	✓	-	-	-	-	-	-	-
EF-TU	✓	✓	-	-	✓	-	-	-
S-layer	✓	✓	-	-	-	-	✓	✓

Identification of targeted *Dhc* biomarkers in tryptic digests of groundwater samples by global proteomics ($n=3$ technical replicates). “✓” identified. “-” not identified.

The results obtained, and observations made in tasks 2 and 7, demonstrated that the application of targeted proteomics via LC-MRM-MS is a feasible approach for monitoring the expression of *Dhc* proteins in contaminated groundwater. Before deploying quantitative MRM-MS workflows that can inform site remediation and management strategies, certain aspects on the detection of *Dhc* biomarkers in groundwater impacted with chlorinated ethenes should be addressed.

The specificity of peptides selected from *Dhc* biomarkers must be considered in a broader microbiological context in groundwater samples compared to axenic or mixed cultures. Thanks to the continued sequencing efforts of functional genes from contaminated environments, database support for making effective *Dhc* peptide selection for targeted proteomics exists. The type of information provided by peptides specific to the *Dhc* species (*i.e.* ALGIVYLDLSQAR in FdhA homologues of multiple strains), or those that can be found in few *Dhc* strains (*i.e.*, AGIIPAPTTASDAYK from the S-layer protein expressed by strain 195) or in multiple strains and other organohalide-respiring bacteria proteomes (*i.e.*, the TceA YFGASSVGAIK peptide), must be used carefully used to derive conclusions. For example, if the goal of an environmental proteomics investigation is to solely demonstrate that *Dhc* mediated reductive dechlorination is occurring at a site, peptides such as those representing FdhA matching multiple strains of *Dhc* can be used. The integration of results from the identification of peptides specific to certain RDases with those from peptides of *Dhc* FdhA (*i.e.*, CISM), or housekeeping proteins found in few strains can provide strain-level resolution and pinpoint *Dhc* strains that are participating in specific dechlorination reactions. qPCR data can provide supporting evidence for the involvement of specific *Dhc* strains in the observed reductive dechlorination reactions. Additional considerations are effective protein extraction and digestion methods, although depending on the biological and geochemical conditions of each sample, it is not guaranteed that similar efficiencies can be obtained. Future studies should also consider transitioning to other MS platforms like the Thermo Scientific Q-Exactive mass spectrometer, which provides the ability to do targeted proteomics with high mass accuracy and resolution and hence the chance to achieve higher sensitivity and better recognition of peptide signals in noisy chromatogram zones.

Task 8: Disseminate new information to practitioners

The results and technical approaches generated in this project are of interest to practitioners because the new information can improve decision-making and ensure that the most promising remedies are being implemented. The new tools advance monitoring regimes and allow more responsive corrective actions (i.e., ASM) at sites where bioremediation performance does not meet expectations. The high-throughput qPCR and proteomics measurements can generate strong evidence that microbial processes are actively attenuating the target contaminants, and provide an additional line of evidence that *in situ* contaminant destruction is occurring. The project also advanced information about specific geochemical conditions that can limit organohalide respiration and contaminant degradation. Further, the project demonstrated the utility of machine learning approaches by linking biogeochemical parameters with detoxification potential (i.e., ethene formation at sites impacted with chlorinated ethenes). This information is obviously relevant for regulators challenged with safeguarding groundwater resources and ensuring that corrective actions achieve remedial goals in desirable time frames. Good decision-making is based on good information and the new tools and process understanding generated in this research project contribute to a richer dataset that will enable knowledge-based site closure decision making.

To achieve this vision, the new information must be communicated to practitioners and regulators, and the project team has engaged in a series of activities to disseminate new information, including the publication of at least 29 peer-reviewed manuscripts, and numerous poster and oral presentations at professional meetings and workshops, webinars, oral presentations at universities and state regulatory agencies, and many direct interactions with practitioners and regulators via phone calls and email.

Brief summary of project deliverables

- Improved understanding of the microbiology contributing to the degradation and detoxification of chlorinated solvents, in particular chlorinated ethenes, chlorinated methanes, and chlorofluorocarbons.
- Unraveled specific reasons for often observed cDCE/VC stalls at sites impacted with chlorinated ethenes. Specifically, the work demonstrated that the presence of N₂O and chlorofluorocarbons can cause inhibition. The work also demonstrated the role of the microbial community to meet the nutritional demands (e.g., cobamides, fixed nitrogen) of *Dhc* and *Dhgm*.
- Discovered new organism- and process-specific biomarkers useful for *in situ* monitoring of chlorinated solvent degradation.
- Demonstrated the utility of targeted proteomics workflows for the measurement of biomarker proteins in groundwater samples.
- Demonstrated that normalized qPCR measurements are useful predictors for ethene formation at sites impacted with chlorinated ethenes.
- Demonstrated that data mining techniques (e.g., machine learning algorithms) can effectively utilize groundwater monitoring data to derive predictive understanding of contaminant degradation (i.e., detoxification potential). This work also revealed key shortcomings in available groundwater datasets, thus providing guidance for the design of monitoring regimes that will facilitate the broader application of data mining techniques. Data mining has great promise to generate predictive understanding of a plume's trajectory and guide decision-making.
- Designed, optimized and validated a new high-throughput qPCR tool (i.e., the RD-qChip) for contaminated site assessment and bioremediation monitoring.
- At least 30 peer-reviewed journal articles, one book (as editor), and four book chapters. More than 90 poster and oral presentations at conferences, worksops and symposia, 41 invited seminars, and 13 webinar presentations.
- New technical information about bioremediation and monitoring tools were disseminated to practitioners, RPMs and regulators.
- Two MS students and six PhD students received degrees, and several undergraduate students and five postdoctoral students received training while working on this project.

Appendix

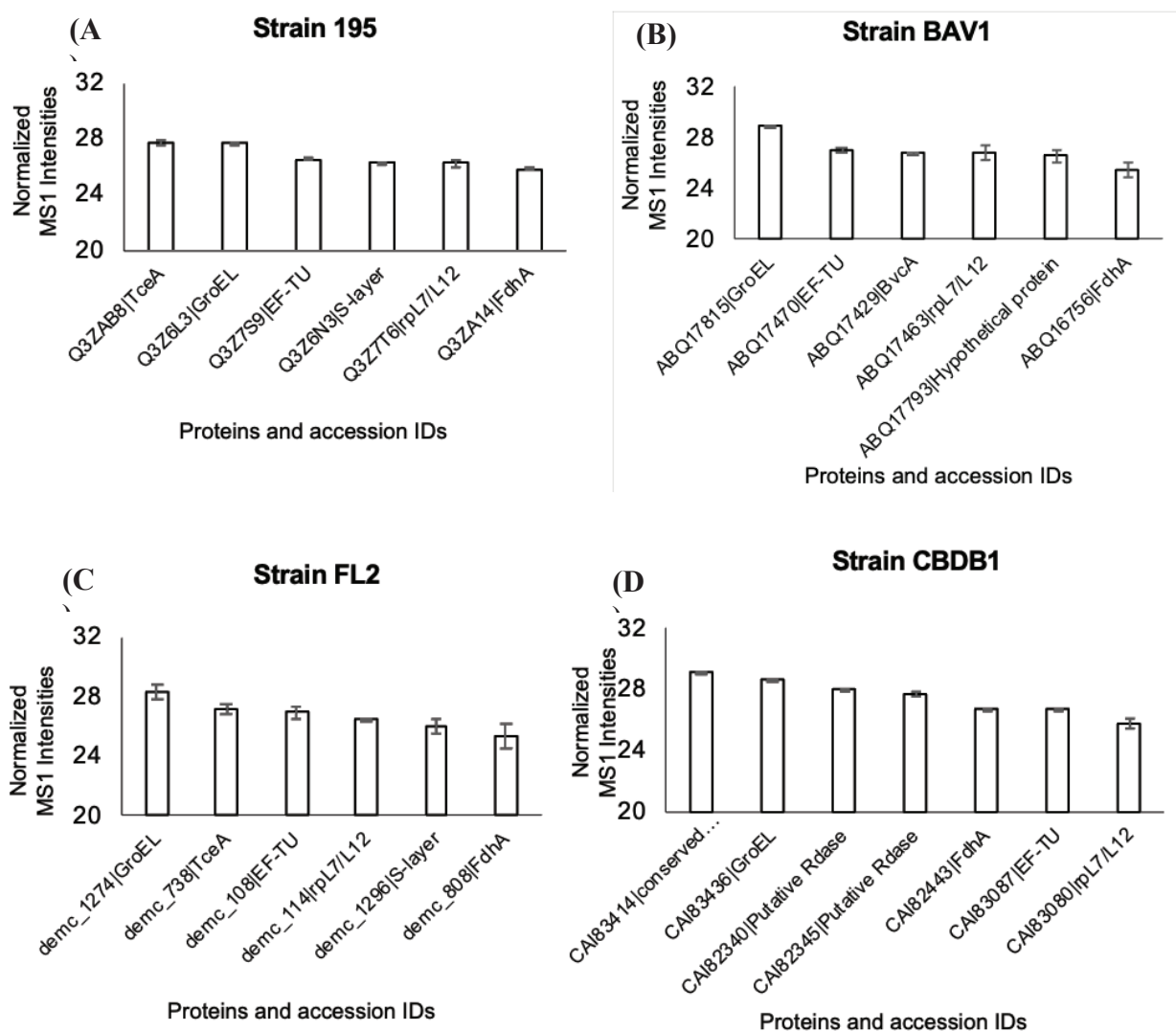


Figure A-73. Targeted protein abundances (log2 normalized MS1 intensities) in 90 mins LC-MS/MS gradients and high mass accuracy and resolution data from measurements of actively dechlorinating axenic cultures. Axenic cultures of (A) strain 195, (B) strain FL2, and (C) strain BAV1 (n=2 biological replicates). (D) Results from a re-analyzed proteomics dataset from *Dhc* strain CBDB1 (PRIDE: PXD003081) are provided as comparison (n=3 aliquots from the same culture). S-layer proteins of strain BAV1 and strain CBDB1 are ABQ17793 and CAI83414, respectively. Error bars are the standard error of the mean.

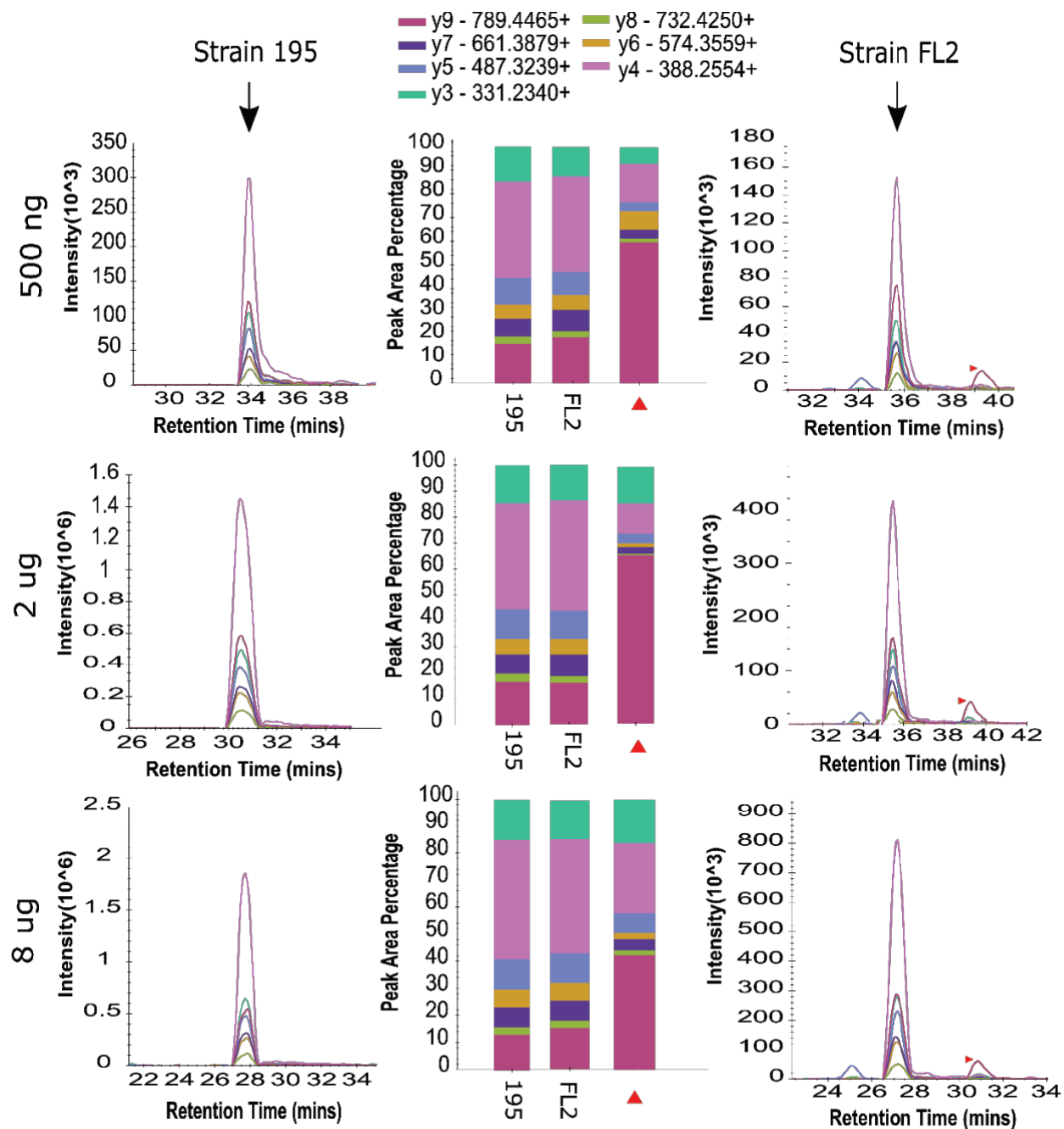


Figure A-74. Example of MRM signal identity validation between shared peptides in *Dhc* protein homologues. The chromatographic traces of peptide YFGASSVGAIK (550.292 m/z ++) found in expressed TceA homologues of *Dhc* strains 195 and FL2, and identified in MRM runs of 0.5 µg, 2 µg and 8 µg of their respective tryptic digests, are shown. The relative contributions of each fragment ion to the total peak areas are displayed with different colors. Other peak groups (*i.e.*, the one marked with a red arrowhead) were also observed in the XICs of strain FL2 but were determined to be interfering signals when the XICs of the same peptide in strain 195 was analyzed.

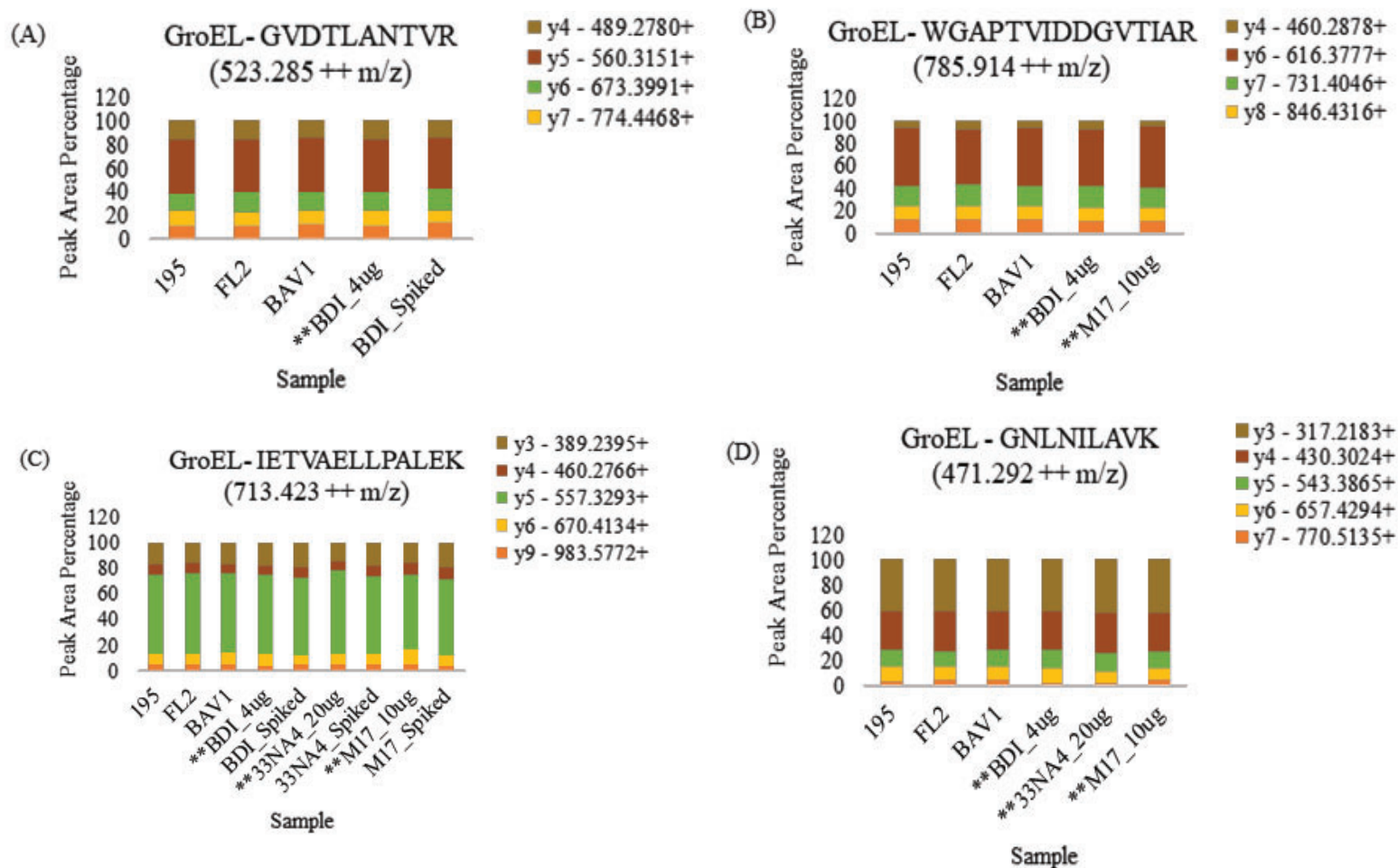


Figure A-75. Relative contribution of *Dhc* fragment ions representing *Dhc* biomarker proteins to total peptide peak areas. The panels show the comparison of the relative contribution of each fragment ion (shown in different colors) to the total peptide peak area between tryptic digests of pure cultures, consortium BDI, and groundwater samples, as well as spiked-in samples for those identified peptides with available unlabeled standards. The double asterisks (**) indicate spiked samples, which received 0.5 pmol of each individual standard. (A-F) GroEL peptides; (G-H) EF-TU peptides; (I-J) S-layer peptides; (K-N) FdhA peptides; (O) BvcA peptide; (P) TceA peptide.

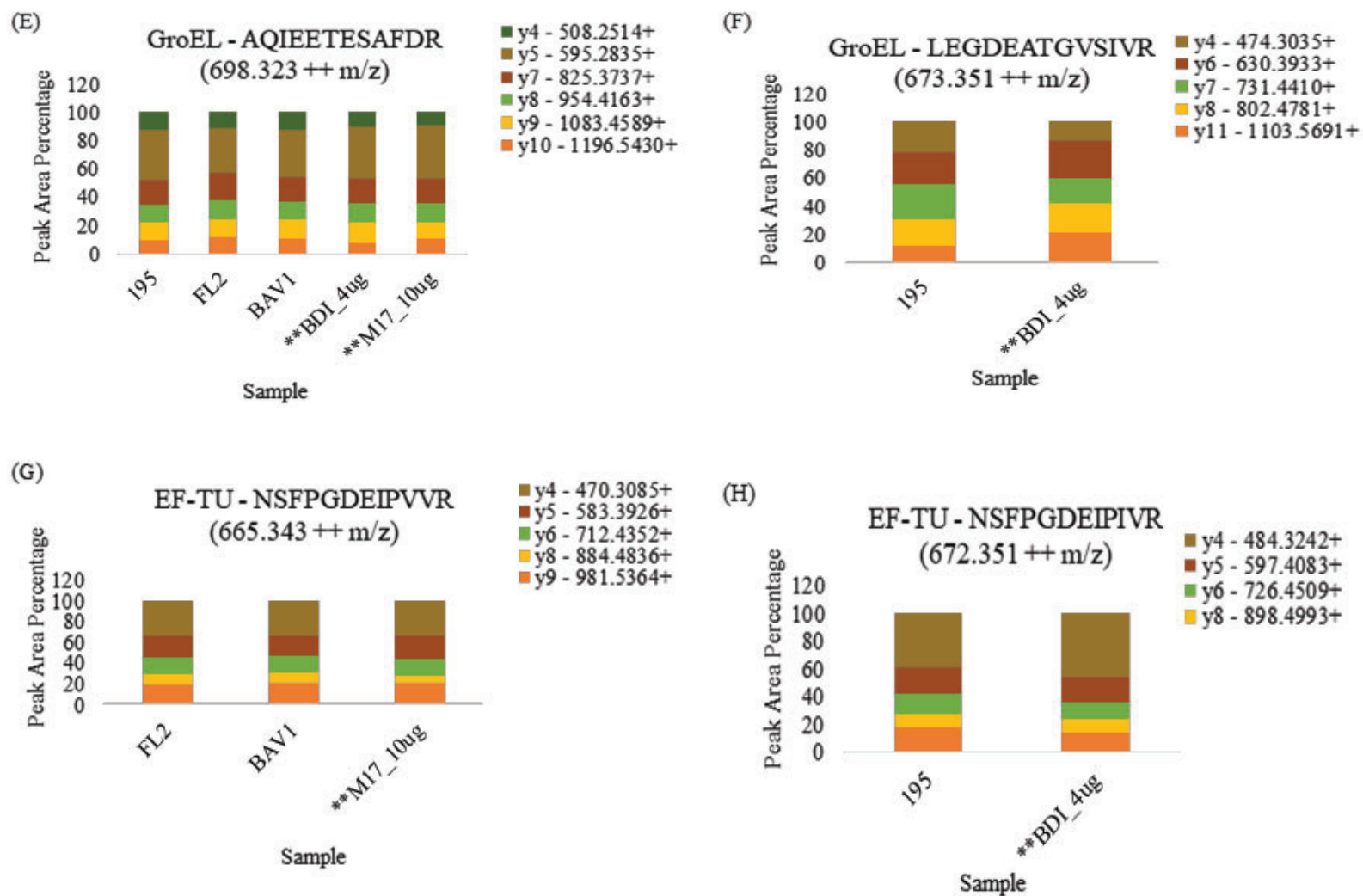


Figure A-72. (continued) (G-H) EF-TU peptides.

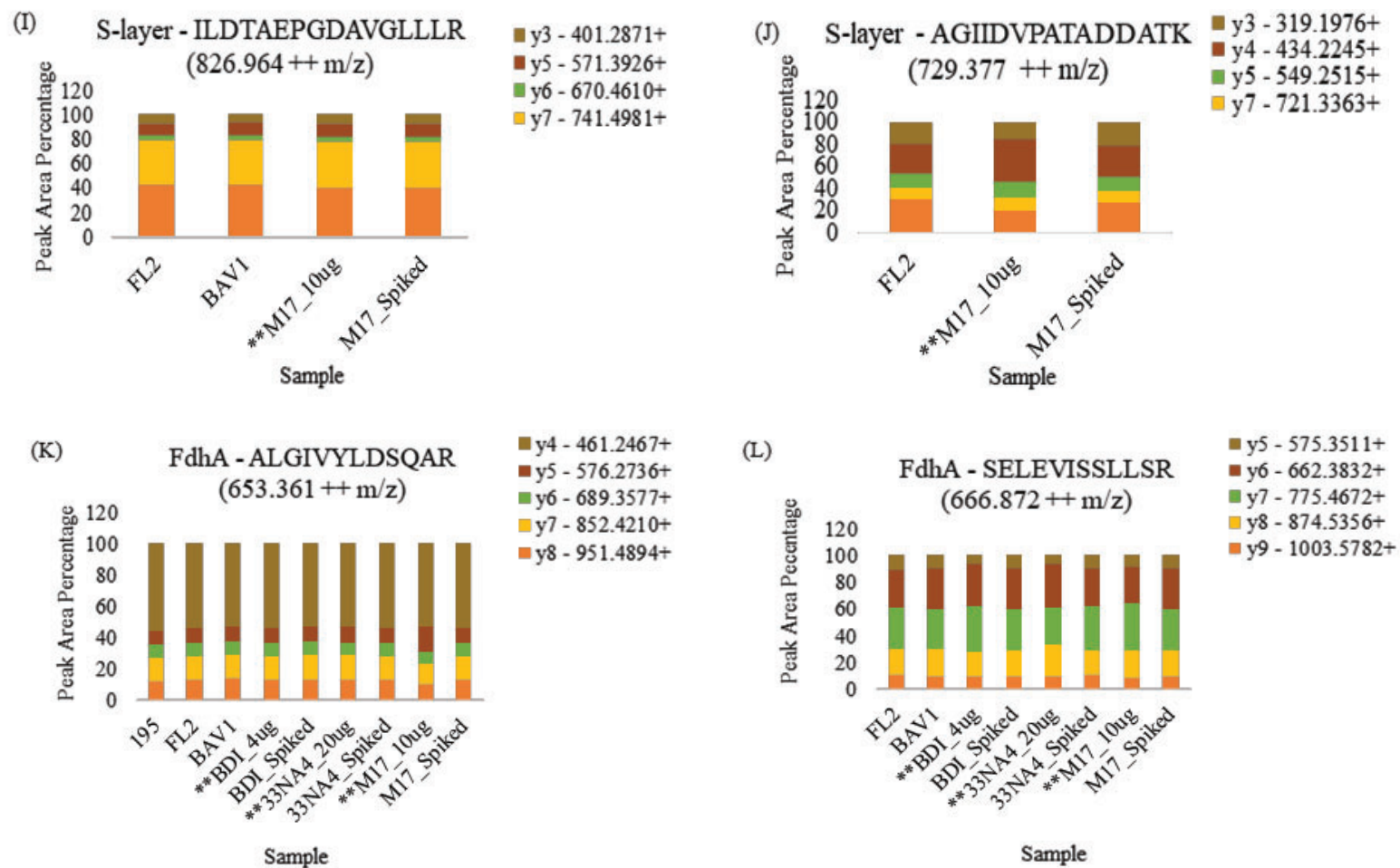


Figure A-72. (continued) (I-J) S-layer peptides; (K-N) FdhA peptides.

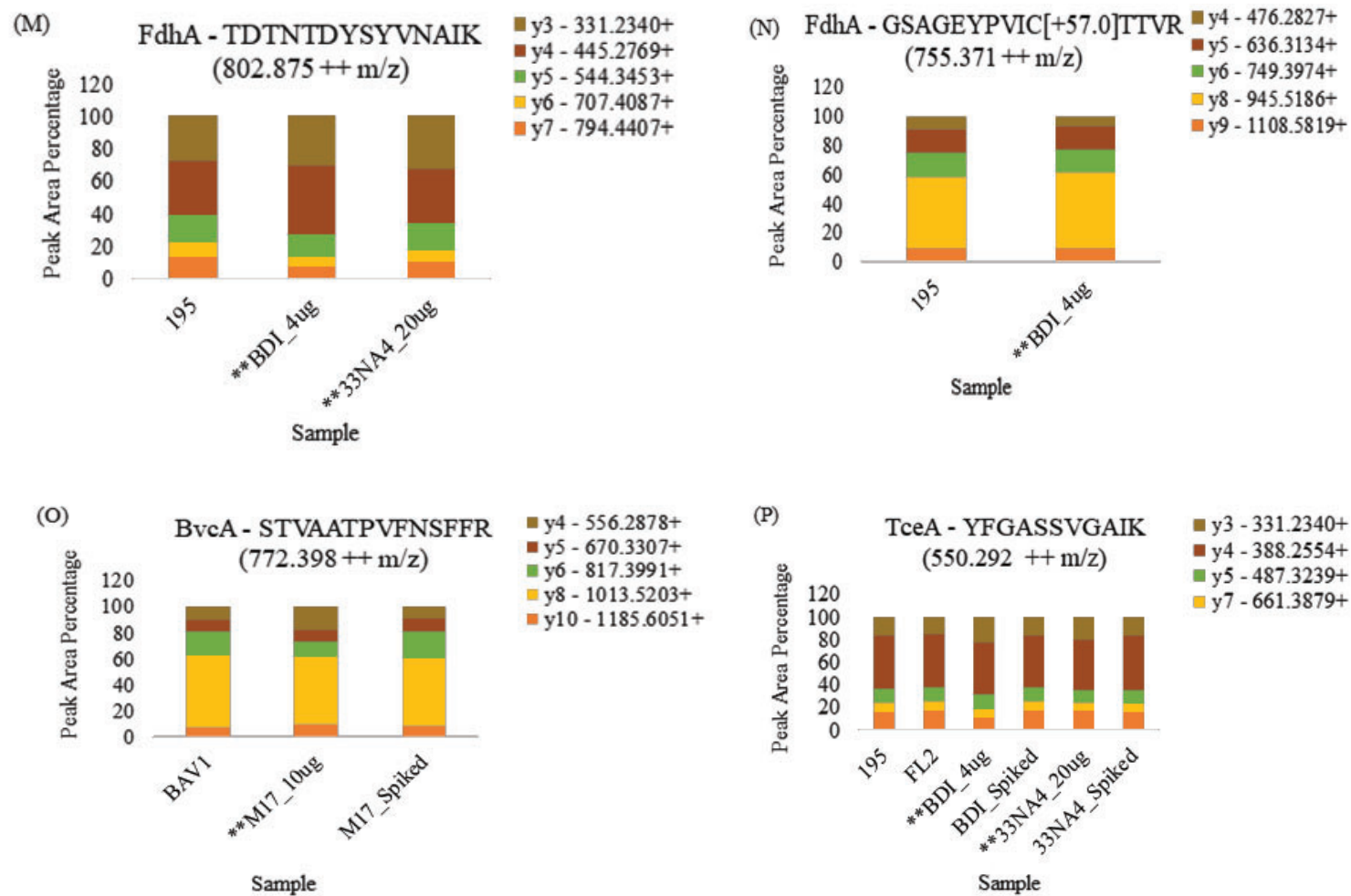


Figure A-72. (continued) (K-N) FdhA peptides; (O) BvcA peptide; (P) TceA peptide.

Table A-46. Top five transitions ranked by contribution to total AUC from the final set of peptides selected from in axenic *Dhc* cultures and then monitored in consortium BDI and groundwater samples.

Unlabeled peptide standards were purchased for peptides marked with ^{AA}. All cysteine residues were carbamidomethylated [+57.0]. NP - Peptide not present in *Dhc* strain.

Protein	Accessions in <i>Dhc</i> 195, FL2 and BAV1 databases	Selected in strain 195?	Selected in strain FL2?	Selected in strain BAV1?	Peptide sequences	Precursors m/z (all doubly charged)	Fragment ions m/z (<i>y</i> -fragment number, all singly charged)
GroEL	Q3Z6L3, demc_1274, ABQ17815	✓	✓	✓	^{AA} GVDTLANTVR	523.285	889.473 (y8), 774.446 (y7), 673.399 (y6), 560.315 (y5), 489.278 (y4)
		✓	✓	✓	WGAPTVIDDGVTIAR	785.914	959.515 (y9), 846.431 (y8), 731.404 (y7), 616.377 (y6), 460.287 (y4)
		✓	✓	✓	^{AA} IETVAELLPALEK	713.413	983.5772 (y9), 783.4975 (y7), 670.4134 (y6), 557.3293 (y5), 460.2766 (y4), 389.2395 (y3)
		✓	✓	✓	GNLNLAVK	471.292	770.5135 (y7), 657.4294 (y6), 543.3865 (y5), 430.3024 (y4), 317.2183 (y3)
		✓	NP	NP	AQIEETESAFDR	698.323	1196.5430 (y10), 1083.4589 (y9), 954.4163 (y8), 825.3737 (y7), 595.2835 (y5), 508.251 (y4)
FdhA	Q3ZA14, demc_808, ABQ16756	✓	NP	NP	LEGDEATGVSIVR	673.351	1103.5691 (y11), 802.4781 (y8), 731.4410 (y7), 630.3933 (y6), 474.3035 (y4)
		✓	NP	NP	SWDWALGEIANK	695.343	815.4621 (y8), 744.4250 (y7), 631.3410+ (y6), 445.2769 (y4), 332.1928 (y3)
		✓	NP	NP	^{AA} ALGIVYLDQAR	653.361	951.4894 (y8), 852.4210 (y7), 689.3577 (y6), 576.2736 (y5), 461.2467 (y4)
		✓	NP	NP	^{AA} GTELISVDC [+57.0]R	575.282	862.4451 (y7), 749.3610 (y6), 636.2770 (y5), 549.2450 (y4), 450.1765 (y3)
		✓	NP	NP	SGSEIAFTGGLIK	640.348	806.4771 (y8), 735.4400 (y7), 588.3715 (y6), 487.3239 (y5), 430.3024 (y4), 373.2809+ (y3)
		✓	NP	NP	SSEQNAASLLK	574.301	716.4301 (y7), 602.3872 (y6), 531.3501 (y5), 460.3130 (y4), 373.2809 (y3)
		✓	NP	NP	TDINTDYSYVNAIK	802.875	794.4407 (y7), 707.4087 (y6), 544.3453 (y5), 445.2769 (y4), 331.2340 (y3)
		✓	NP	NP	SELEVISSLSFR	683.864	1037.5626 (y9), 908.5200 (y8), 809.4516 (y7), 696.3675 (y6), 609.3355 (y5)
		✓	NP	NP	GSAGEYPVIC [+57.0]TTVR	755.371	1108.5819 (y9), 945.5186 (y8), 749.3974 (y6), 636.3134 (y5), 476.2827 (y4)
		NP	✓	✓	LSTASSLEALASFR	790.917	921.4789 (y9), 792.4363 (y8), 608.3151 (y6), 537.2780 (y5), 466.2409 (y4)
		NP	✓	✓	SGSEIAFIGGLIK	646.366	818.5135 (y8), 747.4763 (y7), 600.4079 (y6), 487.3239 (y5), 430.3024 (y4), 373.2809 (y3)
		NP	NP	✓	TDNNTNYSYNAIK	815.889	808.4563 (y7), 721.4243 (y6), 558.3610 (y5), 445.2769 (y4), 331.2340 (y3)
		NP	✓	✓	SELEVISSLSR	66.872	1003.5782 (y9), 874.5356 (y8), 775.4672 (y7), 662.3832 (y6), 575.3511 (y5)
		NP	✓	NP	VCAFFAATGK	536.268	741.3930 (y7), 594.3246 (y6), 447.2562 (y5), 376.2191 (y4), 305.1819 (y3)
TceA	Q3ZAB8, demc_738	✓	✓	NP	DVDDLISAGK	516.764	818.4254+ (y8), 588.371 (y6), 362.203 (y4), 275.171 (y3)
		✓	✓	NP	LEIELQKG	465.268	816.4462 (y7), 687.4036 (y6), 574.3195 (y5), 445.2769 (y4), 332.1928 (y3)
		✓	✓	NP	^{AA} YFGASSVGAIK	550.292	789.4465 (y9), 661.3879 (y7), 487.3239 (y5), 388.2554 (y4), 331.2340 (y3)
BvcA	ABQ17429	NP	NP	✓	DLYLAWAK	490.266	751.4137 (y6), 588.3504 (y5), 475.2663 (y4), 404.2292 (y3)
		NP	NP	✓	^{AA} TPVPIVWEEVDK	706.377	904.4411 (y7), 805.3727 (y6), 619.2933 (y5), 490.2508 (y4), 361.2082 (y3)
		NP	NP	✓	^{AA} STVAATPVFNSFR	772.398	1185.6051 (y10), 1013.5203 (y8), 817.3991 (y6), 670.3307 (y5), 556.2878 (y4)
EF-TU	Q3Z7S9, demc_108, ABQ17463	✓	NP	NP	NSFPGEIPIVR	672.351	995.5520 (y9), 898.4993 (y8), 726.4509 (y6), 597.4083 (y5), 484.3242 (y4)
		✓	NP	NP	ILDSAEPGDAVGLLLR	819.9567	1010.5993 (y10), 741.4981 (y7), 670.4610 (y6), 571.3926 (y5), 401.2871 (y3)
		NP	✓	✓	NSFPGEIPIVVR	665.3435	981.5364 (y9), 884.4836 (y8), 712.4352 (y6), 583.3926 (y5), 470.3085 (y4)
		NP	✓	✓	^{AA} ILDTAEPGDAVGLLLR	826.9645	1010.5993 (y10), 741.4981 (y7), 670.4610 (y6), 571.3926 (y5), 401.2871 (y3)
S-layer	Q3Z6N3, demc_1296, ABQ17793**	✓	NP	NP	YFGNQWNQALC [+57.0]K	815.377	834.4138 (y7), 720.3709 (y6), 592.3123 (y5), 491.2646 (y4), 420.2275 (y3)
		✓	NP	NP	AGHIPAPTTASDAYK	738.39	953.4575 (y9), 755.3570 (y7), 654.3093 (y6), 583.2722 (y5), 381.2132 (y3)
		✓	NP	NP	VAYGTTTGTETTATTLK	858.438	735.4247 (y7), 634.3770 (y6), 533.3293 (y5), 462.2922 (y4), 361.2445 (y3)
		NP	✓	NP	^{AA} FYDVGILEWNADK	785.382	875.4258 (y7), 762.3417 (y6), 633.2991 (y5), 447.2198 (y4), 333.1769 (y3)
		NP	✓	NP	TAVYATAVYDDGDDTLVR	972.962	1168.511 (y10), 1005.448 (y9), 603.346 (y5), 488.319 (y4)
		NP	✓	NP	YFGNQWNQPATC [+57.0]K	815.377	1004.461 (y8), 818.382 (y7), 576.281 (y5), 479.228 (y4), 408.191 (y3)
		NP	✓	NP	TWYSADGLTFTK	695.337	1102.5415 (y10), 939.4782 (y9), 666.3821 (y6), 496.2766 (y4), 395.2289 (y3)
		NP	✓	NP	^{AA} AGHIDVPATADDAK	729.377	889.4262 (y9), 721.3363 (y7), 549.2515 (y5), 434.2245 (y4), 319.1976 (y3)
		NP	✓	NP	VCI [+57.0]YGLPTGTDTIEATTLK	970.487	1347.7002 (y13), 662.3719 (y6), 533.3293 (y5), 462.2922 (y4), 361.2445 (y3)

Table A-47. Statistical parameters used for determining the best-fit inhibition model and inhibition constants in cell suspensions amended with N₂O as the inhibitor.

Culture	Substrate	Inhibitor	Tested models	Statistical Parameters			<i>K</i> ₁ (μM)
				^a R ²	^b AICc	^c Sy.x	
			^d Noncompetitive	0.971	81.338	3.350	40.8 ± 3.8
<i>Geo</i> strain SZ	PCE	N ₂ O	Uncompetitive	0.966	86.477	3.639	29.1 ± 3.1
			Competitive	0.944	102.041	4.678	9.2 ± 1.9
			^d Noncompetitive	0.968	107.060	6.422	21.2 ± 3.5
<i>Dhc</i> strain BAV1	<i>c</i> DCE	N ₂ O	Uncompetitive	0.952	117.928	7.853	2.3 ± 0.5
			Competitive	0.938	124.696	8.902	25.9 ± 2.9
			^d Noncompetitive	0.996	43.791	2.386	9.6 ± 0.4
<i>Dhc</i> strain BAV1	VC	N ₂ O	Uncompetitive	0.986	69.426	4.393	7.0 ± 0.6
			Competitive	0.974	82.243	5.961	1.6 ± 0.3

^a R², the Coefficient of Determination, gives information about the fit of the measured data to the different models tested, and the model with highest R² value provides the best data fit.

^b The AICc (i.e., corrected Akaike's Information Criterion) offers an estimate of the relative quality of tested models, and the model with the lowest AICc value represents the relative best fit among the tested models.

^c The Sy.x represents the Standard Deviation of the Residuals, and the model with the lowest Sy.x value provides best prediction of the data.

^d In all cell suspensions assays, the noncompetitive model (highlighted in bold) gave the highest R² and the lowest AIC and Sy.x values.

Table A-48. RD-qChip v1 Assay Information.

Assay No	Assay Group	Assay Name	Forward Primer	Forward Primer Seq (5'to 3')	Reverse Primer	Reverse Primer Seq (5'to 3')	TaqMan Probe	TaqMan Probe Seq (5'to 3')	Amplicon Size
1	16S	ARCH1 16S	Methgen835F-MI2	GGGRAGTACGKYCGCAAG	Methgen918R-MI2	GAVTCCAATTRARCCGCA	Metg873RP-MI	6FAM-CCAATTCTCTTAAGTTTCA-MGB	84
2	16S	BAC1 16S	Bac 1055YF	ATGGYTGTCTGTCAGCT	Bac 1392R	ACGGGCGGTGTGTAC	Bac16SFam115MGB	6FAM-CAACGAGCGCACC-MGB	65
3	16S	GEOGC 16S	GeoGC-30F	TGCAAGTCGAACGGAATTAGG	GeoGC-114R	CCAGATACCAGGGTAGGTTATCCA	GeoGC-59P-MGB	6FAM-CCTAATTAGTGGCGCACGG-MGB	84
4	16S	ADEH 16S	Ade qF 399F (Amyxo 8F)	GCAACGCCGCGTGTGT	Ade qR 466R (Amyxo 8R)	TCCCTCGCGCACTCCTT	Amyx-425P-MGB	6FAM-ATGAAGGTCTTCGGATCGT-MGB	67
5	16S	DHB 16S	DhbF	GCCGCGAGGTGAAGCA	DhbR	CAGCCTGCAATCCGAACGTG	Dhb-P-MGB	6FAM-ATCCGAGAAAGCCGTTC-MGB	54
6	16S	DFORM 16S	Dforq1205F	CACCACGAAAGTTGGCAACA	Dforq1265R	TTCGGCGACTGCTTCCTT	Dforq1229MGB	6FAM-AAGTCGATGAGCGAACC-MGB	61
7	16S	DHB1 16S	Dhb881F	CGACGCAACGCGAAGAA	Dhb1002R	CGAAGGGCACTCCCATATCTC	DhbCF50-905F-MGB	6FAM-AAGGCTTGACATCCAAC-T-MGB	72
8	16S	DHB2 16S	Dhb123F	CGTGGGTAACTGCCCTTAAG	Dhb160R	CGGCATTAGCAGCCGTTT	Dhb144MGB	6FAM-CCGGAACAACAGCTG-MGB	56
9	16S	DHB3 16S	Dhb1281F	TCCGAGAAAGCCGTTCGTA	Dhb1318R	CGACTTCATGCAGCGAGTT	Dhb1301MGB	6FAM-TTCGATTGCAGGCTG-MGB	57
10	16S	DHC1 16S	Dhc1047qF (Set K)	CGAGCGCAACCTTGTGTG	Dhc1112qR (Set K)	ACCTTCTCCCCGTTTCG	Dhc 1075PF	6FAM-TTTTAGCGAGACTGCC-MGB	65
11	16S	DHC2 16S	Dhc 1200F (Set J)	CTGGAGCTAATCCCCAAGCT	Dhc 1271R (Set J)	CAACTTCATGCAGCGCGG	Dhc-J-MGB	6FAM-TCGGATTGCAGGCTGA-MGB	65
12	16S	DHC3 16S	DhcF-t-Set H	GGTAATACGTAGGAAGCAAGCGT	DhcR-Set H	CCGGTTAAGCCGGGAAATTCCACTTACGCCCAATAAAATC C	Dhc-H-PR1-MGB	6FAM-GACCACCTACGCTCACT-MGB	97
13	16S	DHGM 16S	Dhgm16S-q478F	AGCAGCCGCGGTAAATACG	Dhgm16S-q536R		Dhgm16S-q500P	6FAM-AGGCGAGCGTTATC-MGB	58
14	16S	DSM1 16S	DSM1F	GGAATTTTCGAGTGTAGGGGTGA	DSM1R	TCAGCGTCAGTATCGGTCCA	DSM16S-q1-MGB	6-FAM-GATATTCTGAAGGAACACCAGT-MGB	87
15	16S	DSM2 16S	DSM2F	ACGCCGCGTGAGTGATG	DSM2R	CTGCTGGCACGGAGTTAGC	DSM16S-q2-MGB	6FAM-AGGGTACCCTCAGGC-MGB	129
16	16S	DF1 16S	DF1-335F	AGCAAGGAATTTGGGCAATG	DF1-391R	CCCCACGCGGTGTT	DF1-361P-MGB	6FAM-CGCAAGCCTGACCCA-MGB	55
17	16S	DHB4 16S	Dhbt1378F	CGTTCGCCGGTCTGTACA	Dhbt1415R	TCGGGTGTTGCCAACTTTC	Dhbt1398MGB	6FAM-ACCGCCCGTCACAC-MGB	56
18	16S	ACMB 16S	Acmb963F	GAAGCAACGCGAAGAACCTT	Acmb1001R	GCTGCTAAGCGCCATTCAG	Acmb984MGB	6FAM-CCAGGTCTTGACATCC-MGB	58
19	16S	NTSP 16S	Ntsp965F	CGCAACGCGAAGAACCTTAC	Ntsp1002R	CCCCTTTCGGGTCTTACCA	Ntsp986MGB	6FAM-TGGGCTTGACATGCT-MGB	58
20	16S	SPIRO1 16S	Spiro577F	GCGTGTAGGCGGTTGAAAAA	Spiro618R	CGCAGTCCCTCGGTTGAG	Spiro600MGB	6FAM-AGGTGTGAAATTCTTC-MGB	59
21	16S	SPIRO2 16S	Spiro545F	CGAGCGTGTCCGGAGTTA	Spiro580R	CTGACTTTTCAACCGCTACA	Spiro565MGB	6FAM-TGGGCGTAAAGAGC-MGB	57
22	16S	DSV 16S	DSV1381F	TCCCGGGCCTTGACACA	DSV1417R	GGCTTCGGGTAGAACCAACTT	DSV1402MGB	6FAM-CCCCTCACACCACG-MGB	57
23	16S	SPG 16S	Sphbac110F	CGGGTGCCTAACGCGTAT	Sphbac147R	CCCGAATTTCTCCAGGTTATGT	Sphbac129-MGB	6FAM-CAACCTACCCTTAACCG-MGB	60
24	16S	METSP1 16S	Msp208F	TCCGTCGCTTCAGGATGAG	Msp249R	GGCCGTTACCCCAACAATA	Msp234-MGB	6FAM-CTGCGGCCGATTAG-MGB	55
25	16S	METSP2 16S	Msp809F	GCTGTAAACGATGCGCGTTA	Msp847R	TTCCCTTCGGCACCTCAGT	Msp831-MGB	6FAM-TGTGTCACTGACCACGTG-MGB	61
26	16S	VAD27 16S	Rkn460F	TCGTGAAGGCCGATGAGAGT	Rkn507R	GCTGCTGGCACGGAGTTAG	Rkn488-MGB	6FAM-CCATACGAATAAGCACCG-MGB	59
27	pceA	GEO PCEA	pceA_Geo 833F	GCATGGGCGATGGACTATG	pceA_Geo 891R	AGCAGCACCAGCAACTAACGA	pceA_Geo_852P-MGB	6FAM-TGCAATGAAAACAGCTC-MGB	64

Assay No	Assay Group	Assay Name	Forward Primer	Forward Primer Seq (5'to 3')	Reverse Primer	Reverse Primer Seq (5'to 3')	TaqMan Probe	TaqMan Probe Seq (5'to 3')	Amplicon Size
28	pceA	DHC195_PCEA	Dhc_195_pceA1111F	CCACCCCGGTTCGTTC	Dhc_195_pceA1171R	CGGCGTGGTGGAAACAG	Dhc_195_pceA1134P	6FAM-ACATGACGTAGTCAAGGG-MGB	61
29	pceA	DHCCBDB_PCEA	pceA_cbdb1-1132F	GCCCGCAAGTTCTGTGAAAC	pceA_cbdb1-1191R	GATAGCGCCGAAAGGACAAG	pceA_cbdb1-1153FP	6FAM-TGCGGTATCTGTGCCGAG-MGB	60
30	pceA	SFP_PCEA	Sfp_pceA508F	GGTTAGATAGAAGACCTCCAGATACA	Sfp_pceA584R	GCCATACGTGCAGCAAATTTT	Sfp_pceA508FP	6FAM-CTGATCCTGTGGAACTT-MGB	77
31	pceA	DHCP_HYCE	DhcP_HycE_171F	TGCCCCAATACCTATGCTT	DhcP_HycE_230R	GGAGGACGGGAAGAGCAGAT	DhcP_HycE_193P	6FAM-TCTTCGGCAGTTGAAC-MGB	59
32	pceA	DF_PCEA	Df_pceA_1223f	CGGACAAGCCGAGAAAATTC	Df_pceA_1286R	GCATCCGCACATTTTTTGC	Df-pceA_1223f	6FAM-TACGCGAGTTCTGCCG-MGB	64
33	tceA	DHCFL2_TCEA	tceA-1270qF	ATCCAGATTATGACCTGGTGAA	tceA-1336-qR	GCGGCATATATTAGGGCATCTT	tceAq1294P-MGB	6FAM-TGGGCTATGGCGACCGCAGG-MGB	67
34	cbrA	DHC_CBRA	cbrA_1021qF	ACTGATTGCCTCTCGCTCCTA	cbrA_1080qR	AGCGCAAAAGCGGGTATG	cbrA_1044qPFM	6FAM-CAGGCTATTGATTCTG-MGB	60
35	mbrA	DHC_MBRA1	mbrA_230qF1	CGACAATCGAGTGGCTCACT	mbrA_290qR	GGCAGAGTTCGATATTCATCGTCTA	mbrA_252qPFM	6FAM-ACGCCCCAGTCT-MGB	61
36	mbrA	DHC_MBRA2	mbrA_1237qF	CCCAGGGCATAAAACATGGTAT	mbrA_1303qR	GAGGCTGATTCTCCAGAAC	mbrA_1223FPM	6FAM-TTGATCATTCAAGTCCGGGA-MGB	67
37	dcpA	DHC_DCPA	dcpA491qF	GAAGGCTCCGAAACGGAAA	dcpA549qR	TTCTTGAGGCAATTTGGTGC	dcpA511qP-MGB	6FAM-CGCATTCATGATTTCGAA-MGB	59
38	VC-reductases	DHC_VCRA1	verA401qF	CGGGCGGATGCACTATTTT	verA472qR	GAATAGTCCGTGCCCTTCCTC	verA-q1042PR-MGB	6FAM-TACAGGAAATGGTTGAGTTAC-MGB	72
39	VC-reductases	DHC_BVCA1	bvcA58qF	GCGGGAGCAGGATAGGT	bvcA116qR	TCATCCAAGTCGTGAAAATTCG	bvcA770-MGB	6FAM-CCGCGACTTCAGTTAT-MGB	59
40	VC-reductases	DHC_VCRA2	verAF_Set H (verA_628F)	TGCTGGTGGCGTTGGTGCTCT	verAR-Set H (verA_1068R)	TGCCCCGTCAAAAGTGGTAAAG	verA1324F-P-H-MGB	6FAM-GAGTGGTTGCCATAAC-MGB	440
41	VC-reductases	DHC_BVCA2	bvcAF-Set H (bvcA_545F)	TGCCTCAAGTACAGGTGG	bvcAR-Set H (bvcA_1428R)	ATTGTGGAGGACCTACCT	brcA_P-MGB-H	6FAM-TGCCGAATTTTACGACT-MGB	838
42	Cobalamin	DHCP_CBIZ1	DhcP_cbiZ_410qF	GGATTGGACAGGATAAATCTTCA	DhcP_cbiZ_560q2R	GCAATAGTCTTAGCCTCGGTAGCT	DhcP_cbiZ_440qFP	6FAM-ACCGCAACGGCAGAT-MGB	151
43	Cobalamin	DHCP_CBIZ2	DhcP_cbiZ_410qF	GGATTGGACAGGATAAATCTTCA	DhcP_cbiZ_564qR	CAGACCAATAGTCTTAGCCTCGGTAG	DhcP_cbiZ_440qFP	6FAM-ACCGCAACGGCAGAT-MGB	155
44	Cobalamin	DHCP_COBU	DhcP_cobu_412qF	AATGATGTAGGTGCTGGTCTTATTCC	DhcP_cobu_533R	CCGGCTACCATCAGATAAACG	DhcP_cobu_499qFP	6FAM-AGGGCAAAACCAATG-MGB	122
45	Hydrogenase	ETNE	EtnE-M173qF	GCGGTTCTGCCATCGT	EtnE-MI208qF	TGCCGTCGAAATGATGTC	EtnE-MI177qP	6FAM-ACGACCAAGAAGCGG-MGB	61
46	Hydrogenase	ETNC1	EtnE-MI836qF	CGTGGGCGTACAAGGATGTC	EtnE-MI898qR	TCATGTACGAGCCGACGAAGT	EtnE-MI860qP	6FAM-AAGAGTGGGTCGTCGAC-MGB	63
47	Hydrogenase	ETNC2	EtnE2-M1877qF	TGGAACCGCCATTCGT	EtnE2-M1932qR	GTTTCGGTGATCGTGCTCTTG	EtnE2-MI894-rqP	6FAM-ATCGATGACGCCCCG-MGB	56
48	Hydrogenase	DHC_FHCA	Dhc-fdhA_170F	CCAGCGGTTGCGGATTTA	Dhc-fdhA_239R	TCWGGATCACCTTCCATATTG	Dhc-fdhA189P	6FAM-CTGCTATACCGATTCTCG-MGB	70
49	Hydrogenase	DHCP_VHUA	DhcP_vhuA_440F	CGCCATCTGGCCTCAAAA	DhcP_vhuA_503R	CAGCCGCTACAGGGTGGATA	DhcP_vhuA_468P	6FAM-CCTGCTGGGAAAAG-MGB	64
50	Hydrogenase	DHCP_FEHYMC1	Dhc-FehymC1549F (GT)	CTGAAGAAAAACGCGGTGAA	Dhc-FehymC1607R (GT)	CACCGCTTGGGCATATTC	Dhc-FehymC1571P (GT)	6FAM-CCCTTTACGAAGAAGAC-MGB	59
51	Hydrogenase	DHCP_FEHYMC2	Dhc-FehymC1009F (GT)	GCGGAAAAGGCAGGCATAG	Dhc-FehymC1068R (GT)	AGTACAAGGCATGACCGAAAC	Dhc-FehymC1029P (GT)	6FAM-CCCCAAGACTATTGTCA-MGB	60
52	Hydrogenase	DHC_HUPL	Dhc_hupL69F	CGGCGGCGAGGTTAAAG	Dhc_hupL121R	CAAAGCCGCGGAACAAAG	Dhc_hupL87P MGB	6FAM-TGCCAAATGTGTCGGC-MGB	53
53	Luciferase Assay	MIC_LUCI	Luci qF	TACAACACCCCAACATCTTCGA	Luci qR	GGAAGTTCACCGGCGTCAT	Luci qP-MGB	6FAM-TGGCAGGTCTTCCCGA-MGB	67
54	Cytochrome C	ADEH_CYTC1	2cp-C_1278_505qF	CGGCTGCCGAGAACTC	2cp-C_1278_568qR	CCTGCGACAGCCAGTTGATG	Adeh_1278_532P-M	6FAM-CTCGCCATCGTCTCG-MGB	64

Table A-49. RD-qChip v2 Assay Information.

Assay No	Assay Group	Assay Name	Forward Primer	Forward Primer Seq (5'to 3')	Reverse Primer	Reverse Primer Seq (5'to 3')	TaqMan Probe	TaqMan Probe Seq (5'to 3')	Amplicon Size
1	16S	BAC_16S	Bac16S_1055YqF	ATGGYTGTCGTACAGT	Bac16S_1392qR	ACGGGCGGTGWGTAC	Bac16S_1115qP	6FAM-CAACGAGCGCAACCC-MGB	351
2	16S	METH_16S	Meth16S_835qF	GGGRAGTACGKYCGCAAG	Meth16S_918qR	GAVTCCAATTRARCCGCA	Meth_873qRP	6FAM-CCAATTCCTTTAAGTTTCA-MGB	84
3	16S	DHC_16S	Dhc16S_1200qF	CTGGAGCTAATCCCCAAGCT	Dhc16S_1271qR	CAACTTCATGCAGCGCGG	DhcJ_16S_qP	6FAM-TCGGATTGCAGGCTGA-MGB	66
4	16S	DHB_16S	Dhb16S_qF	GCCGCGAGGTGAAGCA	Dhb16S_qR	CAGCTGCAATCCGAAGT	Dhb16S_qP	6FAM-ATCCGAGAAAGCCGTTC-MGB	54
5	16S	DHCCOR_16S	DhcCor16S_qF	AACTGAAGGTAATACCGCATGTGAT	DhcCor16S_qR	ATCCTCTCAGACCAGTACCGA	DhcCor16S_qP	6FAM-TAAGTCGGTTTCAATAAGC-MGB	146
6	16S	DEFO_16S	Dforq16S_1205qF	CACCACGAAAGTTGGCAACA	Dforq16S_1265qR	TTCGCGGACTGCTTCCTT	Dforq16S_1229qP	6FAM-AAGTCGATGAGCGAACC-MGB	61
7	16S	DHGM_16S	Dhgm16S_478qF	AGCAGCCGCGGTAATACG	Dhgm16S_536qR	CCACTTTACGCCCAATAAATC	Dhgm16S_500qP	6FAM-AGGCGAGCGTTATC-MGB	59
8	16S	DIEL_16S	Diel16S_123qF	CGTGGGTAACCTGCCCTTAAG	Diel16S_160qR	CGGCATTAGCAGCGTTT	Diel16S_144qP	6FAM-CCGGAACAACAGCTG-MGB	56
9	16S	GEO_16S_1	Geo16S_810qF	CGATGAGWACTAGGTGTGTCGG	Geo16S_884qR	CTTCCCAGGCGGAGWACTTAATGC	Geo16S_834qP	6FAM-ATTGACCCCTGCAGTGCC-MGB	75
10	16S	GEO_16S_2	GeoSZ16S_241qF	CGTACCATTAGTAGTGTGTTAGGT	GeoSZ16S_476qR	GGGTATTAACCGACTATCATT	GeoSZ16S_266qP	6FAM-AAGAGCCTACCAAGGCGACGATGTT-MGB	236
11	16S	DF1_16S	DF1-335qF	AGCAAGGAATTTTGGGCAATG	DF1_391qR	CCCCACGCGGTGTT	DF1_361P	6FAM-CGCAAGCCTGACCCA-MGB	55
12	16S	SFP_16S	SFP16S_657qF	GGAATACCSATTGCGAAGGC	SFP16S_753qR	CTAGTGIDCATCGTTTAGGGC	SFP16S_700qP	6FAM-CTGAGGCATGAAAGCGTGG-MGB	117
13	16S	DSF_16S	DSF16S_1308qF	GGTCYCAGTTCGGATTGTTCT	DSF16S_1381qR	GTRTGYTGACCTGCGATTACTAG	DSF16S_1334qP	6FAM-ACTCGAGAACATGAAGTCGG-MGB	74
14	16S	AMYXO_16S	Amyxo16S_399qF	GCAACGCCGCGTGTGT	Amyxo16S_466NgR	TCYCTCSCGACAGTGTCT	Amyx16S_425P	6FAM-ATGAAGGTCTTCGGATCGT-MGB	55
15	16S	FLIPS_16S	FLiPS16S_1131qF	TGGGSACTCAGRCGGAAC	FLiPS16S_1189qR	ACTTGACGTCGTCCCCACCTT	FLiPS16S_1152qP	6FAM-CGGTGACAAACCGGA-MGB	60
16	16S	DCM_ACMB_16S	Acmb963F	GAAGCAACGCGAAGAACCTT	Acmb1001R	GCTGCTAAGCGCCATTACG	Acmb984MGB	6FAM-CCAGGTCTTGACATCC-MGB	58
17	16S	DCM_NTSP_16S	Ntsp965F	CGCAACGCGAAGAACCTTAC	Ntsp1002R	CCCCTTCGGGTCTTACCA	Ntsp986MGB	6FAM-TGGGCTTGACATGCT-MGB	58
18	16S	DCM_SPIRO_16S	Spiro577F	GCGTGTAGGCGGTTGAAAAA	Spiro618R	CGCAGTCCCTCGGTTGAG	Spiro600MGB	6FAM-AGGTGTGAAATCTTC-MGB	59
19	16S	DCM_DSV_16S	DSV1381F	TCCCGGGCCTTGTACACA	DSV1417R	GGCTTCGGGTAGAACCAACTT	DSV1402MGB	6FAM-CCCCTCACACCACG-MGB	57
20	16S	DCM_SPG_16S	Sphbac110F	CGGGTGCCTAACGCGTAT	Sphbac147R	CCCGAATTTCTCCAGGTTATG	Sphbac129-MGB	6FAM-CAACCTACCCTTAACCG-MGB	60
21	16S	DCM_METSP_16S	Msp208F	TCCGTCGCTTCAGGATGAG	Msp249R	GGCCGTTACCCCAACAATA	Msp234-MGB	6FAM-CTGCGGCCGATTAG-MGB	55
22	16S	DCM_VAD27_16S	Rkn460F	TCGTGAAGGCCGATGAGAGT	Rkn507R	GCTGCTGGCACGGAGTTAG	Rkn488-MGB	6FAM-CCATACGAATAAGCACCG-MGB	59
23	RDase	PCEA_195	Dhc195_pceA_1111qF	CCACCCCGGTTTCGTTCA	Dhc195_pceA_1171R	CGGCGTGGTGAAACAG	Dhc195_pceA_1134qP	6FAM-ACATGACGTAGTCAAGGG-MGB	55
24	RDase	CBDDBA1588_DH C_RDHA	cbdb1_1132qF	GCCCGCAAGTTCTGTGAAAC	cbdb1_1191qR	GATAGCGCCGAAAGGACAAG	cbdb1_1153qP	6FAM-TGCGGTATCTGTGCCGAG-MGB	162
25	RDase	PCEA_GEO	pceA_Geo_833qF	GCATGGGCGATGGACTATG	pceA_Geo_891qR	AGCAGCACCAAGCACTAACGA	pceA_Geo_852qP	6FAM-TGCAATGAAACAGCTC-MGB	60

Assay No	Assay Group	Assay Name	Forward Primer	Forward Primer Seq (5'to 3')	Reverse Primer	Reverse Primer Seq (5'to 3')	TaqMan Probe	TaqMan Probe Seq (5'to 3')	Amplicon Size
26	RDase	PCEA_DHB_DSF	pceA_DHB_DSF_326qF	GAAAGACTTGGGATCAAAGGGC	pceA_DHB_DSF_487qR	ATCAAATTCTACAGCCCAGGCG	pceA_DHB_DSF_433qP	6FAM-CTGGACCGGGTTGGATGAG-MGB	60
27	RDase	PCEA_SFP	Sfp_pceA_508qF	GGGTTAGATAGAAGACCTCCAGATACA	Sfp_pceA_584qR	GCCATACGTGCAGCAAATTTT	Sfp_pceA_508qP	6FAM-CTGATCCTGTGGAACCT-MGB	77
28	RDase	TCEA	tceA_1270qF	ATCCAGATTATGACCCTGGTGAA	tceA_1336qR	GCGGCATATATTAGGGCATCTT	tceA_1294qP	6FAM-TGGGCTATGGCGACCGCAGG-MGB	67
29	RDase	VCRA	vcrA_401qF	CGGGCGGATGCACTATTTT	vcrA_472qR	GAATAGTCCGTGCCCTTCCTCTC	vcrA_1042qP	6FAM-TACCAGGAAATGGTTGAGTTAC-MGB	72
30	RDase	BVCA	bvcA_58qF	GCGGGAGCAGGGATAGGT	bvcA_116qR	TCATCCAAGTCGTGAAAATTCG	bvcA_77qP	6FAM-CCGCGACTTCAGTTAT-MGB	59
31	RDase	MBRA	mbrA_230qF	CGACAATCGAGTGGCTCACT	mbrA_290qR	GGCAGAGTTCGATATTCATCGTCTA	mbrA_252qP	6FAM-ACGCCCCAGTCT-MGB	60
32	RDase	CBRA	cbrA_1021qF	ACTGATTGCCTCTCCTCCTA	cbrA_1080qR	AGCGCAAAGCGGGTATATG	cbrA_1044qP	6FAM-CAGGCTATTGATTCTG-MGB	60
33	RDase	DCMB_1041 RDHA	Dhc_dcmb1041_1129qF	GTAAATGCCGAGACCCCAA	Dhc_dcmb1041_1205qR	CCATCTGCTGGCTATCTGG	Dhc1041_1152P	6FAM-TATGGTGA CTATGGACGGGC TA-MGB	76
34	RDase	DCMB_1339 RDHA	Dhc_dcmb1339_521qF	CACCCTTCTGTATAAGCCCG	Dhc_dcmb1339_606qR	GCCCCATAGACTTTGGTGCCT	Dhc1339_546qP	6FAM-ACAAGCATCCGCGAAATAC-MGB	86
35	RDase	DCMB_1434 RDHA	Dhc_dcmb1434_573qF	CCATGAGGGCTCTTTGTCGT	Dhc_dcmb1434_647qR	TTCTGCCATGACTGCGCTAA	Dhc1434_601qP	6FAM-CCTGTGAGACACAAGCTTC-MGB	75
36	RDase	PCB1_CG4_CG5	DhcCG5_PCB1_1845qF	TCGCCAAGCCCAGACATAAC	DhcCG5_PCB1_1946qR	GTGATATAGCACAGTGGCGTTACA	DhcPCB1_1903P	6FAM-CAGCACTTTAAGGAATGC-MGB	121
37	RDase	PCB2_CG1	DhcCG1_PCB2_1134qF	GTTAATCGCACTTGCAGGGC	DhcCG1_PCB2_1027qR	TTGACAGACTTACCTCTCCCA	DhcPCB2_1053P	6FAM-CAATCCCATAGATGCTGGTAT-MGB	108
38	RDase	RDHA-AWM53_01188-RM	rdhA-01188-RM 666qF	GGATAACGGAAGAGAAGTACCTTG	rdhA-01188-RM 809qR	ATATCGTCTTCGCGCATTC	rdhA-01188-RM 763qP	6FAM-CCTCTCAACAATGTAAGTGC-MGB	144
39	RDase	RDHA-AWM53_01801-RM	rdhA-01801-RM 712qF	GGTTACGATGGAATCAGTGCTG	rdhA-01801-RM 853qR	CCATTACTGCGGCATAATTGGTA	rdhA-01801-RM 736qP	6FAM-CAGTCTATGCGTTCTTATC-MGB	142
40	RDase	RDHA-AWM53_00864-RM	rdhA-00864-RM 638qF	GTTGGCTTATGACGGAACCTT	rdhA-00864-RM 850qR	AACCAAAATGCAATAATACCA GTATCG	rdhA-00864-RM 733qP	6FAM-CACGTAGTAGTAGCGTTAT-MGB	213
41	RDase	CFRA	DhbCF_cfrA 685qF	CCCAAGCATGTAATCTCGATGA	DhbCF_cfrA 804qR	GAATGCAGCATATCCAATCTGG	DhbCF_cfrA 722qP	6FAM-CAGAAGGAGTAAAGTGTGAC-MGB	120
42	RDase	CTRA_THMA	Dsf_ctrA_636qF	GCCTGAGGGTGAAAGTCTGAA	Dsf_ctrA_867qR	TGCACTGTCAGCTCCGATTG	Dsf_ctrA_715qP	6FAM-CAGTCGCTAGAAGGAGTAA-MGB	232
43	RDase	CF_ALL	CF_All_202qF	GCAGACTACAAACCGTTTACC	CF_All_348qR	AAGTACAGTAAGACCGGGCGAA	CF_All_275qP	6FAM-CACTTGTAGAGCGTTTGTATG-MGB	147
44	RDase	DCRA_DHB	dcra_DHB_236qF	TATGGGGCCAGGCTTTATTGG	dcra_DHB_381qR	AACGGCCAGCTTGCATGA	dcra_DHB_275qP	6FAM-CACTCAGAGAGCGTTTGTG-MGB	146
45	RDase	DCAA_DSF	dcaA_DSF_387qF	TCAGCATACGGGTGTCATGC	dcaA_DSF_520qR	CCGGACCAATATCTGTTGCGT	dcaA_DSF_411qP	6FAM-CCAGCGCAGTAAAGAAACAG-MGB	134
46	RDase	DCAA_DHB_WL	dcaA_DhbWL_177qF	GTACCAACGTTACAATAGTCTGAG	dcaA_DhbWL_382qR	AGAAAAGAACGCTGTTTCTGCAT	dcaA_DhbWL_214qP	6FAM-AAGGCTTTAGACCCGGAAGC-MGB	206
47	RDase	DCPA	dcpA_491qF	GAAGGCTCCGAAACGGAAA	dcpA_549qR	GCACCAAAATGCTCTCAGGAA	dcpA_511qP	6FAM-CGCATTTCATGATTGCAA-MGB	59
48	RDase	CERA	cerA_Dhgm_1336qF	GCCCTACCAAACCGATTGA	cerA_Dhgm_1421qR	ATGGCTTGTCGGGACATTT	cerA_Dhgm_1364qP	6FAM-TCCGTGAGTTCTGCAAGACC-MGB	86

Assay No	Assay Group	Assay Name	Forward Primer	Forward Primer Seq (5'to 3')	Reverse Primer	Reverse Primer Seq (5'to 3')	TaqMan Probe	TaqMan Probe Seq (5'to 3')	Amplicon Size
49	RDase	DHGM_GP_00862 RDHA	DhgmGP_PR013_00_1338qF	CCACGAGTACTGAAGTCGG	DhgmGP_PR01300_1_401qR	CATGGTGGCAAGGAAGGGAT	DhgmGP_PR01300_1_358qP	6FAM-CAATTCCTACTGTCGGTATCTTCA-MGB	83
50	RDase	DHGM_GP_01297 RDHA	DhgmGP_PR012_97_466qF	TGGACGGGTACGCCTGAAGA	DhgmGP_PR01297_5_18qR	GATATCCGCTGCACCGAAGA	DhgmGP_PR01297_4_95qP	6FAM-AATGATTAGGGCGGCCATGG-MGB	72
51	RDase	DHGM_GP_00862 RDHA	DhgmGP_PR008_62_692qF	ATGAAACCTCGACCAAGCGT	DhgmGP_PR00862_774qR	GGAAAGGTCGTGGCTCATCA	DhgmGP_PR00862_7_17qP	6FAM-ACCGACCAGCCATCAACTACT-MGB	64
52	RDase	HPR1A RDHA P	DhcGT_HPR1a_822qF	GTGTAGGCAGAACCCGAGGTA	DhcGT_HPR1a_929qR	TGCCATCACATGCAGTATTTG	DhcGT_HPR1a_863qP	6FAM-CCGTTATCCACATAGTTG-MGB	108
53	RDase	HPR2A RDHA C	Dhc195_HPR2a_1041qF	ACCTTTTACAACCTGTGGCAATCTG	Dhc195_HPR2a_1122qR	CGATAGTGGCGATAAGCTGATAAC	Dhc195_HPR2a_1072qP	6FAM-CCACCTACAACCTCCTCAGG-MGB	82
54	RDase	ETNC	EtnC-M836qF	CGTGGGCGTACAAGGATGTC	EtnC-M898qR	TCATGTACGAGCCGACGAAGT	EtnC-M860qP	6FAM-AAGAGTGGGTCGTGAC-MGB	63
55	RDase	ETNE	EtnE-M173qF	GCGGTTCTGGCCATCGT	EtnE-M208qR	TGCCGTCGAAATGATGTC	EtnE-M177qP	6FAM-ACGACCAAGAAGCGG-MGB	61
56	RDase	ETNE2	EtnE2-M1877qF	TGGAACCGCCATTCTGT	EtnE2-M1932qR	GTTTCGGTGATCGTGCTCTTG	EtnE2-M894-qP	6FAM-ATCGATGACGCCCGC-MGB	56
57	RDase	EtnE_MB	EtnE_MPO_qF1	TCGTCGAGTTGRATRATGTC	EtnE_MPO_qR1	AAGGCRTTCAAAGAGGAYTT	EtnE_MB_qP	6FAM-CAGCCGTTCTGCTCGATCT-MGB	65
58	RDase	EtnE_MPO	EtnE_MPO_qF1	TCGTCGAGTTGRATRATGTC	EtnE_MPO_qR1	AAGGCRTTCAAAGAGGAYTT	EtnE_MBPO_qP	6FAM-ATTCTGCTCGAGCTCCTG-MGB	65
59	RDase	EtnE_NCD	EtnE_NCD_419qF	TCTCCTAYGTSGGCATCCAGG	EtnE_NCD_557qR	ATGATGTCGACGCCGTTGT	EtnE_NCD_512qP	6FAM-TCAACGAGGACTTCAAGG-MGB	139
60	RDase	SDC9_45582-RDHA	SDC9_rdhA18_673qF	GCGCATTGTCAAGCTCAATC	SDC9_rdhA18_781qR	GCCATACTGCCAATGTTCCG	SDC9_rdhA18_703qP	6FAM-TTATGAACACGCACCCGGAG-MGB	109
61	RDase	SDC9_09280-RDHA	SDC9_rdhA66_1_019qF	CCCTTGAGGTGGATAAGCCCC	SDC9_rdhA66_1135qR	TCTCCCTGGCTCATCATCA	SDC9_rdhA66_1092qP	6FAM-TTGCCCTTCAAAAGCCATTTC-MGB	67
62	RDase	SDC9_23241-RDHA	SDC9_rdhA158_700qF	AGCCAGTCTTCCATTGACGG	SDC9_rdhA158_812qR	AGAAATTCCTGGGTAGCCGC	SDC9_rdhA158_730qP	6FAM-ACCCCTTCATGGATTACGCG-MGB	113
63	RDase	SDC9_07142-RDHA	SDC9_rdhA192_948qF	TACGGACCTTCCTTTGGTGC	SDC9_rdhA192_1_037qR	GGACAGACTTCGGCACACTT	SDC9_rdhA192_979qP	6FAM-ATAGATGCGGGTATCTGGCG-MGB	90
64	RDase	SDC9_07143-RDHA	SDC9_rdhA195_445qF	CAAATGGGTGTGGCCAACTG	SDC9_rdhA195_554qR	TCCGTTATTCCAAGGCGCT	SDC9_rdhA195_504qP	6FAM-CCTCACGTTATCTGGGAGCATG-MGB	110
65	RDase	SDC9_23243-RDHA	SDC9_rdhA52_4_88qF	TAGCCGCCACTAAGGTTGAG	SDC9_rdhA52_587qR	ACTTCGCAGATACCCACCTG	SDC9_rdhA52_513qP	6FAM-TCCCGAAGAAATGACAGCCC-MGB	100
66	RDase	SDC9_48350-RDHA	SDC9_rdhA29_1_488qF	TCGAGACCTGGAACACGAT	SDC9_rdhA29_1547qR	TCATCAAACCTGCGGGCTG	SDC9_rdhA29_1493qP	6FAM-CCAGAATAGCCACCC-MGB	79
67	Corrinoid uptake and modification	CBIZ_P	DheP_cbiZ_410qF	GGATTGGACAGGATAAATCTTCA	DheP_cbiZ_560q2R	GCAATAGTCTTAGCCTCGGTAGCT	DheP_cbiZ_440qFP	6FAM-ACCGCAACGGCAGAT-MGB	151
68	Corrinoid uptake and modification	CBIZ_V_C	DheVS195_cbiZ_415qF	GGGCAGGATAAGTCTTCGGG	DheVS195_cbiZ_676qR	GCCCACGTAGGTTGCCT	DheVS195_cbiZ_488qP	6FAM-TTACCAGTGAAAGCCTGCCG-MGB	261
69	Corrinoid uptake and modification	COBA_DHC_ALL	Dhe_cobA_ALL_416qF	TAGAGCTGATTATGACCGGG	Dhe_cobA_ALL_534qR	ACCTTTACGGGCTTTGATGC	Dhe_cobA_ALL_486qP	6FAM-AAAGGCAGTCAAACACCCGT-MGB	119
70	Corrinoid uptake and modification	COBC_DHC_P	DheGT_cobC_34_6qF	CCTGACGGGAAAGTATGGC	DheGT_cobC_541qR	AACAGTTACCGAACCCACCC	DheGT_cobC_406qP	6FAM-TCCAAGCACAAAAGTGACGA-MGB	195
71	Corrinoid uptake and modification	COBC_DHC_C_V	DheVS195_cobC_408qF	CAAGCACCGGGAGGATGAGA	DheVS195_cobC_526qR	CAACACCCAGCGTAAACTGC	DheVS195_cobC_470qP	6FAM-TCTGCCATTTTCTGGGCATTGA-MGB	119

Assay No	Assay Group	Assay Name	Forward Primer	Forward Primer Seq (5'to 3')	Reverse Primer	Reverse Primer Seq (5'to 3')	TaqMan Probe	TaqMan Probe Seq (5'to 3')	Amplicon Size
72	Corrinoid uptake and modification	COBS_DHC_P1	DhcGT_cobSqF	TGACTGCGCGCTTTGCTGATA	DhcGT_cobSqR2	ACCTCTTGGGCGCGTTCA	DhcGT_cobS_232qP	6FAM-ACAGGCGGTTTACATCTGGA-MGB	123
73	Corrinoid uptake and modification	COBS_DHC_P2	cobSGT_137qF	TCGGGCTGATACTGGTCGGT	cobSGT_293qR	TTATGACCGGCGGCTATTCC	DhcGTcobS_232qP	6FAM-ACAGGCGGTTTACATCTGGA-MGB	157
74	Corrinoid uptake and modification	COBS_DHC_C_V	DhcVS195_cobS_612qF	TATGTCGGCAGCTGGCTG	DhcVS195_cobS_734qR	AATATCAGCGCCGCTACCTC	DhcVS195_cobS_663qP	6FAM-CAGGCTTGGCGGACTAAACG-MGB	124
75	Corrinoid uptake and modification	COBT_DHC	cobT_DhcAll_860qF	TGCGGCGGCWCTTATTGC	cobT_DhcAll_1007qR	CTTACCCAGMCGCATATCCAT	cobT_DhcAll_911qP	6FAM-TTTGCCGCTCACCAGTC-MGB	216
76	Corrinoid uptake and modification	COBT_DHB	cobT_DHB_159qF	CCTCGGAAGCTTAGGTGTCTT	cobT_DHB_229qR	TTGGATGCGTGGTGCCAA	cobT_DHB_191qP	6FAM-CACAACAGCTCGCAGGCA-MGB	71
77	Corrinoid uptake and modification	COBT_DSF	cobT_DSF_468qF	TCACCGCATCATGAACGGAAC	cobT_DSF_598qR	CCAGGACATTYACTCCGGAA TC	cobT_DSF_538qP	6FAM-TCCCTGCTGACCGGAGCTAA-MGB	131
78	Corrinoid uptake and modification	COBU_DHC_P	DhcGT_cobU_476qF	GCAGGGCAAACCAAATGCTG	DhcGT_cobU564qR	ACGCAACAAGGGTTTACCTTG	DhcGT_cobU_519qP	6FAM-TCTGATGGTAGCCGGAAGTGC-MGB	89
79	Corrinoid uptake and modification	COBU_DHC_V	DhcVS_cobU_384qF	GACGGGTGCGTCATTATTGTGG	DhcVS_cobU_491qR	ATCTGGTTTGCCCTTCCCAG	DhcVS_cobU_446qP	6FAM-CCATGGCCAGAGTCTATCGT-MGB	108
80	Corrinoid uptake and modification	COBU_DHC_C	Dhc195_cobU_372qF	AGCTATGGAAGAGCGGGTGCG	Dhc195_cobU_535qR	GTCCGGCCACCATAAGGTAC A	Dhc195_cobU_459qP	6FAM-TTACCGGGATTGCTGGGC-MGB	164
81	Corrinoid uptake and modification	BTUC_DHC_P_V	btuC_GTVS_706qF	CTGGTTATACTCCTGTTTGCCCG	btuC_GTVS_872qR	CCCACAAAACCGATAATACC CAC	btuC_GTVS_818qP	6FAM-CTACCCTGATAACAGCCGC-MGB	167
82	Corrinoid uptake and modification	BTUC_DHC_C	Dhc195_btuC_442qF	ACCACTTTGATACTGGCCGG	Dhc195_btuC_582qR	ACTGTCCCAGGCAGCCATAG	Dhc195_btuC_467qP	6FAM-CACTGGGCTCATTCTTACGG C-MGB	141
83	Corrinoid uptake and modification	BTUD_DHC_V	DhcVS_btuD_31qF	CTGGCATATGGTCCGGTTGA	DhcVS_btuD_174qR	GCCCAGACGCGGTTCTATTA	DhcVS_btuD_53P	6FAM-TTCTGCGGAATGTCAGCTTTA AGAC-MGB	144
84	Corrinoid uptake and modification	BTUD_DHC_P	DhcGT_btuD_659qF	CAGATGGCAGTCCGGTTGAG	DhcGT_btuD_783qR	GATATTACCCAGACGCGGCA	DhcGT_btuD_724qP	6FAM-TATGTCCATACCCACCCACTC-MGB	125
85	Corrinoid uptake and modification	BTUD_DHC_C	btuD_195_547qF	AAATCAGAGTGCCGCGAACG	btuD_195_752qR	AGTCCGCTTAGGGGATGAGT	btuD_195_666qP	6FAM-CAGTCCGGCCGAGGTAATAA-MGB	118
86	Corrinoid uptake and modification	BTUF_DHC_ALL	btuF_All_622qF	GAMAGTCAGAAACCGCGTGTT	btuF_All_738qR	AGCAGTAATACTAACGCYTC C	btuF_All_692qRP	6FAM-AAGGCAGTAGAACCAAC-MGB	206
87	Hydrogenases	HYCE_DHC_ALL_1	HycEAll_1257qF	GCATTTTCAAAYTCACAGCCG	HycEAll_1465qR	GGGCTTATCGGSTTATTCGG	HycEAll_1356qP	6FAM-CGGCGGATAATTACCA-MGB	209
88	Hydrogenases	HYCE_DHC_ALL_2	HycEAll_1274qF	GAVGGGAAAATGGCGGCAA	HycEAll_1449qR	CCGAAGCTGGCYGAAGA	HycEAll_1356qP	6FAM-CGGCGGATAATTACCA-MGB	176
89	Hydrogenases	ECHE_DHC_ALL_1	echEAll_288qF	TGCCTGGGCWGAGCTTTC	echEAll_410qR	ATATCCAGRATAATCTCACGG GC	echEAll_314qP	6FAM-ACAGCCATAGCCTCT-MGB	123
90	Hydrogenases	ECHE_DHC_ALL_2	EchE_All_3qF	GGCACGVACAGTAATACCT	EchE_All_147qR	CTCACAKGCYTTTTCTACCCC G	EchE_All_44qP	6FAM-AATACCGGATGCTGTGGC-MGB	145
91	Hydrogenases	HYMB_DHC_AL_L	HymBAll_449qF	GCCATAGACCAGGCAACCG	HymBAll_531qR	TTGTAAAACCTGRATATCCAGC GAGA	HymBAll_469qP	6FAM-ACGCGGTTTCTGGG-MGB	83
92	Hydrogenases	HYMC_DHC_AL_L1	hymCAll_638qF	TGCCCGGTGGGTGCTA	hymCAll_690qR	GCCGCCCAGACAGCATC	hymCAll_655qP	6FAM-CAAGGAACGCTCGGC-MGB	53
93	Hydrogenases	HYMC_DHC_AL_L2	HymCAll_848qF	GCTGACCTGACCATHTHTGGAAGA	HymCAll_960qR	TCCATAAACTTGACCCAGCC	HymAll_875qP	6FAM-TCGGAAGTGGTGAACGG-MGB	110
94	ETC	CISM (fdhA)_DHC_ALL_1	fdhA_All_2944qF	AAAGACCAGCTGTGGCAAAGA	fdhA_All_3022qR	ATGAGGTCTCAGTTTAGTAGG CGAG	fdhA_All_2977qP	6FAM-CTCCGCTGATCTTGA-MGB	79

Assay No	Assay Group	Assay Name	Forward Primer	Forward Primer Seq (5'to 3')	Reverse Primer	Reverse Primer Seq (5'to 3')	TaqMan Probe	TaqMan Probe Seq (5'to 3')	Amplicon Size
95	ETC	CISM (fdhA)_DHC_ALL 2	fdhA_All_2891qF	ATGGGTCCACGGAAAAGACC	fdhA_All_2952qR	CTCAAGATCAGCGGAGGGAC	fdhA_All_2911qP	6FAM-AGCTGTGGCAAAGAGACCC-MGB	62
96	Hydrogenases	VHUA_DHC_ALL	vhuA_All_122qF	GTATCTGATGCCCGCATGC	vhuA_All_317qR	CACGCAGTTTGC GGCC	vhuA_All_197qP	6FAM-ATGCCCGCTATTACC-MGB	196
97	Hydrogenases	HUPL_DHC_ALL 1	hupL_1425qF	CAGCATCACGCGGGTCA	hupL_1502qR	TTAAAGATGCCAAATGTGTC GG	hupL_1450qP	6FAM-CATAAATATTTCAAAGCCGCG-MGB	78
98	Hydrogenases	HUPL_DHC_AL L2	hupL_DhcAll_13 61qF	TTTAAAGCGGCGGTAGTGC	hupL_DhcAll_1560qR	CCCATAACGAGGATTGAAGG TCA	hupL_DhcAll_1490qP	6FAM-ATTTCAAAGCCGCGAACA-MGB	200
99	Dehydrogenases	FDHA1_DHGM_B L-DC-9	DHGM_fdhA1_1 161qF	CAACTTCTGGAARCGCCCC	DHGM_fdhA1_1291q R	TGTAGCGCCAYTGCATCC	DHGM_fdhA1_1243q P	6FAM-AAGGAAGTTTCGGTCACCA-MGB	131
100	Dehydrogenases	FDHA2_DHGM_B L-DC-9	DHGM_fdhA2_7 23qF	CCCTCCGGTGT TTTTGCAT	DHGM_fdhA2_809qR	GCCGATGTTTCGAGTGAAACG	DHGM_fdhA2_752qP	6FAM-CTCAGGATAAAGGGGCGACA-MGB	87
101	Dehydrogenases	FDHA3_DHGM_B L-DC-9	DHGM_fdhA3_1 489qF	ATCCTSTACGCCATGGGC	DHGM_fdhA3_1569q R	GGTSAGCATGGCCAGRTTG	DHGM_fdhA3_1521q P	6FAM-CCACGGCACCGACAATG-MGB	81
102	Nitrogenases	NIFD_DHC_C	nifD195_650qF	ATAACCCGTTCCGTCTGTTC	nifD195_769qR	TACGGCATTCCCTGGATTAAG	nifD195_687qP	6FAM-CGAAATAACGGGAATCTTGC GCA-MGB	119
103	Nitrogenases	NIFK_DHC_C	nifK_195_37qF	ATCATTTCCAGCAAACGCATT	nifK_195_119qR	GGCTTCCCCATTCTTGACC	nifK_195_64qP	6FAM-CGATAGCCACAACC-MGB	83
104	Nitrogenases	NIFH_DHC_C	nifH_195_69qF	GCCCGGTATTCTGCAGCC	nifH_195_147qR	ATGTCCAGCGGGCTGAAAT	nifH_195_96qP	6FAM-TGCGGATTCCATTCG-MGB	79
105	Nitrogenases	NIFH_DHC_P	nifH_DhcP_362q F	AGCATGCCAGATTCAATACCG	nifH_DhcP_466qR	GGCTGCAACTGAACATAGGT CC	nifH_DhcP_400qP	6FAM-TCCAAGACTATGGGTGTGAC-MGB	105
106	Nitrogenases	NIFD_DHV	nifD_DVH_697q F	GACGCCTACGAGATTGAAC	nifD_DVH_820qR	CATGACGAGGTTGAGGTTG	nifD_DVH_751qP	6FAM-ACCTTCTCCGGCAACAGCGT-MGB	123
107	Control assay	RPOB_DHC_ALL	rpoB_Dhc_784q F2	AACGCCCGCAARCTTATTAA	rpoB_Dhc_875qR	TCCAGACGGCGTTAACCT	rpoB_Dhc_816qP	6FAM-TGACCCCCAGCACTAT-MGB	92
108	Control assay	LUC	Luci qF	TACAACACCCCAACATCTTCGA	Luci qR	GGAAGTTCACCGGCGTCAT	Luci qP-MGB	6FAM-TGGCAGGTCTTCCCGA-MGB	67

Reporting and Dissemination

Reporting

Project Task 7 included non-experimental subtasks including submission of interim reports and a final report. The submission of the final report was delayed because numerous unforeseen factors impacted the construction, testing and validation phases of the RD-qChip. During the active phase of the project, monthly financial reports and quarterly progress reports were submitted as required by SERDP. In progress review (IPR) presentations were given as requested by SERDP. The findings of this research were disseminated through peer-reviewed journal publications, oral presentations, posters, and webinars. This SERDP project supported the training of two MS students, six doctoral students, and five postdocs. All students have completed their degrees and pursue professional careers in industry or academia.

Dissemination

Over the course of the project, all research personnel actively engaged in the dissemination of research findings through preparation of peer-reviewed journal articles, oral presentations and webinars, poster presentation and many discussions with practitioners and RPMs at professional meetings.

Posters and/or oral presentations at national and international professional meetings and symposia

1. Murdoch, R.W., G. Chen, F. Kara-Murdoch, and F.E. Löffler. 2019. Novel gene cluster responsible for involved in anoxic metabolism of dichloromethane. American Geophysical Union Fall Meeting, San Francisco, CA, December 9-13, 2019.
2. Chen, G., R.W. Murdoch, F. Kara-Murdoch, S.R. Campagna, and F.E. Löffler. 2019. Anaerobic metabolism of dichloromethane: Implications for current and early Earth microbial communities. American Geophysical Union Fall Meeting, San Francisco, CA, December 9-13, 2019.
3. Kara-Murdoch, F., R.W. Murdoch, C. Swift, Y. Yin, J. Yan, B. Simsir, K.M. Ritalahti, and F.E. Löffler. 2019. The reductive dechlorination qChip: A high-throughput qPCR tool for bioremediation monitoring. SERDP/ESTCP Symposium, Washington, DC, 3-5 December 2019.
4. Leitner KD, K.F. Murdoch, G. Chen, R.W. Murdoch, and F.E. Löffler. 2019. Using heterologous expression techniques to explore chlorinated organic compound degradation by novel haloacid dehalogenases identified in pristine environments. The 11th Annual Undergraduate Research Conference at the Interface of Biology and Mathematics, Knoxville, TN, November 26, 2019.
5. Kaya, D., B. V. Kjellerup, K. Chourey, R. L. Hettich, D. M. Taggart, and F.E. Löffler. 2019. Effect of fixed nitrogen limitation on reductive dechlorination activity of *Dehalococcoides mccartyi*. AES-1067, ASM Microbe 2019, San Francisco, CA, June 20-24, 2019.
6. Hatzinger, P.B., M. Annable, A. Haluska, F.E. Löffler, and H. Anderson. 2019. Evaluation of factors that influence the long-term success or failure of chlorinated solvent source zone bioremediation. Battelle Fifth International Symposium on Bioremediation and Sustainable Environmental Technologies, Baltimore, DE, April 15-18, 2019.
7. m, J., E.E. Mack, E.S. Seger, and F.E. Löffler. 2019. Biotic and abiotic Degradation of 1,1,2-trichloro-1,2,2-trifluoroethane (CFC-113): Implications for detoxification of chlorinated

- ethenes. Battelle Fifth International Symposium on Bioremediation and Sustainable Environmental Technologies, Baltimore, DE, April 15-18, 2019.
8. Kristy B.D., J.S. Dixon, K.D. Leitner, M.E. Landon, N. Jiang, D.C. Garcia, R.W. Murdoch, G. Chen, and F.E. Löffler. 2019. Genetically engineering *E. coli* to degrade toxic contaminants using dehalogenating mechanisms. Annual Exhibition of Undergraduate Research and Creative Achievement (EURēCA). The University of Tennessee at Knoxville, Knoxville, TN, USA, April 17th, 2019.
 9. Jiang N, J. Yan, and F.E. Löffler. 2019. The lower base of corrinoid small molecules regulates reductive dehalogenase enzyme function in *Dehalococcoides* species. American Society for Biochemistry and Molecular Biology (ASBMB) Spotlight Talk – Enzymes and Enzyme Cofactors Session. Orlando, FL, USA (oral and poster presentation), April 9th, 2019.
 10. Jiang N, J. Yan, and F.E. Löffler. 2019. Bacteria need their vitamins, too! American Society for Biochemistry and Molecular Biology (ASBMB), Science in a Flash. Orlando, FL, USA, (selected top 10 finalist from ~60), April 8th, 2019.
 11. Jiang N, SS. Date, N.C. Mucci, M.A. Romero, J.A.F. Kreig, R.M. Wittman, N.D. Phillip, K.L. Cross, J.M. Vélez, E.C.Z. Suárez, J.M. Parks, A. Johs, J. Yan, R.L. Zaretski, M.L. Simpson, L.L. Riedinger, and F.E. Löffler. 2019. How vitamins impact microbial and social community engagement. ESE 599 Seminar. The University of Tennessee at Knoxville, Knoxville, TN, March 28th, 2019.
 12. Jiang N, J. Yan, and F.E. Löffler. The interdisciplinary effects of taking your vitamins. Annual Bredesen Center Retreat. The University of Tennessee at Knoxville, Knoxville, TN, USA, January 8th, 2019.
 13. Seus, L., J. Yan, S.R. Campagna, and F.E. Löffler. 2018. Resolving the interplay between redox condition, corrinoid pool, and activity of corrinoid-auxotrophic dechlorinators. Annual Meeting of NIEHS Superfund Research Centers in Sacramento, CA, November 28-30, 2018.
 14. Dixon JS, M.E. Landon, B.D. Kristy, K.D. Leitner, N.A. Reavill, C.E. Young, M.T. Street, R.P. Penumadu, K.E. Glass, M.A. Bian, W. Gullledge, T.E. Keyes, E.B. List, J. Pan, SS. Patel, M.M. Payne, G.T. Statum, R.B. Laurel, J.C. Clements, G. Chen, R.W. Murdoch, D.C. Garcia, M. Jiang, S.A. Ripp, and F.E. Löffler. 2018. Developing a biosensor for dichloromethane. 4th Annual Discovery Day. The University of Tennessee, Knoxville, TN, USA, August 30th, 2018.
 15. Jiang N, Yan J, and F.E. Löffler. 2018. Corrinoid lower base structure as a determinant of community function. 17th International Symposium on Microbial Ecology (ISME) – Poster Pitch. Congress Center Leipzig, Leipzig, Saxony, Germany, August 12th-17th, 2018.
 16. Jiang N., J. Yan, and F.E. Löffler. 2018. Tackling chlorinated solvent contamination using corrinoid cofactor engineering in *Geobacter sulfurreducens*. Nipping Science Forum (NSF). Oak Ridge National Laboratory, Oak Ridge, TN, USA, June 29th, 2018.
 17. Villalobos-Solis, M. I., P. E. Abraham, C. M. Swift, K. Chourey, F. E. Löffler, R. L. Hettich. 2018. Selection of *Dehalococcoides mccartyi* protein biomarkers for LC-MRM-MS monitoring of contaminated groundwater. 66th ASMS Conference on Mass Spectrometry and Allied Topics. June 3-7, 2018. San Diego, CA.
 18. Jiang N., J. Yan, and F.E. Löffler. 2018. Critical impacts of vitamins on the biogeochemical cycling and fate of chlorinated organic compounds. 67th Annual Meeting of the Geological Society of America – Southeastern Section (Geomicrobiology of Microbes and Minerals: Influence Across Ecosystem Scales Session). Knoxville Convention Center, Knoxville, TN USA, April 12th-13th, 2018.

19. Yin, Y., J. Yan, G. Chen, and F.E. Löffler. 2018. Kinetic analysis implicates nitrous oxide as a potent inhibitor of the bacterial reductive dehalogenation process. Battelle Eleventh International Conference on Remediation of Chlorinated and Recalcitrant Compounds, Palm Springs, CA, April 8-12, 2018.
20. Seus, L., J. Yan, B. Simsir, A. Bourdon, A.T. Farmer, S.R. Campagna, and F.E. Löffler. 2018. Aquifer geochemistry affects microbial corrinoid production and the activity of corrinoid-auxotrophic, organohalide-respiring bacteria. 4th Annual Women in STEM Symposium, March 4th, 2018. University of Tennessee, Knoxville TN.
21. Jiang N., J. Yan, and F.E. Löffler. 2018. Engineering the corrinoid cofactor of *Geobacter sulfurreducens* to combat environmental toxins. 4th Annual Cynthia B. Peterson Poster Competition. March 2nd, 2018. The University of Tennessee Knoxville, Knoxville, TN, USA.
22. Jiang N, Friel KD, Yan J, and Löffler F. E. The microscopic and macroscopic effects of vitamins leading to environmental cleanup and public engagement opportunities. Annual Bredesen Center Retreat. January 9th, 2018. The University of Tennessee at Knoxville, Knoxville, TN, USA.
23. Jiang N., J. Yan, and F.E. Löffler. 2017. Environmental remediation: A natural solution to extraordinary challenges. Oak Ridge Postgraduate Association Your Science in a Nutshell Ignite Talk. December 4th, 2017. Oak Ridge National Laboratory, Oak Ridge, TN, USA.
24. Seus, L., J. Yan, A.K. Bourdon, A.T. Farmer, S.R. Campagna, and F.E. Löffler. 2017. Does aquifer geochemistry determine the corrinoid pools and control the activity of corrinoid-auxotrophic, organohalide-respiring bacteria? 30th Annual NIEHS Superfund Research Program Meeting, December 6-8, 2017 in Philadelphia, PA.
25. Bourdon, A.K., A.T. Farmer, S.R. Campagna, J. Yan, Meng Bi, and F.E. Löffler. 2017. Purinyl-cobamide serves as cofactor of tetrachloroethene reductive dehalogenases in *Desulfitobacterium*. 30th Annual NIEHS Superfund Research Program Meeting, December 6-8, 2017 in Philadelphia, PA.
26. ara-Murdoch, F., D. Kaya, M.I. Villalobos Solis, C.M. Swift, R.L. Hettich, and F.E. Löffler. 2017. Advanced monitoring of reductive dechlorination biomarkers and implications for contaminated site management. 2017 SERDP-ESTCP Symposium, November 28-30, 2017, Washington, DC.
27. Jiang N., J. Yan, and F.E. Löffler. 2017. Vitamins in our environment: The effects of corrinoids on chlorinated solvent remediation. Kentucky-Tennessee Branch of the American Society for Microbiology Fall 2017 Meeting. November 10th-11th, 2017. Tennessee Technological University, Cookeville, TN, USA.
28. Jiang N., J. Yan, and F.E. Löffler. 2017. Benefiting our beneficial bacteria: Microbes need their vitamins, too! Oak Ridge Postgraduate Association Town Hall Lightning Talk. September 19th, 2017. Oak Ridge National Laboratory, Oak Ridge, TN, USA.
29. Friel K.D., S.R. Barr, M.L. Boyd, D.R. Corbett, M.A. Mynatt, N. Jiang, and F.E. Löffler. 2017. The *mgsD* gene from *Dehalococcoides* and *Dehalogenimonas* and their effects on the potential to adapt to salty environments. NIMBioS 9th Annual Undergraduate Research Conference at the Interface of Biology and Mathematics. November 11th-12th, 2017. The University of Tennessee at Knoxville, Knoxville, TN, USA.
30. Murdoch, R.W., and F.E. Löffler. An initial pangenomic survey of the Peptococcaceae. 2017. Joint Genome Institute Microbial Genomics & Metagenomics Workshop, Sep. 18 – 22, 2017, Walnut Creek, CA.

31. Kara-Murdoch, F., D. Kaya, M. I. Villalobos Solis, C.M. Swift, R.L. Hettich, and F.E. Löffler. 2017. High-throughput quantitative monitoring of reductive dechlorination biomarkers at chlorinated solvent sites. 5th Annual Oak Ridge Postgraduate Research Symposium (ORPA), August 18, Knoxville, TN.
32. Worrell DN, Jiang N, Wang P, Chen G, Yan J, Edwards EA, and Löffler FE. Functional analysis of the nicotinate mononucleotide: 5,6-dimethylbenzimidazole phosphoribosyltransferase (CobT) of *Desulfitobacterium*. 5th Annual Oak Ridge Postgraduate Association Research Symposium. August 18th, 2017. Oak Ridge, TN, USA.
33. Jiang N., J. Yan, and F.E. Löffler. 2017. Pathway modification in *Geobacter sulfurreducens* alleviates corrinoid auxotrophy in *Dehalococcoides* species and sustains reductive dechlorination. 5th Annual Oak Ridge Postgraduate Association Research Symposium (ORPA). August 18th, 2017. Oak Ridge, TN, USA.
34. Villalobos-Solis, M. I., C. M. Swift, K. Chourey, F. E. Löffler, R. L. Hettich. 2017. Identification of peptides from *Dehalococcoides mccartyi* protein biomarkers in chloroethene contaminated groundwater samples through a standard free LC-MRM-MS approach. 65th ASMS Conference on Mass Spectrometry and Allied Topics. June 4-8, 2017. Indianapolis, IN.
35. Yin, Y., J. Yan, G. Chen, and F.E. Löffler. 2017. Nitrous oxide affects corrinoid-dependent reductive dehalogenation. ASM Microbe 2017, June 1-5, 2017, New Orleans, LA, USA.
36. Jiang, N., J. Yan, P. Wang, P.-H. Wang, E.A. Edwards, and F.E. Löffler. 2017. Synthetic corrinoid biosynthesis pathway in *Geobacter sulfurreducens* supports organohalide-respiration in *Dehalococcoides*. ASM Microbe 2017, June 1-5, 2017, New Orleans, LA, USA.
37. Jiang N., J. Yan J, and F.E. Löffler. 2017. Genetic modification reveals corrinoid biosynthesis pathway in *Geobacter* species ^[L]_{SEP} and creates corrinoid partner to sustain reductive dechlorination in *Dehalococcoides*. Department of Biology, University of Konstanz. March 30th, 2017, Konstanz, Germany. ^[L]_{SEP}
38. Yan, J., Y. Yang, K. Chourey, I. Villalobis Solis, R. Hettich, and F. Löffler. 2017. TceA of *Dehalococcoides mccartyi* is a vinyl chloride to ethene reductive dehalogenase. DehaloCon II, Leipzig, March 26-29, 2017.
39. Chen, G. S. Kleindienst, and F. Löffler. 2017. Interspecies hydrogen transfer enables concomitant dichloromethane and chloromethane degradation in an anaerobic consortium. DehaloCon II, Leipzig, March 26-29, 2017.
40. Jiang, N., J. Yan, and and F. Löffler. 2017. Engineered corrinoid biosynthesis pathway in *Geobacter sulfurreducens* sustains reductive dechlorination in *Dehalococcoides*. DehaloCon II, Leipzig, March 26-29, 2017.
41. Jiang, N., L. Huo, H. Jin, J. Wang, X. Li, X. Zeng, J. Yan, F.E. Löffler, and Y. Song. 2017. Treading international waters: Harnessing native microbial communities to bioremediate contamination in China's groundwater. Annual Bredesen Center Retreat. January 10th, 2017. Knoxville, TN, USA.
42. Yan, J., B. Simsir, K. Fullerton A. Bourdon, A. Farmer, S. Campagna, and F. Löffler. 2016. Aquifer redox geochemistry determines corrinoid pools that affect activity of corrinoid-auxotrophic, organohalide-respiring bacteria. NIEHS Environmental Health Science FEST, Durham, NC, December 5-8, 2016.
43. Bourdon, A., A. Tester, S. Campagna, J. Yan, M. Bi, and F. Löffler. 2016. Purinyl-cobamide serves as cofactor of tetrachloroethene reductive dehalogenases in *Desulfitobacterium*. NIEHS Environmental Health Science FEST, Durham, NC, December 5-8, 2016.

44. Yan, J., and F.E. Löffler. 2016. Corrinoid-controlled reductive dechlorination activity in organohalide-respiring *Dehalococcoides mccartyi*. China-US Symposium on Environmental Science and Pollution Control, International Nexus of Food, Energy, Water, and Soil, Yixing, Jiangsu Province, China, October 26-29, 2016.
45. Jiang, N., J. Yan, P. Wang, E.A. Edwards, Y. Song, and F.E. Löffler. 2016. O-methyltransferase expression engineers corrinoid partner for *Dehalococcoides mccartyi*. China-US Symposium on Environmental Science and Pollution Control, International Nexus of Food, Energy, Water, and Soil, Yixing, Jiangsu Province, China, October 26-29, 2016.
46. Yan, J., M. Bi, A.K. Bourdon, A. Tester, S.R. Campagna, and F. E. Löffler. 2016. Isolation and functional characterization of a novel corrinoid cofactor of tetrachloroethene reductive dehalogenases in *Desulfitobacterium*. The 16th ISME meeting. August 21-26, 2016, Montreal, Quebec, Canada.
47. Chen, G., and F.E. Löffler. 2016. Mutualistic interaction between dichloromethane and chloromethane metabolism in an anaerobic consortium. The 16th ISME meeting. August 21-26, 2016, Montreal, Quebec, Canada.
48. Jiang, N., J. Yan, P. Wang, E.A. Edwards, and F.E. Löffler. 2016. Functional heterologous expression of a key methyltransferase reveals cobamide biosynthesis in *Geobacter* species. 16th International Symposium on Microbial Ecology. August 21-26, 2016. Montreal, Quebec, Canada.
49. Jiang, N and F.E. Löffler. 2016. Employing genetics to elucidate gene functionality in supporting chlorinated solvent remediation in groundwater systems. School of Water Resources and Environment, China University of Geosciences. July 6th, 2016. Beijing, China.
50. Jiang, N and F.E. Löffler. 2016. Genetically modified *Geobacter* species supports bioremediation of chlorinated compounds. College of Land and Environment, Shenyang Agricultural University (SYAU). June 24th, 2016. Shenyang, Liaoning, China.
51. Jiang, N and F.E. Löffler. 2016. Discovering microbial potential to remediate groundwater contamination. The Institute of Applied Ecology (IAE) at the Chinese Academy of Sciences (CAS). June 21st, 2016. Shenyang, Liaoning, China.
52. Villalobos, M., K. Chourey, F. Löffler, and R. Hettich. 2016. Selection of protein biomarkers in *Dehalococcoides mccartyi* strains enables an MRM-MS approach for monitoring dechlorination activities in environmental samples. 64th ASMS Conference on Mass Spectrometry and Allied Topics, American Society for Mass Spectrometry, San Antonio, TX, June 5-9, 2016.
53. Yan, J., B. Şimşir, K. Chourey, R.L. Hettich, and F.E. Löffler. 2016. TceA of *Dehalococcoides mccartyi* is a vinyl chloride-reductive dehalogenase. Poster presentation at the Tenth International Conference on Remediation of Chlorinated and Recalcitrant Compounds, Palm Springs, CA, May 22-26, 2016.
54. Şimşir, S., J. Yan, and F.E. Löffler. 2016. Geochemical conditions affect corrinoid pools that control *Dehalococcoides mccartyi* reductive dechlorination activity. Platform presentation at the Tenth International Conference on Remediation of Chlorinated and Recalcitrant Compounds, Palm Springs, CA, May 22-26, 2016.
55. Kaya, D., K. Chourey, R. Hettich, D.M. Ogles, F.E. Löffler. 2016. *Dehalococcoides mccartyi* nitrogenase expression indicates fixed-nitrogen limitation. Poster presentation at the Tenth International Conference on Remediation of Chlorinated and Recalcitrant Compounds, Palm Springs, CA, May 22-26, 2016.

56. Chourey, K., M.I. Villalobos Solis, R. Hettich, B. Şimşir, J. Yan, D. Kaya, B. Baldwin, D.M. Ogles, and F.E. Löffler. 2016. High-throughput proteomics and qPCR pipelines enumerate reductive dechlorination biomarkers in groundwater. Platform presentation at the Tenth International Conference on Remediation of Chlorinated and Recalcitrant Compounds, Palm Springs, CA, May 22-26, 2016.
57. Yang, Y., S.A. Higgins, B. Şimşir, K. Chourey, R.L. Hettich, B. Baldwin, D.M. Ogles, and F.E. Löffler. 2016. Reductive dechlorination of vinyl chloride to ethene in the absence of *Dehalococcoides mccartyi*. Poster presentation at the Tenth International Conference on Remediation of Chlorinated and Recalcitrant Compounds, Palm Springs, CA, May 22-26, 2016.
58. Lee, J., J. Im, U. Kim, and F.E. Löffler. 2016. Prediction of *in situ* chlorinated ethene detoxification potential using a data-mining approach. Poster presentation at the Tenth International Conference on Remediation of Chlorinated and Recalcitrant Compounds, Palm Springs, CA, May 22-26, 2016.
59. Chourey, K., M.I. Villalobos Solis, R. Hettich, B. Şimşir, J. Yan, D. Kaya, B. Baldwin, D.M. Ogles, and F.E. Löffler. 2016. High-throughput proteomics and qPCR pipelines enumerate reductive dechlorination biomarkers in groundwater. Platform Presentation at Tenth International Conference on Remediation of Chlorinated and Recalcitrant Compounds, Palm Springs, CA, May 22-26, 2016.
60. Jiang, N, J. Yan, and F.E. Löffler. 2016. Methyltransferases, corrinoids and chlorinated solvents: Factors that impact bioremediation and perhaps your career. East Tennessee Collegiate Division Meeting of the Tennessee Academy of Science (TAS). April 22nd, 2016. Knoxville, TN, USA.
61. Şimşir, B., J. Yan, A.K. Bourdon, S.R. Campagna, and F.E. Löffler. 2016. Biogeochemical controls over corrinoid bioavailability to organohalide-respiring *Chloroflexi*. Southeastern Biogeochemistry Symposium. Knoxville, TN, USA, March 11-13, 2016.
62. Jiang N., J. Yan, and F.E. Löffler. 2016. Comparative genomics and heterologous expression studies reveal cobamide biosynthesis in *Geobacter* species. 3rd Annual Southeastern Biogeochemistry Symposium. Knoxville, TN, USA, March 11-13, 2016.
63. Lee, J., J. Im, U. Kim, and F. E. Löffler. 2016. A data mining approach to predict *in situ* chlorinated ethene detoxification potential. 2016 International Meeting of the Microbiological Society of Korea. Kimdaejeung Convention Center, Gwangju, South Korea, April 20-22, 2016.
64. Lee, J., J. Im, U. Kim, and F. E. Löffler. 2015. A data mining approach to predict *in situ* chlorinated ethene detoxification potential. American Geophysical Union Fall Meeting 2015, San Francisco, CA, December 14-18, 2015.
65. Yan, J., B. Şimşir, A. T. Farmer, M. Bi, Y. Yang, S. R. Campagna, and F.E. Löffler. Cobamide lower bases control dechlorination rates and extents in organohalide-respiring *Dehalococcoides mccartyi*. NIEHS Superfund Research Program Annual Meeting. San Juan, PR, November 18-20, 2015.
66. Şimşir, B., J. Yan, F.E. Löffler. 2015. Geochemical conditions affect corrinoid pools that control *Dehalococcoides mccartyi* reductive dechlorination activity. NIEHS Superfund Research Program Annual Meeting, San Juan, Puerto Rico, November 18-20, 2015.
67. Şimşir, B., J. Yan, C. Lebron and F.E. Löffler. 2015. The B₁₂-qChip: a high-throughput qPCR tool for monitoring and predicting reductive dechlorination activity of organohalide-respiring *Chloroflexi*. NIEHS Superfund Research Program Annual Meeting. San Juan, PR, November 18-20, 2015.

68. Bi, M., J. Yan, A.K. Bourdon, A. Tester, S.R. Campagna, and F.E. Löffler. 2015. Identification of a novel corrinoid cofactor of tetrachloroethene reductive dehalogenases in *Desulfitobacterium* spp. NIEHS Superfund Research Program Annual Meeting, San Juan, Puerto Rico, November 18-20, 2015.
69. Barr, S.R., M.L. Boyd, D.R. Corbett, K.D. Friel, M.A. Mynatt, N. Jiang, and F.E. Löffler. 2015. Heterologous expression of mannosylglycerate synthase from *Dehalococcoides mccartyi* strain 195 and *Dehalogenimonas lykanthroporepellens* strain BL-DC-9 to characterize potential adaptation to salt stress. 3rd Annual ORNL Faculty and Postgraduate Poster Session. August 4th, 2015. Oak Ridge, TN, USA.
70. Jiang, N., J.G. Elkins, and F.E. Löffler. 2015. Synthetic biology approaches to expand the spectrum of electron acceptors used by *Geobacter lovleyi* strain SZ. 3rd Annual ORNL Postdoc Research Symposium. July 30th, 2015. Oak Ridge, TN, USA.
71. Villalobos-Solis, M. I., K. Chourey, and R. Hettich. 2015. Development of a targeted proteomics approach for the validation of dechlorination biomarker proteins from organohalide-respiring bacteria strains. 63rd. ASMS Conference on Mass Spectrometry and Allied Topic in St. Louis, MO, May 31-June 4, 2015.
72. Yang, Y., S.A. Higgins, J. Yan, K. Chourey, R. Hettich, and F.E. Löffler. 2015. Reductive dechlorination of vinyl chloride in the absence of *Dehalococcoides mccartyi*. 115th General Meeting of the American Society for Microbiology, New Orleans, LA, USA, May 30-June 2, 2015.
73. Yang, Y., J. Yan, and F.E. Löffler. 2015. Reductive dechlorination of vinyl chloride in the absence of *Dehalococcoides mccartyi*. Third International Symposium on Bioremediation and Sustainable Environmental Technologies, Miami, FL, USA, May 18-21, 2015.
74. Şimşir, B., K. Chourey, R. Hettich, K.M. Ritalahti, and F.E. Löffler. The molecular tool box: current and future applications to improve microbial remedies. Third International Symposium on Bioremediation and Sustainable Environmental Technologies, Miami, FL, USA, May 18-21, 2015.
75. Şimşir, B., Yan, J., Chourey, K., Villalobos Solis, M. I., Ritalahti, K., Hettich, R. L., F. E. Löffler. High-throughput qPCR and environmental proteomics to assess microbial activities at complicated chlorinated solvent sites. SERDP/ESTCP Chlorinated Solvents in Groundwater Technical Exchange Meeting, Arlington, VA, December 10-12, 2014.
76. Kleindienst, S., S. Higgins, K. Chourey, R. Hettich, and F. E. Löffler. 2014. Dichloromethane dehalofermentation by a novel group of *Peptococcaceae*. 9th International Symposium on Subsurface Microbiology, Pacific Grove, CA, USA October 5-10, 2014.
77. Şimşir, B., D. Tsementzi, K. Cusick, K.M. Ritalahti, K.T. Konstantinidis, and F.E. Löffler. 2014. Mutualistic interactions between free-living, pleomorphic spirochetes (FLiPS) and *Dehalococcoides mccartyi*. 9th International Symposium on Subsurface Microbiology (ISSM), Pacific Grove, CA, USA, October 5-10, 2014.
78. Lee, J., J. Park, and F. E. Löffler. 2014. Adaptation of reductively dechlorinating microbes to saline conditions. 15th International Symposium on Microbial Ecology, Seoul, South Korea, August 24-29, 2014.
79. Kleindienst, S., S. Higgins, K. Chourey, R. Hettich, E. Mack, F.E. Löffler. 2014. Dichloromethane dehalofermentation. GRC - Molecular Basis of Microbial One-Carbon Metabolism, South Hadley, MA, USA, August 10-15, 2014.

80. Jiang, N., A.M. Guss, and F.E. Löffler. 2014. Investigations into the bioremediation capabilities of *Geobacter lovleyi* strain SZ. 2nd Annual ORNL Postdoc Research Symposium, Oak Ridge, TN, USA, July 10, 2014.
81. Ogles, D., A. Biernacki, B.R. Baldwin, K.M. Ritalahti, and F.E. Löffler. 2014. Next-generation qPCR: High-throughput, highly parallel qPCR arrays (QuantArrays) for comprehensive site assessment. Battelle 9th International Conference on Remediation of Chlorinated and Recalcitrant Compounds, Monterey, CA, USA, May 19-22, 2014.
82. Chourey, K., R. Hettich, K.M. Ritalahti, B. Şimşir, and F.E. Löffler. 2014. Development of a metaproteomics platform to detect reductive dechlorination biomarker proteins in environmental samples. Battelle 9th International Conference on Remediation of Chlorinated and Recalcitrant Compounds, Monterey, CA, USA, May 19-22, 2014.
83. Yan, J., B. Şimşir, K.M. Ritalahti, and F.E. Löffler. 2014. Exploring *Dehalococcoides mccartyi* corrinoid requirement to enhance reductive dechlorination activity. Battelle 9th International Conference on Remediation of Chlorinated and Recalcitrant Compounds, Monterey, CA, USA, May 19-22, 2014.
84. Koenigsberg, S., M. Kram, R. Harwood, L. Dodge, R. Kelley, F. Lakhwala, J. Sohl, F.E. Löffler, D. Ogles, R. Pirkle, G. Gustafson, and S. Britt. 2014. A multivariate diagnostic tools-amended pilot test model. Battelle 9th International Conference on Remediation of Chlorinated and Recalcitrant Compounds, Monterey, CA, USA, May 19-22, 2014.
85. Justicia-Leon, S.D., K.W. Chu, S.O.C. Mundle, G. Lacrampe-Couloume, B. Sherwood Lollar, E.E. Mack, and F.E. Löffler. 2014. *Dehalobacter* sp. strain RM1 in consortium RM induces large fractionation of stable carbon isotopes during dichloromethane fermentation. Battelle 9th International Conference on Remediation of Chlorinated and Recalcitrant Compounds, Monterey, CA, USA, May 19-22, 2014.
86. Şimşir, B., J. Yan, K.M. Ritalahti, and F.E. Löffler. 2014. Reductively dechlorinating consortia: Unraveling community interactions that control *Dehalococcoides* activity. Battelle 9th International Conference on Remediation of Chlorinated and Recalcitrant Compounds, Monterey, CA, USA, May 19-22, 2014.
87. Şimşir, B., D. Tsementzi, K. T. Kontantinidis, and F. E. Löffler. 2013. Comparative metagenomics unravel adaptive evolution processes in *Dehalococcoides mccartyi*. 2nd Thünen Symposium on Soil Metagenomics “Mining and learning from metagenomes”, Thünen Institute, 11-13 December, 2013, Braunschweig, Germany.
88. Koenigsberg, S., M. Kram, R. Harwood, F. Lakhwala, F. Löffler, D. Ogles, R. Pirkle, G. Gustafson, and S. Britt. 2013. A multivariate diagnostic strategy to examine product performance claims. Second International Symposium on Bioremediation and Sustainable Environmental Technologies, Jacksonville, FL, USA, June 10-13, 2013.
89. Padilla-Crespo, E., B. Şimşir, K. M. Ritalahti, and F. Löffler. 2013. Cultures and tools to initiate and monitor of 1,2-dichloropropane detoxification at contaminated sites. Second International Symposium on Bioremediation and Sustainable Environmental Technologies, Jacksonville, FL, USA, June 10-13, 2013.
90. Padilla-Crespo, E., and F. E. Löffler. Discovery of *dcpA* and design of molecular biology tools for natural attenuation or enhanced bioremediation. 2013. NSF Offices, MSPHDs' Student Poster Symposium. Arlington, VA. March 14, 2013.
91. Şimşir, B., J. Yan, K. M. Ritalahti, and F. E. Löffler. 2013. Defining the essential community supporting *Dehalococcoides* reductive dechlorination activity. Remediation Technology Summit (RemTEC13), March 4-6, 2013, Westminster, CO.

92. Padilla-Crespo E., J. Yan, C. Swift, K. Chourey, K.M. Ritalahti, R.L. Hettich, and F.E. Löffler. 2013. Identification and environmental distribution of the 1,2-dichloropropane-to-propene reductive dehalogenase gene. Seventh International Conference on Remediation of Contaminated Sediments. Dallas, TX. February 4-7, 2013. *Best Student Paper*.
93. Şimşir, B., K. M. Ritalahti and F. E. Löffler. 2012. Defining the essential community supporting *Dehalococcoides* reductive dechlorination activity. American Society for Microbiology KY-TN Branch Meeting, Maryville, TN, October 26-27, 2012.

Oral presentations (Invited)

1. Löffler, F.E., 24-February-2020, Microbial Ecology Meets Bioremediation Practice. Invited talk, Department of Microbiology and Molecular Genetics, Oklahoma State University.
2. Löffler, F.E., 12-November-2019, Exploiting Nature's Blueprint for Bioremediation: Challenges and Opportunities. Department of Civil and Environmental Engineering, University of California Los Angeles.
3. Löffler, F.E. 14-November-2018. Anaerobic Microbial Degradation of Dichloromethane and Chloromethane, Dupont, Dow, Corteva, and Chemours Corporate Remediation Group, Wilmington, DE.
4. Löffler, F.E. 21-September-2018. Natural Attenuation as a Productive Site Remedy. U.S. Naval Air Station North Island, Department of Toxic Substance Control (DTSB) and the San Diego Regional Water Quality Control Board, San Diego, CA.
5. Löffler, F.E. 27-April-2018. Microbiome Design: Lessons Learned from Bioremediation, Microbiome Engineering Workshop, April 27, 2018, University of Wisconsin, Madison, WI.
6. Löffler, F.E. 25-April-2018. Corrinoids Modulate Microbiome Function. Madison Microbiome Meeting, Unmasking Common Principles Governing the Microbiome, April 25-26, 2018, University of Wisconsin, Madison, WI.
7. Löffler, F.E. 13-October-2017. N₂O as a Modulator of Corrinoid-Dependent Microbial Metabolism. International Workshop of Geomicrobiome: Subsurface Microbial Composition and Function and Microbial Interactions with Subsurface Environment, October 13-15, 2017, Wuhan, China.
8. Löffler, F.E. 12-September-2017. Systems Biology: Climate Feedback Loops: Why Microbial Ecology Matters. NOAA ATDD – UTK Science Workshop, September 12, 2017, Knoxville, TN, USA.
9. Löffler, F.E. 23-August-2017. Systems Biology: A Pathway to Precision Bioremediation. 254th ACS National Meeting & Exposition, August 20-24, 2017, Washington, DC, USA.
10. Löffler, F.E. 03-June-2017. No Laughing Matter: N₂O as Inhibitor of Corrinoid-Dependent Pathways. ASM Microbe 2017, June 1-5, 2017, New Orleans, LA, USA.
11. Löffler, F.E. 23-May-2017. Next Generation MBTs: A Pathway to Precision Bioremediation. Battelle Fourth International Symposium on Bioremediation and Sustainable Environmental Technologies, Miami, FL, May 22-25, 2017.
12. Löffler, F.E. 28-March-2017. Interspecies Hydrogen Transfer Enables Concomitant Dichloromethane and Chloromethane Degradation in an Anaerobic Consortium. DehaloCon II, Leipzig, March 26-29, 2017.
13. Löffler, F.E. 28-February-2017. Success by the Numbers: Pathways to Precision Bioremediation, Civil & Environmental Consultants, Inc., Knoxville, TN.
14. Löffler, F.E. 6-Dec-2016. Sustained *In Situ* Detoxification of Priority Chloroorganic Pollutants. Session "From Bench to the Field: Technology-Based Solutions to Reduce

- Environmental Exposures". NIEHS Environmental Health Science FEST, Durham, NC, December 5-8, 2016.
15. Löffler, F.E. 2-Nov-2016. Science-Driven Engineering of the Contaminated Subsurface. Harbin Institute of Technology, Harbin, Heilongjiang Province, China.
 16. Löffler, F.E. 10/06/2016. Microbes to the Rescue: Contaminant Detoxification in Groundwater. University de los Andes, Bogotá, Colombia.
 17. Löffler, F.E. 09/30/2016. Contaminants on the Menu: Keep Gobbling! Annual Geosyntec Consultants Sediment Action Group Conference, September 29-October 1, 2016, Knoxville, TN.
 18. Löffler, F.E. 04/21/2016. Must Have Your Vitamins: B₁₂ as a Modulator of Reductive Dechlorination Activity. University of Kentucky Superfund Research Center, University of Kentucky, Lexington, KY. April 21, 2016.
 19. Löffler, F.E. 04/20/2016. Minerals to the Rescue: Anaerobic Degradation of Chlorinated Methanes. Kentucky Department for Environmental Protection, Frankfort, KY. April 20, 2016.
 20. Löffler, F.E. 03/29/2016. Must Have Your Vitamins: B₁₂ as a Modulator of Reductive Dechlorination Activity. Department of Microbiology and Plant Biology, University of Oklahoma, Norman, OK. April 4, 2016.
 21. Löffler, F.E. 03/15/2016. They Can't Do It On Their Own: Community Control Over Organohalide-Respiring *Chloroflexi*. Annual Conference 2016 of the Association for General and Applied Microbiology (VAAM), Jena, Germany, March 16-19, 2016 (Keynote).
 22. Löffler, F.E. 03/29/2016. Degradation of Chlorinated Methanes: Making the Simple Complicated. Chemours, Wilmington, DE. March 29, 2016.
 23. Löffler, F.E. 01/19/2016. Natural Attenuation of Chlorinated Solvents in Streambed Sediment. University of Jena, Collaborative Research Centre AquaDiva (CRC AquaDiva), Friedrich Schiller University Jena, Jena, Germany.
 24. Löffler, F.E. 11/25/2015. Systems Biology Approach for Managing Groundwater Resources. Center for Applied Geochemistry, University of Tübingen, Tübingen, Germany.
 25. Löffler, F.E. 11/25/2015. Emerging Contaminants. Center for Applied Geochemistry, University of Tübingen, Tübingen, Germany.
 26. Löffler, F.E. 08/19/2015. Corrinoid Quantity and Quality Determine Reductive Dechlorination Rates and Extents. 250th ACS National Meeting & Exposition, August 16-20, 2015, Boston, MA.
 27. Löffler, F. E. 08/06/2015. Detection and Quantification of Contaminant Degradation in the Environment. CAMPOS Workshop, University of Tübingen, Germany.
 28. Löffler, F. E. 06/02/2015. Breaking Carbon-Chlorine Bonds as a Lifestyle, Divisions N, Q, R Lecturer, 115th General Meeting of the American Society for Microbiology, May 30-June 2, 2015, New Orleans, LA.
 29. Löffler, F. E. 05/20/2015. The Molecular Tool Box: Current and Future Applications to Improve Microbial Remedies. Third International Symposium on Bioremediation and Sustainable Environmental Technologies, Miami, FL, USA, May 18-21, 2015.
 30. Löffler, F. E. 03/03/2015 (Keynote). Processes Leading to Vinyl Chloride Detoxification. Remediation Technology Summit (RemTEC13), March 2-4, 2015, Westminster, CO.
 31. Löffler, F. E. 12/11/2014. Advanced Environmental Molecular Diagnostics to Assess, Monitor, and Predict Microbial Activities at Complicated Chlorinated Solvent Sites.

- SERDP/ESTCP Chlorinated Solvents in Groundwater Technical Exchange Meeting, Ft. Myers, Arlington, VA, December, 10-12 2014.
32. Löffler, F. E. Foundation for Environmental Biotechnology: Background and Principles. United Nations University, Biotechnology Programme for Latin America and the Caribbean (BIOLAC), Facultad de Química, Universidad de la República, Montevideo, Uruguay. September 22 - October 3, 2014.
 33. Löffler, F. E. Foundation for Environmental Biotechnology: Bioremediation Applications. United Nations University, Biotechnology Programme for Latin America and the Caribbean (BIOLAC), Facultad de Química, Universidad de la República, Montevideo, Uruguay. September 22 - October 3, 2014.
 34. Löffler, F. E. Environmental Controls over $\text{NO}_3^-/\text{NO}_2^-$ Fate. United Nations University, Biotechnology Programme for Latin America and the Caribbean (BIOLAC), Facultad de Química, Universidad de la República. Montevideo, Uruguay. Facultad de Química, Universidad de la República. Montevideo, Uruguay, September 22 - October 3, 2014.
 35. Löffler, F. E. 2014. The Extant Microbial Community Controls the Activity of Organohalide-Respiring *Chloroflexi*. 15th International Symposium on Microbial Ecology, Seoul, South Korea, 24-29 August 2014.
 36. Löffler, F. E. 8/10/2014. Chlorinated C1 Compound Remediation in the Environment. Gordon Research Conference “Molecular Basis of Microbial One-Carbon Metabolism”, Mount Holyoke College, South Hadley, MA, 8-15 August 2014.
 37. Löffler, F. E. 2014. T Contaminated Sites – Much More Than a Legacy Issue. 2014 STEM Graduate Summit “Towards a clean and sustainable future: Green technologies, restoration and management of contaminated sites”. Congressional Hispanic Caucus Institute, Capitol Hill, Senate Meeting Room, Washington, D.C., April 9th, 2014.
 38. Löffler, F. E. 12/12/2013. Cultivation and Metaomics Approaches Characterize Organohalide-Respiring Communities. 2nd Thünen Symposium on Soil Metagenomics “Mining and learning from metagenomes”, Thünen Institute, Braunschweig, Germany, 11-13 December 2013.
 39. Löffler, F. E. 07/19/2013. Corrinoid Requirement of Organohalide-Respiring *Chloroflexi*. DFG 1530 PhD Conference “Anaerobic Biological Dehalogenation: Organisms, Biochemistry, and (Eco-) Physiology”, Helmholtz Centre for Environmental Research, Leipzig, Germany.
 40. Löffler, F. E. 06/13/2013. Towards Knowledge-Based Bioremediation: Monitoring Tools for Keystone Bacteria. Second International Symposium on Bioremediation and Sustainable Environmental Technologies, Jacksonville, FL, USA, June 10-13, 2013.
 41. Löffler, F. E. 05/31/2013. *Dehalococcoides* and Knowledge-Driven Bioremediation. China-US Workshop on Advances in Environmental Microbiology and Biotechnology. Nanjing University, Nanjing, China.

Webinars

1. Löffler, F. E. 10-September-2019. Microbial Transformation of Fluorinated Alkanes and Analytical Procedures to Quantify Poly- and Perfluoroalkyl Substances. Tennessee Department of Environmental Quality (TDEQ) webinar.
2. Löffler, F.E., 22-April-2019, Biogeochemical Controls Over Organohalide-Respiring *Chloroflexi*, presented in the webinar series “Biogeochemical Interactions Affecting Bioavailability for In Situ Remediation: Session I - Innovative Approaches for Chlorinated Compound Bioremediation in Groundwater” sponsored by NIEHS Superfund Research Program. Attended by more than 330 people.

3. Löffler, F. E. 14-November-2018. Approaches to Assess Bioremediation Potential. DuPont, Dow Chemical, Corteva Agriscience, Chemours and contractors webinar.
4. Löffler, F. E. 21-June-2018. Anaerobic Microbial Transformation and Degradation of Bisphenol A, American Chemical Council webinar.
5. Löffler, F. E. 18-July-2017. Next Generation MBTs: A Pathway to Precision Bioremediation. DuPont, Chemours and contractors webinar.
6. Löffler, F. E. 13-March-2017. Sustained In Situ Detoxification of Priority Chloroorganic Pollutants. EPA CLU-IN Internet Seminar, <https://clu-in.org/live/archive/>
7. Löffler, F. E. 14-February-2017. Molecular Biology for Groundwater Scientists Part 2, Microbial Insights Webinar Series. <http://www.microbe.com/webinars/>
8. Löffler, F. E. 7-February-2017. Molecular Biology for Groundwater Scientists Part 1, Microbial Insights Webinar Series. <http://www.microbe.com/webinars/>
9. Löffler, F. E. 01/13/2016. Mythbusters - Misconceptions in Environmental Remediation, Microbial Insights Webinar Series. <http://www.microbe.com/webinars/>
10. Löffler, F. E. 09/23/2015. Fate of Vinyl Chloride in Aquifers: Organisms, Pathways, and Monitoring Tools, EOS Remediation Webinar Series.
11. Löffler, F. E. 04/14/2015. Mythbusters - Misconceptions in Environmental Remediation, Microbial Insights Webinar Series. <http://www.microbe.com/webinars/>
12. Löffler, F. E. 01/14/2014. Microbial Degradation of Vinyl Chloride: Organisms, Pathways and Monitoring Tools, Microbial Insights Webinar Series. <http://www.microbe.com/webinars/>
13. Löffler, F. E. 02/12/2013. Microbial Degradation of Chlorinated Pollutants: Principles and Applications, Microbial Insights webinar. <http://www.microbe.com/webinars/>

Peer-reviews publications (published)

1. Yang, Y., L. Huo, X. Li, J. Yan, and F.E. Löffler. **2020**. Genome sequence of *Sulfurospirillum* sp. strain ACS_{DCE}, an anaerobic bacterium that respire tetrachloroethene under acidic pH conditions. Microbiol. Resour. Announc. In Press.
2. Yang, Y., J. Yan, X. Li, Y. Lv, Y. Cui, F. Kara-Murdoch, G. Chen, and F.E. Löffler. **2020**. Complete genome sequence of ‘*Candidatus Dehalogenimonas etheniformans*’ strain GP, a vinyl chloride-respiring anaerobe isolated from grape pomace. Microbiol. Resour. Announc. 50:e01212-20 | doi.org/10.1128/MRA.01212-20
3. Huo, L., Y. Yang, Y. Lv, X. Li, F.E. Löffler, and J. Yan. **2020**. Complete genome sequence of *Sulfurospirillum* sp. strain ACS_{TCE}, a tetrachloroethene-respiring anaerobe isolated from contaminated soil. Microbiol. Resour. Announc. 9:e00941-20 | doi.org/10.1128/MRA.00941-20
4. Chen, G., A.R. Fisch, C.M. Gibson, E.E. Mack, E.S. Seger, S.R. Campagna, and F.E. Löffler. **2020**. Mineralization versus fermentation: Evidence for two distinct anaerobic bacterial degradation pathways for dichloromethane. The ISME Journal. 14:959-970 | doi.org/10.1038/s41396-019-0579-5
5. Yang, Y., R.A. Sanford, N.L. Cápiro, J. Yan, G. Chen, X. Li, and F.E. Löffler. **2020**. Roles of organohalide-respiring Dehalococcoidia in carbon cycling. mSystems 5:e00757-19 | doi.10.1128/mSystems.00757-19

6. Kaya, D., B.V. Kjellerup, K. Chourey, R.L. Hettich, D.M. Taggart, F.E. Löffler. **2019**. Impact of fixed nitrogen availability on *Dehalococcoides mccartyi* reductive dechlorination activity. Environ. Sci. Technol. 53:14548-14558 | doi: 10.1021/acs.est.9b04463
7. Im, J., E.E. Mack, E.S. Seger, and F.E. Löffler. **2019**. Biotic and abiotic degradation of 1,1,2-trichloro-1,2,2-trifluoroethane (CFC-113): Implications for bacterial detoxification of chlorinated ethenes. Environ. Sci. Technol. 53:11941-11948. | doi: 10.1021/acs.est.9b04399
8. Lawson, C.E., W.R. Harcombe, R. Hatzenpichler, S.R. Lindemann, F.E. Löffler, M.A. O'Malley, H. García-Martin, B.F. Pfeleger, L. Raskin, O.S. Venturelli, D.G. Weissbrodt, D.R. Noguera, and K.D. McMahon. **2019**. Unmasking common principles and practices for microbiome engineering. Nature Microbiology, 17:725-741. | doi: 10.1038/s41579-019-0255-9
9. Yan, J., Y. Yang, X. Li, and F.E. Löffler. **2019**. Complete genome sequence of *Dehalococcoides mccartyi* strain FL2, a trichloroethene-respiring anaerobe isolated from pristine freshwater sediment. Microbiol. Resour. Announc. 8:e00558-19. | doi.org/10.1128/MRA.00558-19.
10. Villalobos Solis, M.I., P.E. Abraham, K. Chourey, CM Swift, F.E. Löffler, and R.L. Hettich. **2019**. Targeted detection of *Dehalococcoides mccartyi* microbial protein biomarkers as indicators of reductive dechlorination activity in contaminated groundwater. Scientific Reports, 9:10604 | doi.org/10.1038/s41598-019-46901-6
11. Yin, Y., Yan, G. Chen, F. Kara Murdoch, N. Pfisterer, and F.E. Löffler. **2019**. Nitrous oxide is a potent inhibitor of bacterial reductive dechlorination. Environ. Sci. Technol. 53:692-701. | doi: 10.1021/acs.est.8b05871
12. Kleindienst, S., K. Chourey, G. Chen, R. Iyer, R.L. Hettich, S.R. Campagna, E.E. Mack, E.S. Seger, and F.E. Löffler. **2019**. Proteogenomics reveals novel reductive dehalogenases and methyltransferases expressed during anaerobic dichloromethane metabolism. Appl. Environ. Microbiol. 85:e02768-18 | doi: 10.1128/AEM.02768-18
13. Clark, K., D.M. Taggart, B.R. Baldwin, K.M. Ritalahti, R.W. Murdoch, J.K. Hatt, and F.E. Löffler. **2018**. Normalized quantitative PCR measurements as predictors for ethene formation at sites impacted with chlorinated ethenes. Environ. Sci. Technol. 52:13410-13420. | doi: 10.1021/acs.est.8b04373
14. Yan, J., M. Bi, A.K. Bourdon, A.T. Farmer, P.-H. Wang, O. Molenda, A. Quaille, N. Jiang, Y. Yang, Y. Yin, B. Şimşir, S.R. Campagna, E.A. Edwards, and F.E. Löffler. **2018**. Purinylcobamide is a native prosthetic group of reductive dehalogenases. Nat. Chem. Biol. 14:8-14. | doi:10.1038/nchembio.2512
15. Yang, Y., N.L. Cápiro, J. Yan, T.F. Marcet, K.D. Pennell, and F.E. Löffler. **2017**. Resilience and recovery of *Dehalococcoides mccartyi* following low pH exposure. FEMS Microbiol. Ecol. 93(12). | doi: 10.1093/femsec/fix130
16. Yang, Y., S.A. Higgins, J. Yan, B. Şimşir, K. Chourey, R. Iyer, R.L. Hettich, B. Baldwin, D.M. Ogles, and F.E. Löffler. **2017**. Grape pomace compost harbors organohalide-respiring *Dehalogenimonas* species with novel reductive dehalogenase genes. The ISME Journal. 11:2767-2780. | doi: 10.1038/ismej.2017.127
17. Şimşir, B., J. Yan, J. Im, D. Graves, and F. E. Löffler. **2017**. Natural attenuation in streambed sediment receiving chlorinated solvents from underlying fracture networks. Environ. Sci. Technol. 51:4821-4830. | doi: 10.1021/acs.est.6b05554
18. Kleindienst, S. S.A. Higgins, D. Tsementzi, G. Chen, K.T. Konstantinidis, E.E. Mack, F. E. Löffler. **2017**. '*Candidatus* Dichloromethanomonas elyunquensis' gen. nov., sp. nov., a

- dichloromethane-degrading anaerobe of the *Peptococcaceae* family. Syst. Appl. Microbiol. 40:150-159. | doi: 10.1016/j.syapm.2016.12.001
19. Chen, G., S. Kleindienst, D.R. Griffiths, E.E. Mack, E.S. Seger, and F.E. Löffler. **2017**. Mutualistic interaction between dichloromethane- and chloromethane-degrading populations in an anaerobic consortium. Environ. Microbiol. 19:4784-4796. | doi: 10.1111/1462-2920.13945
 20. Yang, Y., N.L. Cápiro, T.F. Marcet, J. Yan, K.D. Pennell, and F.E. Löffler. **2017**. Organohalide respiration with chlorinated ethenes under low pH conditions. Environ. Sci. Technol. 51:8579-8588. | doi: 10.1021/acs.est.7b01510
 21. Wang, P. H., S. Tang, K. Nemr, R. Flick, J. Yan, R. Mahadevan, A. Yakunin, and F. E. Löffler, and E. A. Edwards. **2017**. Refined experimental annotation reveals conserved corrinoid autotrophy in chloroform-respiring *Dehalobacter* isolates. ISME J. 11:626-640. | doi:10.1038/ismej.2016.158
 22. Lee, J., J. Im, U. Kim, and F. E. Löffler. **2016**. A data mining approach to predict *in situ* detoxification potential of chlorinated ethenes. Environ. Sci. Technol. 50:5181-5188. | doi: 10.1021/acs.est.5b05090
 23. Kleindienst, S., S.A. Higgins, D. Tsementzi, K.T. Konstantinidis, E.E. Mack, and F.E. Löffler. **2016**. Draft genome of a strictly anaerobic dichloromethane-degrading bacterium. Genome Announcements. 4:2 e00037-16. | doi: 10.1128/genomeA.00037-16
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 25. Cápiro, N. L., F. E. Löffler, and K. D. Pennell. **2015**. Spatial and temporal dynamics of organohalide-respiring bacteria in a heterogeneous PCE-DNAPL source zone. J. Contam. Hydrol. 182:78-90. | doi: 10.1021/es501320h
 26. Cápiro, N. L., Y. Wang, J. K. Hatt, C. A. Lebrón, K. D. Pennell, and F. E. Löffler. **2014**. Distribution of organohalide-respiring bacteria between solid and aqueous phase. Environ. Sci. Technol. 48:10878-10887. | doi: 10.1021/es501320h.
 27. Justicia-Leon, S. D., S. Higgins, E. Erin Mack, D. R. Griffiths, S. Tang, E. A. Edwards, and F. E. Löffler. **2014**. Bioaugmentation with distinct *Dehalobacter* strains achieves chloroform detoxification in microcosms. Environ. Sci. Technol. 48:1851-1858. | doi: 10.1021/es403582f
 28. Padilla-Crespo, E., J. Yan, C. Swift, D. D. Wagner, K. Chourey, R. L. Hettich, K. M. Ritalahti, and F. E. Löffler. **2014**. Identification and environmental distribution of *dcpA* encoding a 1,2-dichloropropane-to-propene reductive dehalogenase in *Dehalococcoides mccartyi*. Appl. Environ. Microbiol. 80:808-818. | doi: 10.1128/AEM.02927-13
 29. Yan, J., J. Im, Y. Yi, and F. E. Löffler. **2013**. Guided cobalamin biosynthesis supports *Dehalococcoides mccartyi* reductive dechlorination activity. Phil. Trans. R. Soc. B. 368, 20120320. | doi: 10.1098/rstb.2012.0320
 30. Löffler, F. E., J. Yan, K. M. Ritalahti, L. Adrian, E. A. Edwards, K. T. Konstantinidis, J. A. Müller, H. Fullerton, S. Zinder, and A. M. Spormann. **2013**. *Dehalococcoides mccartyi* gen. nov., sp. nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen cycling and bioremediation, belong to a novel bacterial class, *Dehalococcoidia* classis nov., order *Dehalococcoidales* ord. nov. and family *Dehalococcoidaceae* fam. nov., within the phylum *Chloroflexi*. Int. J. Syst. Evol. Microbiol. 63:625-635. | doi: 10.1099/ijs.0.034926-0

Books and book chapters (peer-reviewed and published)

1. Moe, W.M. and F.E. Löffler. **2020**. Dehalococcoidaceae. Bergey's Manual Trust. Accepted.
2. Adrian, L. and F.E. Löffler (Eds). Organohalide-Respiring Bacteria. **2016**. Springer-Verlag, Berlin Heidelberg. ISBN 978-3-662-49873-6. | doi: 10.1007/978-3-662-49875-0 <http://link.springer.com/book/10.1007/978-3-662-49875-0>
3. Sanford, R.A., J. Chowdhary, and F.E. Löffler. Organohalide-respiring *Deltaproteobacteria*. In A Adrian, L. and F.E. Löffler (Eds). Organohalide-Respiring Bacteria. **2016**. Springer-Verlag, Berlin Heidelberg. ISBN 978-3-662-49873-6. | doi: 10.1007/978-3-662-49875-0
4. Adrian, L. and F.E. Löffler. Organohalide-respiring bacteria - an introduction. In A Adrian, L. and F.E. Löffler (Eds). Organohalide-Respiring Bacteria. **2016**. Springer-Verlag, Berlin Heidelberg. ISBN 978-3-662-49873-6. | doi: 10.1007/978-3-662-49875-0
5. Adrian, L. and F.E. Löffler. 2016. Outlook - The next frontiers for research on organohalide-respiring bacteria. In A Adrian, L. and F.E. Löffler (Eds). Organohalide-Respiring Bacteria. 2016. Springer-Verlag, Berlin Heidelberg. ISBN 978-3-662-49873-6. | doi: 10.1007/978-3-662-49875-0
6. Löffler, F. E., K. M. Ritalahti and S. H. Zinder. **2013**. *Dehalococcoides* and reductive dechlorination. SERDP ESTCP Environmental Remediation Technology, Vol. 5. H. F. Stroo, A. Leeson, and H. C. Ward (Eds.). Bioaugmentation for Groundwater Remediation. Springer, New York. ISBN 978-1-4614-4114-4. | doi: 10.1007/978-1-4614-4115-1

Peer-reviewed publications (In Preparation or In Revision)

Yan, J., J. Wang, M.I. Villalobos Solis, H. Jin, K. Chourey, X. Li, Y. Yang, Y. Yin, R.L. Hettich, and F.E. Löffler. 2020. Respiratory vinyl chloride reductive dechlorination to ethene in TceA-expressing *Dehalococcoides mccartyi*. In Revision.

Kara Murdoch, F., R. Murdoch, C. Swift, Y. Yin, B. Simsir, J. Yan, K.M. Ritalahti, and F.E. Löffler. 2020. Nanofluidic high-throughput qPCR to assess, monitor and predict microbial activities at chlorinated solvent sites. In Preparation.

Kara Murdoch F., L. Yan, R. Murdoch, Y. Yin, and F.E. Löffler. 2020. Design and validation of a new standard approach for high-throughput quantitative PCR. In Preparation.

Jiang, N., J. Yan, B. Şimşir, and F.E. Löffler. 2020. Engineered lower base biosynthesis pathway in *Geobacter sulfurreducens* sustains reductive dechlorination activity in corrinoid-auxotrophic *Dehalococcoides mccartyi*. In Preparation.

Student Theses

Yongchao Yin, PhD, July. **2019**. The Overlooked Modulating Role of Nitrous Oxide for Corrinoid-Dependent Microbial Processes. University of Tennessee, Knoxville.

Nannan Jiang, PhD, May **2019**. On the Influence of Cobamides on Organohalide Respiration and Mercury Methylation. University of Tennessee, Knoxville.

Laurel Seus, MS, July **2019**. Redox Conditions Determine Cobamide Production in Hyporheic Sediment.

Şimşir, Burcu, PhD, December **2016**. Ecology of Organohalide-Respiring *Dehalococcoides mccartyi*: Corrinoid Cofactor-Related Community Interactions and Controls over Strain Selection. University of Tennessee, Knoxville.

Yang, Yi, PhD, December **2016**. Bioremediation of Chlorinated Ethenes: pH Effects, Novel Dechlorinators and DecisionMaking Tools. University of Tennessee, Knoxville.

Elizabeth Padilla Crespo, PhD, April **2015**. Integrated Application of Genomic, Biochemical and Cultivation Approaches to Characterize 1,2-Dichloropropane Dichloroelemination in Organohalide-respiring *Chloroflexi*. University of Tennessee, Knoxville.

Amanda Devolk, July **2018**. An Investigation of the Effects of Corrinoid Structure on Methanogenesis Activity in *Methanosarcina barkeri* Fusaro.

Meng Bi, MS, December **2015**. Characterization of a Novel Corrinoid from *Desulfotobacterium* spp.

Awards/Other Impacts

- The project PI, Dr. Frank Löffler was elected to fellowship in the American Academy of Microbiology in 2016.
- Doctoral student Yongchao Yin won the 9th Geosyntec Groundwater Practice Student Paper Competition, 2018, Geosyntec Consultants.
- The OpenArray plate technology was adopted by Microbial Insights, Inc., a woman-owned business and leading biotechnology laboratory offering microbial diagnostic solutions through innovative Molecular Biological Tools (MBTs).

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