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# Bioremediation of Chlorinated Ethenes: pH Effects, Novel Dechlorinators and Decision-Making Tools

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To the Graduate Council:

I am submitting herewith a dissertation written by Yi Yang entitled "Bioremediation of Chlorinated Ethenes: pH Effects, Novel Dechlorinators and Decision-Making Tools." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Civil Engineering.

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# Bioremediation of Chlorinated Ethenes: pH Effects, Novel Dechlorinators and Decision-Making Tools

**A Dissertation Presented for the**
**Doctor of Philosophy**
**Degree**
**The University of Tennessee, Knoxville**

**Yi Yang**
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# DEDICATION

***To my parents and my beloved wife Jia Xue.***

應無所住而生其心。

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# ABSTRACT

Chlorinated solvents have been widely used in different areas of modern society. Usage of these chlorinated solvents was not necessarily accompanied with proper handling and disposal of these hazardous compounds, which caused a variety of environmental problems and continues to affect human health. Remediation of chlorinated ethenes contaminated sites has high priority for state regulators and site owners. Among the available treatment technologies, bioremediation shows great promise as a cost-effective corrective strategy for a variety of environmental pollutants. Prerequisites are that the microbiology involved in contaminant degradation and geochemical factors, such as pH, are understood, so that bioremediation technologies can be confidently implemented. The aims of this dissertation work were 1) to enrich and isolate PCE dechlorinators under low pH conditions, 2) to investigate how pH fluctuations affect the microbial community of a PCE-to-ethene consortium, 3) to determine the pH tolerance of *Dehalococcoides mccartyi* (*Dhc*), 4) to identify a non-*Dehalococcoides* type microorganism responsible for reductive dechlorination of vinyl chloride, 5) to identify and characterize a novel vinyl chloride reductase gene and 6) to develop an Excel-based tool to guide remedial practitioners to select suitable remediation strategies. Only one enrichment culture out of total sixteen sites samples showed PCE dechlorination activity at pH 5.5 and stoichiometric conversion to *c*DCE occurred after repeated transfers. The analysis of 16S rRNA gene sequencing data revealed the genera *Desulfovibrio, Sulfurospirillum,* and *Megasphaera* were most abundant in pH 5.5 enrichment. Two PCE-dechlorinating

isolates (strains PLC-TCE and PLC-DCE) were obtained from a pH 5.5 enrichment, and identified as members of the genus *Sulfurospirillum*. Experiments with a *Dhc*-containing consortium demonstrated that exposure time affected *Dhc* ability to recover reductive dechlorination activity following low pH exposure. Low pH conditions affected *Dhc* strains differently, and *Dhc* strains carrying the *vcrA* gene responsible for reductive dechlorination of the human carcinogen vinyl chloride (VC) were least tolerant to low pH. Enrichment and isolation efforts led to the discovery of a *Dehalogenimonas* (*Dhgm*) species capable of respiring chlorinated ethenes, including VC. These research findings advance understanding of the microbial reductive dechlorination process and will improve the implementation of *in situ* bioremediation.

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| | |
|---|---|
| ATSDR | Agency for Toxic Substances and Disease Registry |
| BES | Bromoethanosulfonic Acid |
| BvcA | BAV1 VC Reductive Dehalogenase |
| *bvcA* | BAV1 VC Reductive Dehalogenase Encoding Gene |
| cDCE | 1,2-*cis*-dichloroethene |
| CERCLA Act | Comprehensive Environmental Response, Compensation, and Liability |
| *Dhgm* | *Dehalogenimonas* |
| *Dhb* | *Dehalobacter* |
| *Dhc* | *Dehalococcoides mccartyi* |
| EPA | Environmental Protection Agency |
| MCL | Maximum Contamination Limit |
| OUTs | Operational Taxonomic Units |
| PCE | Tetrachloroethene (Perchloroethene) |
| PCR | Polymerase Chain Reaction |
| qPCR | quantitative PCR |
| RDase | Reductive Dehalogenase |
| *Rdh* | Reductive Dehalogenase Homologous Gene |
| sp. | Species (singular) |
| spp. | Species (plural) |

| | |
|---|---|
| TCE | Trichloroethene |
| tDCE | 1,2-*trans*-dichloroethene |
| TceA | TCE Reductive Dehalogenase |
| *tceA* | TCE Reductive Dehalogenase Encoding Gene |
| VC | Vinyl Chloride |
| *vcrA* | Vinyl Chloride Reductase |
| BioPIC | Biological Pathway Identification Criteria |

# CHAPTER I INTRODUCTION

Chlorinated solvents are a group of aliphatic hydrocarbons with one to three carbons where at least one of the hydrogen atoms is substituted by a chlorine atom. Commonly used chlorinated solvents include chlorinated methanes, chlorinated ethanes and chlorinated ethenes. Chlorinated solvents have been widely used in different areas of modern society, such as cleaning of machinery in manufacturing, *etc*. (1-3). Usage of these chlorinated solvents was not necessarily accompanied with proper handling and disposal of these hazardous compounds, which caused a variety of environmental problems and affected human health. A major issue is the contamination of groundwater at thousands of government-owned and private sites in the United States alone. The Environmental Protection Agency (EPA) was guided by the National Priorities List (NPL) to determine which contaminated sites need further investigation and remediation (4, 5). Required by the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), the Agency for Toxic Substances and Disease Registry (ATSDR) and EPA have been working together "to prepare a list, in order of priority, of substances that are most commonly found at facilities on the National Priorities List (NPL) and which are determined to pose the most significant potential threat to human health due to their known or suspected toxicity and potential for human exposure at these NPL sites" (http://www.atsdr.cdc.gov/spl/). According to this 2015 Substance Priority List (SPL), chlorinated aliphatic hydrocarbons (CAHs), such as tetrachloroethene (PCE), trichloroethene (TCE), 1,2-*cis*-dichloroethene (cDCE), 1,1-dichloroethene (1,1-DCE),

1,2-*trans*-dichloroethene (*t*DCE), and vinyl chloride (VC) were ranked #33, #16, #267, #177, #82, #4, respectively (http://www.atsdr.cdc.gov/spl/). Formulas, nomenclature and properties of chlorinated ethenes are compiled in Table 1.1.

Since detoxification and remediation of chlorinated ethenes has high priority for the agencies and site managers, different technologies and strategies have been developed and tested for in situ remediation of chlorinated solvent-contaminated sites (e.g. *in situ* thermal desorption, *in situ* chemical oxidation or reduction, and *in situ* bioremediation). Among these available treatment technologies, bioremediation shows great promise as a cost-effective removal strategy for a wide variety of environmental pollutants. Major advantages of in situ bioremediation include cleanup without transportation of hazardous waste and additional cost, innocuous end products (e.g., $H_2O$, $CO_2$, inorganic chloride) of biodegradation, minimum land and environmental disturbance; and environmentally friendly with public acceptance (7, 8). However, some disadvantages and specific requirements limited a more widespread implementation, such as intractable heavy metal waste; long and extensive period of performance monitoring; high concentrations of contaminants inhibiting microorganisms; detrimental geochemical conditions including low pH and accumulation of toxic biodegradation products (6, 7). To succeed in applying bioremediation technologies to contaminated sites, many requirements and factors need to be taken into consideration, such as energy source, electron donors, pH, and presence of microorganisms capable of degrading chlorinated solvents (Figure 1.1).

**Table 1.1** Properties of chlorinated ethenes*

| Chlorinated ethenes | Common names | Abbreviation | Formula | Carbon oxidation state | Density (g/mL) | Solubility (mg/L) | Henry's Law Constant (25°C) (Dimensionless) | National Priorities List frequency (Total: 1,770 sites) | MCL (mg/L) |
|---|---|---|---|---|---|---|---|---|---|
| **Tetrachloroethene** | Perchloroethene, Tetrachloroethylene | PCE | $C_2Cl_4$ | +2 | 1.63 | 150 | 0.711 | 1077 | 0.005 |
| **Trichloroethene** | Trichloroethylene, Trethylene, Triclene, Acetylene Trichloride | TCE | $C_2HCl_3$ | +1 | 1.46 | 1,100 | 0.372 | 1046 | 0.005 |
| **1,2-*cis*-dichloroethene** | *cis*-dichloroethene | *c*DCE | $C_2H_2Cl_2$ | 0 | 1.28 | 3,500 | 0.158 | 541 | 0.07 |
| **1,2-*trans*-dichloroethene** | *trans*-dichloroethene | *t*DCE | $C_2H_2Cl_2$ | 0 | 1.26 | 6,260 | 0.384 | 594 | 0.1 |
| **1,1-Dichloroethene** | Vinylidene chloride, 1,1-dichloroethylene | 1,1-DCE | $C_2H_2Cl_2$ | 0 | 1.22 | 3,344 | 1.08 | 610 | 0.007 |
| **Chloroethene** | Vinyl chloride | VC | $C_2H_3Cl$ | -1 | 0.91 | 2,763 | 1.07 | 593 | 0.002 |

*Data obtained from EPA site (http://water.epa.gov/drink/contaminants/basicinformation/), ATSDR(http://www.atsdr.cdc.gov/spl/) and Ward CH, *etc*. (6).

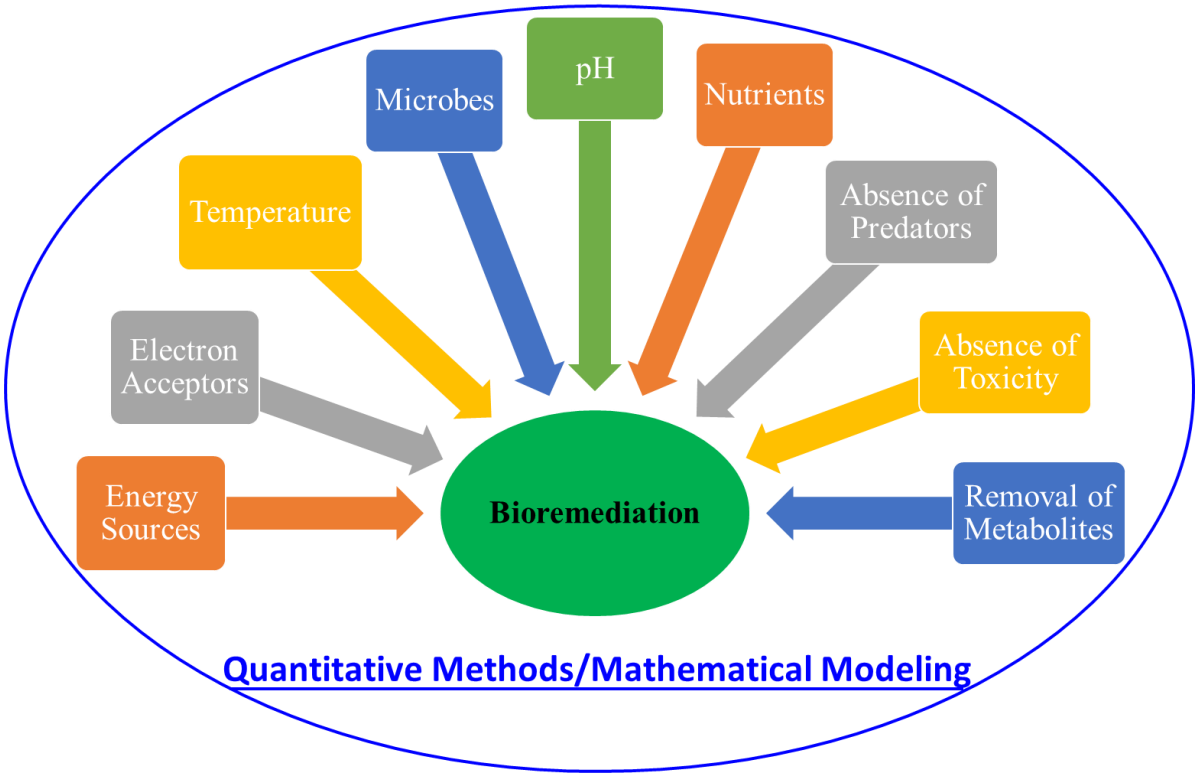


**Figure 1.1** Requirements for bioremediation. (Modified from Cookson A.M. (8))

Although scientific understanding and *in situ* remedial technologies have advanced greatly, further improvements are necessary for widespread and cost-effective remediation. For example, microorganisms capable of degrading chlorinated solvents require a circumneutral pH environment (pH 6.8-7.8); low pH conditions inhibit the microbial reductive dechlorination process (9). At contaminated sites with low pH groundwater, buffers such as sodium carbonate, lime (CaO), and hydroxide can be added to raise and/or neutralize the pH (10); however, precipitation of calcite from lime addition and subsequent aquifer clogging as well as high cost limit their wide application (11). Decades of anaerobic reductive dechlorination research resulted in the isolation of several

anaerobic bacterial species responsible for different steps of PCE reductive dechlorination. Remarkably, all of the cultures that are able to dechlorinate the intermediary daughter compound vinyl chloride (VC) contain strains of the species *Dehalococcoides mccartyi* (*Dhc*) (12). Although comprehensive diagnostic molecular biological tools (MBTs) have been developed and applied for site assessment, more research is required to support and conclusively assess dechlorinating capabilities at a specific site. For example, a key question is why the ability of VC to ethene reductive dechlorination is restricted to *Dhc*, or do other microorganisms with this capability exist? Finally, protocols and guidelines are required to be continually updated to match the advancement of bioremediation research, so that achievements are effectively implemented at field sites. In 1996, Wiedemeier and Wilson developed a protocol to quantify natural attenuation during the remedial investigation process, which relies on naturally occurring physical, chemical, and biological processes to clean up or attenuate pollution in soil or groundwater (http://toxics.usgs.gov/definitions/natural_attenuation.html, http://www.clu-in.org/techfocus/default.focus/sec/Natural_Attenuation/cat/Overview/). This protocol has been highly used by researchers and site managers (13-15); however, this protocol has not been revised since its initial publication. Further, efforts of incorporating latest technical and scientific findings into the previous protocol will be conducive for characterizing the contaminated sites and selecting the most effective bioremediation approach for that particular site. Based on the three problems listed above, we are planning to design and conduct a series of experiments to enhance our understanding of

how we can apply these three factors: pH, microorganism and modeling, to achieve a successful remediation of chlorinated solvents sites.

## Physical Requirement for Bioremediation: pH

Hydronium ion concentration is a very important parameter affecting essentially all biochemical reactions. The pH is the negative logarithm of hydronium ion concentration, which is a major determinant affecting the microbial diversity of aquifers. The majority of soil microorganisms thrive in neutral pH and changes in pH may cause essential microbial enzymes to be inactive and/or denature proteins within cells (16). Decreasing pH could also increase the solubility of toxic metals and metalloids, and mobile metal concentrations may exceed regulatory standards and subsequently affect microbial activities (17, 18). Different microbial groups including nitrate reducers, sulfate reducers, metal reducers and methanogens are all affected by pH and toxic metal species (19-24). Furthermore, dechlorinating bacteria responsible for chlorinated ethene detoxification are active at circumneutral pH, but dechlorination activities are severely inhibited when the pH drops below 6.0 (10, 25-27). The cleavage of a carbon-chlorine bond releases hydrochloric acid (HCl), which deprotonates and releases protons causing acidification (Eq. (1)-(5)).

PCE→TCE reaction: $CCl_2=CCl_2 + H_2 \rightarrow CHCl=CCl_2 + H^+ + Cl^-$ (1)

TCE→DCE reaction: $CHCl=CCl_2 + H_2 \rightarrow CHCl=CHCl + H^+ + Cl^-$ (2)

DCE→VC reaction: $CHCl=CHCl + H_2 \rightarrow CH_2=CHCl + H^+ + Cl^-$ (3)

VC→Ethene reaction: $CH_2=CHCl + H_2 \rightarrow CH_2=CH_2 + H^+ + Cl^-$ (4)

Net Reaction: $CCl_2=CCl_2 + 4H_2 \rightarrow CH_2=CH_2 + 4H^+ + 4Cl^-$ (5)

Large amounts of HCl may be liberated by dechlorinating bacteria when remedial actions are applied to the contaminated sites. In aquifers with low buffering capacity, pH decreases result in the stalling of the dechlorination process (10, 25, 28). Further, biostimulation with fermentation of substrates such as alcohols, organic acids (lactate, formate, acetate, *etc*.), emulsified vegetable oil (EVO) and other organic materials (e.g. molasses, corn cobs, newsprint, wood chips, microbial biomass, *etc*.) causes the release of organic acids (Eq. (6)) and acidification (29-32).

Fermentation of glucose: $C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CH_3COOH + 2CO_2$

Low pH conditions caused by fermentation or enhanced dechlorination inhibit the activity of dechlorinating microorganisms. Most of the conducted research to date investigates the addition of buffer systems or alkaline chemicals to maintain the pH at a suitable level for dechlorinating bacteria (pH>6.5). Robinson *et al.* took a modeling approach, that included parameters such as amount of chlorinated solvent degraded, site water chemistry, electron donor, alternative terminal electron-accepting processes, gas release and soil mineralogy, to investigate pH control during enhanced DNAPL source zone dechlorination. The results indicated that significant bicarbonate addition may be necessary even to soils that are naturally well buffered with calcite and iron oxides (10). Delgado *et al.* suggested that bicarbonate as a pH buffer is an important variable for

bioremediation of chlorinated ethenes (33). Lacroix *et al.* proposed to use silicate minerals to buffer subsurface pH as a long-term source of alkalinity and tested several silicate minerals for pH control during reductive dechlorination in batch cultures (34-36). Philips *et al.* suggested that the significant impact of acidification on bio-enhanced DNAPL dissolution can be overcome by the amendment of a pH buffer or by applying a non-acidifying electron donor like formate (30).

Based on the hypothesis that microbial reductive dechlorination can occur at low pH conditions, several experiments were conducted with several specific aims listed as below:

1) Screening existing isolates and mixed cultures for dechlorinating activity in the lower pH range (e.g., at what pH value does reductive dechlorination cease?).
2) How does low pH affect the microbial community structure in terms of the abundance of key dechlorinators?
3) Enriching and isolating PCE dechlorinators that perform under lower pH conditions and characterizing the isolates.
4) Determining the pH tolerance of consortium BDI capable of dechlorinating PCE to ethene at circumneutral pH.

## Microbial Requirement for Bioremediation: Dechlorinators

Frequently, incomplete reductive dechlorination of PCE and TCE results in the formation of VC, which is a major risk driver at contaminated sites. A range of microorganisms,

such as *Desulfitobacterium* spp., *Sulfurospirillum* spp., *Dehalobacter* sp., *Desulfuromonas* spp., *Desulfomonile* spp., *Geobacter* spp., and *Dehalococcoides mccartyi* (*Dhc*) (37-39) were demonstrated to degrade PCE to TCE or *c*DCE anaerobically by the process of reductive dechlorination.

Reductive dechlorination can be classified into metabolic reductive dechlorination (i.e., organohalide respiration) and co-metabolic reductive dechlorination (e.g., *Desulfomonile*) (12, 38, 40). Only some *Dhc* strains are capable of further degrading *c*DCE or VC to ethene (41-44). Recently, it was demonstrated that in the consortium WBC-2, containing *Dhc*, *Dehalobacter* (*Dhb*), and *Dehalogenimonas* (*Dhgm*) strains, a *Dehalogenimonas* population was responsible for the dechlorination of *t*DCE to VC (45). However, this Dhgm strain was unable to dechlorinate VC and a *Dhc* population was responsible for reductive dechlorination of VC to ethene in this consortium.

So far, two species of *Dehalogenimonas* were isolated, *Dhgm alkenigignens* and *Dhgm lykanthroporepellens* (46, 47), which are distinct but phylogenetically related to the previously cultured *Dhc* (48). Both *Dhgm lykanthroporepellens* strains were reported only to couple growth with the dechlorination of polychlorinated alkanes, such as 1,2,3-trichloropropane (1,2,3-TCP), 1,2-dichloropropane (1,2-DCP), 1,2-dichloroethane (1,2-DCA), 1,1,2-trichloroethane (1,1,2-TCA), and 1,1,2,2-tetrachloroethane (1,1,2,2-TeCA); while chlorinated alkenes (PCE, TCE, *c*DCE, *t*DCE, and VC) and chlorinated benzenes could not be dechlorinated (47-49). For both species of the *Dhgm* genus, chlorobenzenes,

chloroform, 1-chloropropane, 2-chloropropane, 1,2-dichlorobenzene, 1,1-dichloroethane, dichloromethane, tetrachloromethane, 1,1,1-trichloroethane, PCE, TCE, *c*DCE, *t*DCE, or VC did not serve as electron acceptors (46, 50, 51).

All *Dhgm* and *Dhc* strains possess different numbers of reductive dehalogenase (RDase) genes identified from published genome sequences (52-54). But many of these RDases are not characterized in terms of their structure and function. Up to date, RDases responsible for VC-to-ethene detoxification were identified and characterized in two *Dhc* strains: VcrA from *Dhc* strain VS (55) and BvcA from *Dhc* strain BAV1 (56). *bvcA* and *vcrA* genes have been proposed as biomarkers to indicate potential *in situ* VC biodegradation. Both of these VC reductive dehalogenase genes are absent in *Dhgm* strains, which served as an explanation for the inability of *Dhgm* to degrade VC to ethene.

While PCE to *c*DCE transformation can be performed by different microbial species, only some *Dhc* strains are capable of reductively degrading VC to benign ethene under anoxic conditions. We hypothesized that VC-to-ethene dechlorination was not restricted to *Dehalococcoides*, and other microorganisms that can perform VC-to-ethene detoxification also exist. Then a series of experiments are conducted with the following objectives:

1) Enriching microbes capable of degrading VC to ethene.

2) Identifying the microorganisms responsible for VC degradation by utilizing molecular tools (e.g., PCR and qPCR) and sequencing techniques (e.g., 16S rRNA gene amplicon and metagenome sequencing).
3) Isolating and characterizing the VC-degrading microorganism(s).
4) Identifying and characterizing novel VC reductive dehalogenase genes that are involved in the dechlorination of VC to ethene.

## Summary

Results from these research topics are intended to support remediation project managers to choose more efficient and economical strategies and technologies to remediate contaminated sites. Investigation into the pH effects on the microbial dechlorination will inform the site managers about the microorganisms that degrade chlorinated solvents under low pH conditions. At low pH contaminated sites, the presence of such low pH tolerant dechlorinators indicates the feasibility of chlorinated solvents biodegradation, which will save the cost of adjusting and buffering low pH contaminated aquifers. Moreover, exploring the response of consortium BDI to low pH will demonstrate whether dechlorinators can recover from low pH exposure, which will help site managers to decide whether bioaugmentation is needed or not. From a scientific point of view, it is of interest to investigate how dechlorinators adjust to the pressure of a decreasing pH caused by their own metabolic or co-metabolic activities. Biomarkers such as *Dhc* 16S rRNA gene and VC RDase genes (*bvcA* and *vcrA*) are considered capable of providing rapid and reliable measurements indicating natural attenuation and in situ ethene formation.

However, this approach only targets known gene sequences and can therefore not detect novel dechlorinating microorganisms. Whether dechlorinating microorganism(s) besides *Dhc* exist that are capable of degrading VC to ethene is still unknown. And this research is also trying to provide an answer to this question.

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# CHAPTER II REDUCTIVE DECHLORINATION OF CHLORINATED ETHENES UNDER LOW PH CONDITIONS

A version of this chapter is going to be submitted for publication. Yang, Y., N.L. Cápiro, T.F. Marcet, J. Yan, K.D. Pennell, and F.E. Löffler. Reductive dechlorination of chlorinated ethenes under low pH conditions. In preparation.

## Abstract

Bioremediation treatment (e.g., fermentable substrate additions) often leads to groundwater acidification due to enhanced dechlorination (i.e., release of $H^+$ + $Cl^-$) and organic acids released from fermentation. The reductive dechlorination process achieves robust detoxification of chlorinated ethenes at circumneutral pH, but pH decreases below 6.0 are generally associated with declining activity. To find dechlorinators that could maintain chlorinated ethene dechlorination activity below pH 6.0, available pure cultures (*Geobacter lovelyi* strain SZ, *Desulfuromonas michiganensis* strain BB1, *Desulfitobacterium* sp. strain Viet1, *Desulfitobacterium* sp. strain JH1) and a PCE-to-ethene-dechlorinating consortium were tested at pH values of 5.5, 6.0, and 7.2. All cultures dechlorinated tetrachloroethene (PCE) at circumneutral pH but only *Sulfurospirillum multivorans* was able to dechlorinate PCE to *cis*-1,2-dichloroethene (cDCE) at pH 5.5 and maintain this activity upon transfers in pH 5.5 medium. Low pH PCE dechlorination was further explored in microcosms using solid materials collected from 4 pristine and 12 chlorinated solvent-contaminated sites. In microcosms from six locations, ethene formation was observed in pH 7.2 and pH 5.5 microcosms. While PCE-to-ethene reductive dechlorination activity could be maintained in pH 7.2, PCE and

cDCE dechlorination ceased in pH 5.5 transfer cultures. Only the transfer cultures derived from pristine acidic peat bog soil microcosms dechlorinated PCE to stoichiometric amounts of cDCE at pH 5.5 and a similar dechlorination pattern was observed at pH 7.2. Analysis of 16S rRNA gene sequencing data revealed distinct differences in community structure between pH 7.2 and pH 5.5 enrichment cultures. In the pH 7.2 microcosms, the genera *Dehalococcoides, Acetobacterium*, *Spirochaetaceae, Caldisericum, Desulfuromonadales,* and vadinBC27 (a wastewater-sludge group) dominated in the pH 7.2 enrichment. By comparison, the major genera in the pH 5.5 enrichment were *Desulfovibrio, Sulfurospirillum*, *Megasphaera*, *Propionibacterium* and *Pelosinus*. Two PCE dechlorinating isolates were obtained from the pH 5.5 enrichment, one of which dechlorinated PCE to TCE (strain PLC-TCE) and the other produced *c*DCE from PCE or TCE (strain PLC-DCE). 16S rRNA gene sequencing identified the isolates as member of the species *Sulfurospirillum multivorans* with 16S rRNA gene similarities of 98.6% and 98.5%. This study suggested *Sulfurospirillum* may play a significant role in *in situ* bioremediation of chlorinated ethenes under low pH conditions.

## Introduction

*In situ* bioremediation involving anaerobic dechlorinating microorganisms has shown success as a cost-effective removal strategy for a variety of chlorinated pollutants.(1) A number of anaerobic bacterial isolates responsible for different steps of PCE reductive dechlorination have been identified including *Desulfitobacterium*, *Sulfurospirillum*,

*Dehalobacter*, *Desulfuromonas*, *Geobacter*, *Dehalogenimonas* and *Dehalococcoides* (*Dhc*) (2, 3). These dechlorinating bacteria were all enriched and isolated in medium at circumneutral pH. Subsequent characterization revealed that maximum reductive dechlorination activity occurred at circumneutral pH but activity was severely inhibited below pH 6.0 and no dechlorination was reported at pH 5.5 (4-6).

Dechlorination processes release hydrochloric acid, which, under typical *in situ* conditions, rapidly dissociates into chloride anions and protons. Depending on the buffering capacity of the aquifer, extensive dechlorination may generate excessive hydrochloric acid to affect the groundwater pH. Furthermore, biostimulation with fermentable substrates such as alcohols, emulsified vegetable oil (EVO) and other organic materials (e.g., molasses, corn cobs, newsprint, wood chips, and microbial biomass) causes acidification due to the formation of organic acids (7). In aquifers with low buffering capacity, pH decreases can slow down and stall microbial dechlorination processes. Acidification can have other undesirable secondary effects such as increased solubility of toxic metals and metalloids, which may affect microbial activities and/or impair groundwater quality (i.e., exceed regulatory standards) (8).

A common response to groundwater pH reductions following *in situ* biostimulation is the addition of buffer or alkaline chemicals to maintain the pH in a suitable range for dechlorinating bacteria (pH > 6.5). For example, the addition sodium bicarbonate and colloidal $Mg(OH)_2$ has been successfully used to manipulate groundwater pH *in situ* (9,

10). Calcite plays an important role in buffering the pH of calcareous soils, but the amount of calcite varies for different soil types (11). Despite the buffering capacity from calcite, large amounts of bicarbonate may be required to buffer the groundwater pH during enhanced DNAPL source zone dechlorination; and increased concentration of carbonate may result in the precipitation of calcite rather than dissolution of calcite ($CaCO_3\downarrow \leftrightarrow Ca^{2+}+CO_3^{2-}$) (12). A low cost, self-regulating (i.e., pH-dependent dissolution rate) approach using silicate minerals was proposed to buffer groundwater, but further experiments indicated silicate minerals and their dissolution products may inhibit reductive dechlorination of chlorinated ethenes (5). Several studies applied a modeling approach to estimate the buffer requirements for stabilizing groundwater pH (10, 12-17). Although feasible, *in situ* pH adjustments are challenging and the current approaches have limitations.

An alternate solution would be reductively dechlorinating microorganisms that are active under low pH conditions. Some dechlorinating isolates of the genera *Desulfuromonas*, *Geobacter*, *Desulfitobacterium*, *Sulfurospirillum* and *Dehalococcoides* were tested for dechlorinating activity at low pH but all reports indicated that growth and dechlorination activity ceased at pH values below 6 (Table 2.1). Apparently, the known dechlorinators are neutrophils limited to sustained dechlorination in neutral pH environments, and no microbes capable of growth with chlorinated ethenes at pH 5.5 have been described. Also, limited information is available how pH shifts affect microbial community structure. To fill these knowledge gaps, a series of experiments were conducted to screen

the pH range of existing pure and mixed cultures capable of dechlorinating chlorinated ethenes, to enrich and isolate PCE dechlorinators capable of PCE dechlorination under low pH conditions, and to investigate the response of the bacterial community, including *Dhc*, to low pH conditions.

**Table 2.1** pH values for optimal growth of dechlorinating bacteria

| Bacteria | Optimal pH range | Reference |
|---|---|---|
| ***Geobacter lovleyi* strain SZ** | 6.5~7.5 | (22) |
| ***Desulfitobacterium* sp. strain Y51** | 6.5~7.5 | (49) |
| ***Desulfuromonas chloroethenica* TT4B** | 6.5~7.4 | (19) |
| ***Desulfuromonas michiganensis* BB1** | 6.8~8 | (19) |
| ***Sulfurospirillum multivorans*** | 7~7.5 | (23) |
| ***Dehalococcoides mccartyi*** | 6~8 | (50) |

## Materials and Methods

**Chemicals.** PCE and TCE were purchased from Acros Organics (Distributed by VWR international, West Chester, PA, USA). *c*DCE, VC and ethene were bought from Sigma-Aldrich Chemicals (St. Louis, MO, USA). HOMOPIPES (Homopiperazine-1,4-bis(2-ethanesulfonic acid)) and MES (2-(N-morpholino)ethanesulfonic acid) was purchased from Acros Organics. Sodium bicarbonate was purchased from Fisher Scientific (Pittsburgh, PA, USA). Di-water was used to prepare solutions and mineral salts medium.

**Analytical methods.** The pH of bulk liquid phase was measured by transferring 1 mL liquid aliquots from a culturing vessel into a 2-ml plastic tube. After centrifuging the tube

for 30 seconds at 14,000 rpm, the pH of the supernatant was measured with Fisher Scientific Accumet Glass AgCl pH electrode (Pittsburgh, PA, USA). Total chlorinated solvent mass or concentrations of chlorinated compounds were measured by analyzing 100 µL headspace gas samples on a gas chromatograph (GC). The concentrations of chlorinated ethenes were calculated by normalizing the peak area values to standard curves generated by adding known amounts of chlorinated ethenes into the bottles with same gas to liquid ratio. The total moles of polychlorinated ethenes per bottle was calculated by the formula: total moles = (volume x density) / molecular weight. Gas samples (100 µL) were removed from the headspace of 160 mL serum bottles using a gastight 250 µL Hamilton SampleLock syringe and then injected into the GC manually. Samples were measured with an Agilent 7890A GC equipped with an Agilent DB624 column (30 m x 0.53 mm I.D., 3 µm.) with a flame ionization detected (FID). The retention times were determined by injecting neat compounds into the GC. The retention time was used as the identity for the specific chlorinated compounds.

**Medium preparation.** Reduced mineral salts medium was prepared following established protocols (18). Vitamin stock solution was added by passing through sterile 0.22 µm membranes filters after the medium had been autoclaved (18). Lactate (5 mM) and hydrogen gas (10 mL) were added into 160 mL serum bottles as carbon source and electron donor, respectively. The pH 7.2 mineral salts medium was buffered with 30 mM bicarbonate. For pH 4.5 mineral salts medium, 30 mM Homopiperazine-1,4-bis(2-ethanesulfonic acid) (HOMOPIPES; $pK_a$=4.84 at 20 °C) was used instead of bicarbonate.

For pH 5.5 or 6 mineral salts medium, 30 mM bicarbonate was replaced with 30 mM 2-(N-morpholino)ethanesulfonic acid (MES; $pK_a$=6.15 at 20 °C).

**Screening existence of dechlorinating isolates and mixed cultures at different pH.** Several PCE-dechlorinating isolates (*Desulfuromonas michiganensis* strain BB1 (19), *Desulfitobacterium* sp. strains Viet1 (20), *Desulfitobacterium* sp. strain JH1 (21), *Geobacter lovleyi* strain SZ (22), *Sulfurospirillum multivorans* (23)) and a *Dhc*-containing consortium BDI (Bio-Dechlor Inoculum) (24) were tested for PCE dechlorination at pH 5.5, 6 and 7.2. These dechlorinating isolates and mixed cultures have been maintained in the lab fed with PCE (18). *Desulfuromonas michiganensis* strain BB1, *Desulfitobacterium* sp. strains Viet1 and JH1, *Geobacter lovleyi* strain SZ, *Sulfurospirillum multivorans* and a *Dhc*-containing consortium BDI were cultivated in 160 mL serum bottles containing 100 mL pH 7.2 mineral salts medium amended with 5 µL neat PCE, 10 mL hydrogen and 5 mM lactate. Triplicate serum bottles containing 100 mL salts medium were inoculated with 3 mL culture grown at pH 7.2.

**Sampling sites, microcosms setup and transfer cultures.** Samples from a total of sixteen sites were used to set up microcosms for enriching PCE dechlorinators at pH 5.5 and pH 7.2 (Table S2.1). Groundwater, soil and sediment samples were transferred to the lab and stored at 4°C. Before setting up microcosms, the groundwater, soil and sediment samples were moved into glove box (filled with nitrogen and 3% hydrogen). Following opening 160 mL serum bottles with 100 mL mineral salts medium in the glove box, soil

or sediment samples (about 10 g wet weight) were added into the bottles with autoclaved spatulas; or 50 mL groundwater was mixed with 50 mL mineral salts medium. The serum bottles were closed with autoclaved black rubber stoppers (Geo-Microbial Technologies, Inc., Ochelata, Okla.) and crimped with aluminum caps. After removing the serum bottles from the glove box, neat liquid PCE (5 µL) were added into serum bottles by 5 µL Hamilton micro-syringes (Hamilton Company, Rena, Nevada). All microcosms were established in duplicate or triplicate, and incubated at room temperature (21°C). Time zero measurements were conducted after a 24-hour equilibration period. After VC and ethene were detected in the original microcosms, the microcosms were shaken vigorously, and 3 mL inocula were removed with nitrogen-flushed 3-mL syringes. The withdrawn culture suspension was immediately injected into a new bottle with fresh mineral salts medium (pH 7.2 or 5.5) amended with 5mM lactate as carbon source, 10 mL hydrogen as electron donor. For enriching microbes under different pH conditions, various buffer systems were used: 30 mM HOMOPIPES for pH 4.5, 30 mM MES for pH 5.5 or 6 mineral salts medium, and 30 mM bicarbonate for pH 7.2 mineral salts medium. Aseptic techniques were applied to all steps.

**DNA extraction.** Microbial biomass was collected from 2-mL liquid culture suspension by vacuum filtration through 0.22 µm membrane filters (Millipore GVWP025000). Filter-trapped microbial cells were suspended in the PowerSoil® bead tubes (Mo Bio Laboratories Inc., Carlsbad, CA) and ruptured with a high efficiency Bead Ruptor Homogenizer (Omni International, Kennesaw, GA, USA) at a speed of 3.25 m/s for 5

minutes. Genomic DNA was extracted using the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA) following the manufacturer's recommendations. DNA concentrations were quantified with a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE). DNA samples extracted from replicate cultures were pooled and stored at -20°C.

**16S rRNA gene amplicon sequencing and analysis.** MiSeq 16S rRNA gene amplicon sequencing was used to analyze the taxonomic compositions of the dechlorinating enrichment cultures maintained at pH 5.5 and pH 7.2 by targeting the V4 variable regions of the 16S rRNA gene. Amplification was performed in 50 µL assays, consisting of 5 µL DNA sample, 1 µL barcoded-primer (10 µM), and 44 µL mixture of 31 µL de-ionized water (5 PRIME, Gaithersburg, MD, USA), 5 µL Invitrogen Pfx50™ buffer (Invitrogen, Carlsbad, CA, USA), 1 µL CAP 515F primer (10 µM), 1 µL dNTP, 1 µL Invitrogen Pfx50™ Polymerase and 5 µL of MgCl2 (25mM) (Invitrogen, Carlsbad, CA, USA). Thermo cycling program was set as following: denaturation at 94°C for 3 min followed by 35 cycles at 94°C for 45 sec, annealing at 55°C for 60 sec, and extension at 72°C for 90 sec, and final extension at 72°C for 10 min. Quality (size) of produced amplicons was checked using High Sensitive DNA Kit on a model 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Relative concentrations of the individual samples were estimated based on the peak height at the appropriate size, and pooled to equal amounts. Pooled samples were purified with SPRI magnetic beads (Beckman Coulter, Inc., Indianapolis, IN, USA). The products from purification step were analyzed again with High Sensitive DNA kit for

quality assurance and verification of the removal of primer dimers. Before sequencing, concentrations of pooled amplicons were determined using Illumina Library Quantification kit (KAPA Biosystems, Boston, MA, USA) following the manufacturer's protocol. Quantification of each sample was determined based on amplicon adaptors. The amplicon library was diluted to a starting concentration of 10 nM, followed by sequencing on the Illumina MiSeq desktop sequencer (Illumina, Inc., San Diego, CA, USA)Sequencing was conducted in the sequencing facility of Center of Environment Biotechnology (CEB, University of Tennessee, and Knoxville) following the published methods (25). Sequence files were then paired and analyzed using Mothur software following the analysis pipeline MiSeq SOP (26). After quality control, 69030 sequences (17441054 total base pairs) from pH 5.5 sample and 103503 sequences (26171881 total base pairs) from pH 7.2 sample were obtained. Two samples, one is pH 5.5 and the other is pH 7.2). These trimmed and paired sequences were uploaded to Silva-NGS server for comparison analysis based on high-quality SILVA alignment (27).

**Isolation procedures of dechlorinators at low pH.** Isolation efforts focused on the PCE-dechlorinating cultures that maintained dechlorinating activity for at least 10 consecutive transfers in pH 5.5 medium. Dilution to extinction series were established following the published protocol (18). Colony formation was monitored weekly. Once colonies were visualized, 8 colonies were selected and picked up from $10^{-4}$ and $10^{-5}$ dilution agar tubes. These colonies were then transferred to fresh pH 5.5. medium to test for PCE dechlorination.

**Identification of isolates and phylogeny.** PCR assays were applied to amplify 16S rRNA genes using general bacterial primers set 8F/1541R (8F-AGA GTT TGA TCC TGG CTC AG and1541R-AAG GAG GTG ATC CAG CCG CA) using the published protocol (18). The PCR products were cleaned using DNA Clean & Concentrator™-5(Zymo Research Corp., Irvine, CA, U.S.A.). The cleaned PCR products were sequenced by Sanger method using general bacterial primers set 8F/1541R. Nearly full-length 16S rRNA gene sequences were obtained and analyzed using DNA Baser software to trim low quality reads and correct ambiguities in the contigs (Heracle BioSoft SRL, Romania). The 16S rRNA gene sequences were then blasted against NCBI NT database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify phylogenetically related microorganisms. Sequences (Table S2.3) were imported into Geneious software (Biomatters, Auckland, New Zealand) and aligned by MAFFT (28). Then a 16S rRNA gene phylogenetic tree was built using Geneious Tree Builder with the default settings.

## Results

**Screening existence of dechlorinating isolates and mixed cultures.**

*Desulfuromonas michiganensis* strain BB1, *Desulfitobacterium* sp. strain Viet1, *Desulfitobacterium* sp. strain JH1 and the *Dhc*-containing consortium BDI dechlorinated PCE to TCE, cDCE and ethene at pH 7.2, respectively, while no PCE dechlorination occurred at pH 5.5 and 6.0. *Geobacter lovleyi* strain SZ could perform PCE dechlorination to cDCE at pH 6 and 7.2, but not at pH 5.5. *Sulfurospirillum multivorans*

was reported to grow between pH 7 and 7.5 (23), but our efforts demonstrated that the organism dechlorinated PCE to *c*DCE at pH 5.5 (Figure S2.1). All screened dechlorinating cultures could only perform dechlorination at circumneutral pH except *Sulfurospirillum multivorans*, which could dechlorinate PCE to *c*DCE at pH 5.5.

**Microcosms and enrichment of dechlorinators at pH 5.5.**

PCE-to-ethene reductive dechlorination was observed in both pH 5.5 and pH 7.2 microcosms established with samples collected from six sampling sites (#5, 6, 7, 11, 13 and 16 in Table S2.1). Soil samples from Axton Cross site (Holliston, MA) could degrade PCE to VC at pH 5.5, and ethene at pH 7.2. Microcosms set up with acidic peat bog soil sample from the pristine Nature Conservancy located at Shady Valley (TN) showed PCE to cDCE dechlorination at pH 5.5 and pH 7.2. The tidal flat sample degraded PCE to TCE at pH 7.2, but not at pH 5.5. No PCE dechlorination activity was detected in the other samples listed in the Table S2.1. Among 16 investigated sites, only the cultures derived from the Axton Cross sample material (designated as ACS, #13 in Table S2.1) maintained PCE dechlorination activity at pH 5.5 after repeated transfers. At pH 5.5 and 7.2, VC and ethene were the predominant daughter products of PCE reductive dechlorination in the original ACS microcosms (Figure 2.1). PCE dechlorination activity in the original pH 4.5 microcosms was lost when the culture was transferred to a new bottle with pH 4.5 medium. The pH 5.5 ACS enrichment maintained its ability to degrade PCE to cDCE, but dechlorination of cDCE to VC/ethene was lost after the second transfer. By comparison, PCE-to-ethene dechlorination activity was stably maintained in

subsequent transfers at pH 7.2. Repeated transfers resulted in a consortium capable of degrading PCE to cDCE at pH 5.5, and a consortium capable of degrading PCE-to-ethene reductive dechlorination at pH 7.2 (Figure 2.1). Attempts to establish stable enrichment cultures following continuous transfers at pH 5.5 were not successful for any of the ethene-producing microcosms. PCE dechlorination to cDCE and VC occurred in the first transfer cultures at pH 5.5, and PCE dechlorination to cDCE was observed in the second transfer cultures, and PCE dechlorination ceased in third transfer cultures at pH 5.5. By comparison, transfer cultures derived from the active microcosms maintained their PCE-to-ethene dechlorination activities when cultivated at pH 7.2.

**pH effects on community structure.**

In all tested 16 samples, only ACS sample demonstrated PCE dechlorination at both pH 5.5 and pH 7.2. To investigate the differences between dechlorinating community maintained at pH 5.5 and pH 7.2, and identify the dechlorinators responsible for PCE dechlorination, 16S rRNA gene amplicon sequencing was applied to the two enrichment cultures (maintained at pH 5.5 and pH 7.2, respectively) derived from continuous transfers of the ACS microcosms. A total of 172,409 sequences from two samples (pH 5.5 and pH 7.2) were classified into 815 operational taxonomic units (OTUs), and only 41 sequences could not be assigned into any OTUs. Rarefaction analysis of sequences showed more OTUs were identified for the pH 7.2 enrichment compared to the pH 5.5 enrichment (Figure S2.2). *Firmicutes*, *Bacteroidetes* and *Proteobacteria* were the major phyla in both pH 5.5 and pH 7.2 enrichments (Figure 2.2). At pH 5.5, the phylum

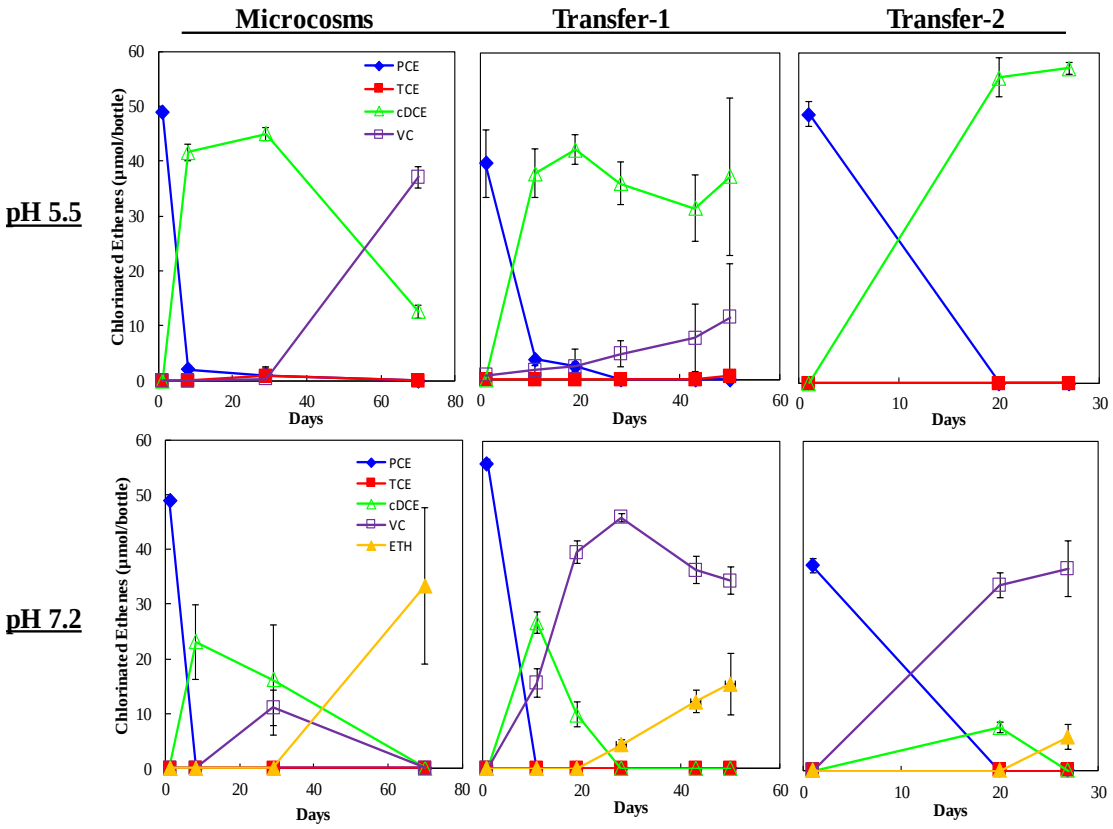


**Figure 2.1** PCE dechlorination by microcosms and transfer enrichments of ACS sample under different pH conditions (First row-pH 5.5, demonstrating PCE could be degraded to VC under microcosm condition, but only PCE-to-cDCE dechlorination could be repeatedly transferred; Second row-pH 7.2, demonstrating PCE-to-ethene dechlorination could be maintained). All figures show the results from average of triplicate serum bottles.

*Actinobacteria* was relatively enriched, while at pH 7.2, the phyla *Caldiserica*, *Chloroflexi* and *Spirochaetes* were more abundant (Figure 2.2). The dominant genera in pH 5.5 enrichments were very different from those predominating in the pH 7.2 enrichment. *Dehalococcoides* and *Acetobacterium* dominated in the pH 7.2 enrichment, and accounted for 22.6% and 57.6% of the microbial community, respectively. By comparison, the major genera in the pH 5.5 enrichment consisted of *Desulfovibrio* (33.0%), *Sulfurospirillum* (25.2%), and *Megasphaera* (19.9%) (Table 2.2).

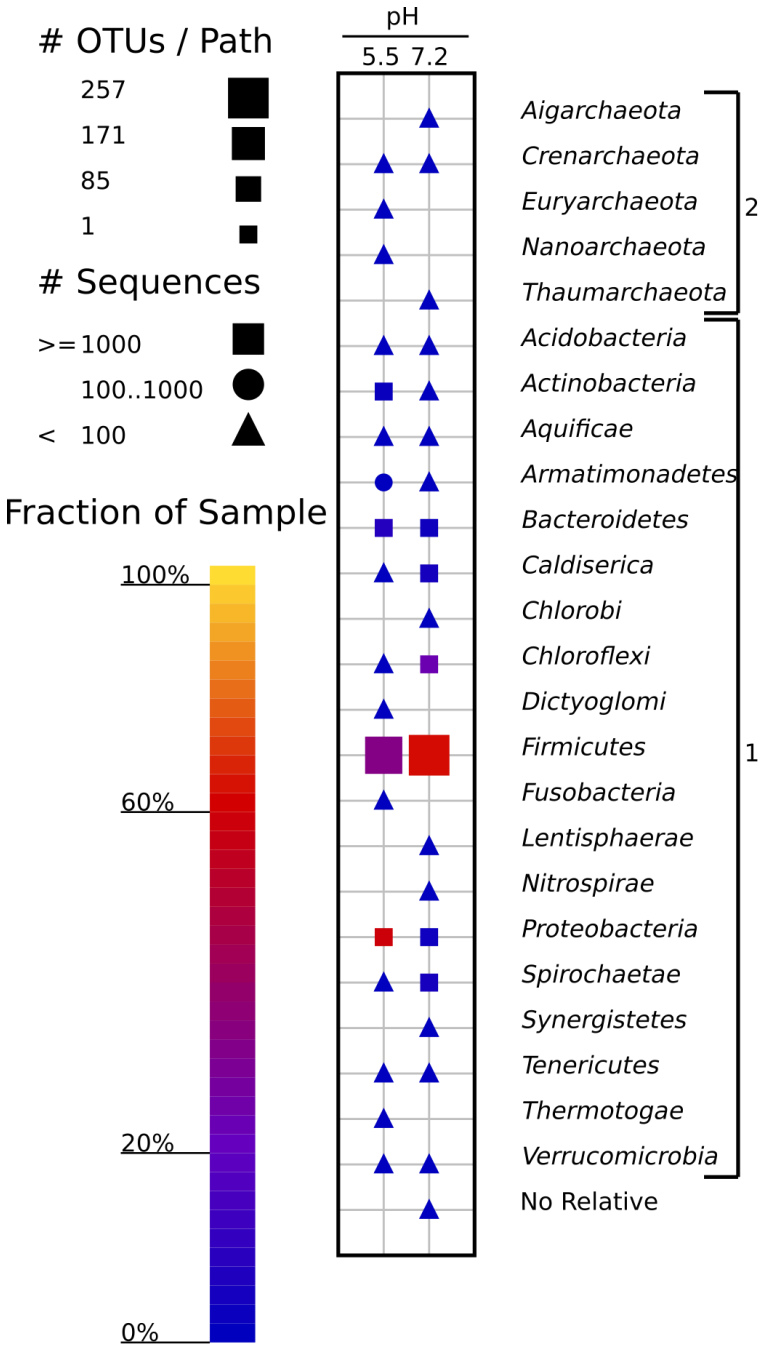


**Figure 2.2** Taxonomic fingerprints comparison between pH 5.5 and 7.2 enrichments at phylum level. The size of the shape represents the number OTUs in the phylum divided by the number of path (for phylum level, path equals 2). Different shapes represent the number of sequences in the phylum group (Square indicates more than 1000 sequences; circle indicates between 100 and 1000 sequences; triangle indicates less than 100 sequences). Different colors represent the fraction of sequences in the sample.

**Low pH PCE reductive dechlorination by two *Sulfurospirillum* isolates**

From the ACS enrichment, which is the only active enrichment at pH 5.5, two isolates were obtained. Isolate PLC-TCE dechlorinated PCE to TCE and isolate PLC-DCE dechlorinated PCE to cDCE (Figure 2.3). Both isolates dechlorinated PCE and grew in defined mineral salts medium at pH 5.5, and rates of 2.65±0.21 µmol $Cl^-$ released per day for PLC-TCE and 6.00±0.25 µmol $Cl^-$ released per day for PLC-DCE were measured. Higher dechlorination rates of 3.74±0.07 µmol $Cl^-$ released per day for isolate PLC-TCE and 9.26±1.59 µmol $Cl^-$ released per day for isolate PLC-DCE at pH 7.2 (Figure 2.3). Sanger sequencing, applied to PCR products amplified with general bacterial primers, yielded a single sequence for each isolate. Only uniform spirillum-shaped bacteria were observed under light microscope using phase contrast and 100X magnification. BLAST analysis using the nucleotide sequences of partial 16S rRNA genes of two dechlorinating isolates revealed highly similar sequences (99.7% identity) that affiliated with the genus *Sulfurospirillum* within the ε-*Proteobacteria*. The 16S rRNA gene sequences of *Sulfurospirillum* sp. strains PLC-TCE and PLC-DCE shared 98.6% and 98.5% similarities with the 16S rRNA gene sequence of *Sulfurospirillum multivorans* (NR_121740.1). A phylogenetic analysis, based on available *Sulfurospirillum* 16S rRNA gene sequences, demonstrated that *Sulfurospirillum* sp. strains PLC-TCE and PLC-DCE were most closely related to the PCE dechlorinator *Sulfurospirillum* sp. strain JPD-1 (AY189928.1) (Figure 2.4).

**Table 2.2** Comparing the dominant genera between pH 5.5 and pH 7.2 enrichments. (Percentages indicate the abundance of representative OTUs by 16S rRNA gene amplicon sequencing analysis.)

| Major Genera (%) | **pH 7.2** | **pH 5.5** |
|---|---|---|
| *Dehalococcoides* | 22.6 | 0.0 |
| *Acetobacterium* | 57.6 | 0.0 |
| *Spirochaetaceae* Uncultured | 4.6 | 0.1 |
| *Caldisericum* | 4.2 | 0.1 |
| *Desulfuromonadales* BVA18 | 2.6 | 0.0 |
| vadinBC27 | 1.1 | 0.0 |
| *Desulfovibrio* | 0.1 | 33.0 |
| *Sulfurospirillum* | 0.2 | 25.2 |
| *Megasphaera* | 0.0 | 19.9 |
| *Propionibacterium* | 0.0 | 1.5 |
| *Pelosinus* | 0.0 | 1.00 |
| Others | 7.0 | 19.2 |
| Total | 100.0 | 100.0 |

## Discussion

Sustained *in situ* bioremediation of chlorinated solvents under acidic pH conditions (pH < 6.0) has been challenging (12). Different approaches tackle decreasing pH problem (e.g., adjusting pH by adding different sodium bicarbonate (12)) have been explored but manipulations of groundwater remain challenging and costly. An obvious alternative solution would be the application of organisms that show robust dechlorination activity below pH 6.0. Efforts to enrich PCE-to-ethene-dechlorinating cultures at pH 5.5 were not successful, but 6 out 16 sites samples showed PCE-to-ethene dechlorination at circumneutral pH. Although *c*DCE, VC and ethene formation by the PCE-to-ethene consortium SL2-PCEa was observed at pH 4.8, 5.3 and 5.9 (5), respectively, the optimal pH for SL2-PCEa consortium was around 7 and it is not clear whether different steps of

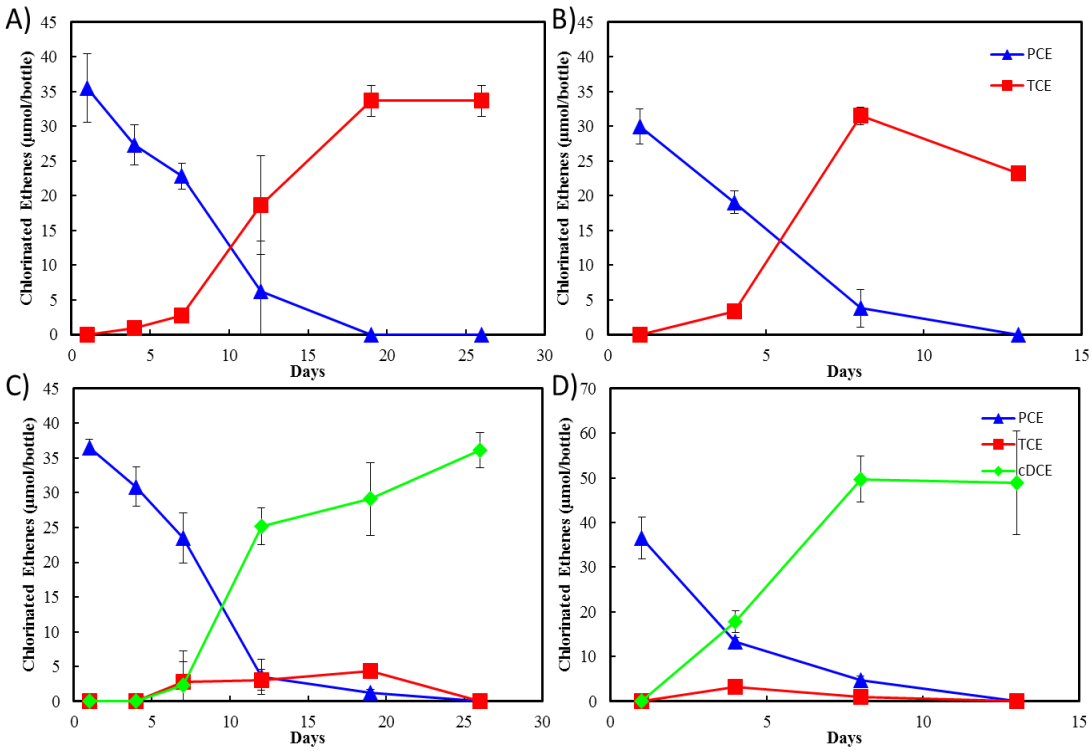


**Figure 2.3** *Sulfurospirillum* isolates strain PLC-TCE and PLC-DCE, which dechlorinate PCE to TCE (A-pH 5.5; B-pH 7.2) and *c*DCE at pH 5.5 (C-pH 5.5; D-pH 7.2), respectively. The isolates were cultivated with lactate and hydrogen as carbon source and electron donor. Error bars represent one standard deviation below or above the average of duplicate samples. (Blue Triangle-PCE, Red Square-TCE and Green Diamond-cDCE).

PCE-to-ethene dechlorination activity can be maintained after continuous transfer at low pH. Moreover, whether dechlorination activity was coupled with microbial growth was not demonstrated.

To achieve successful *in situ* bioremediation of chlorinated solvents under acidic pH condition has been a challenge. Different approaches have been investigated to tackle decreasing pH problem (e.g. adjusting pH by adding different sodium bicarbonate). An alternative approach is to enrich PCE-to-ethene dechlorinating consortium at acid pH.


**Figure 2.4** Phylogenetic tree of 16S rRNA genes sequences, showing strain PLC-TCE and PLC-DCE clustered with *Sulfurospirillum* sp. JPD-1. Sequences were aligned by MAFFT(23) in Geneious Software. Then the phylogenetic tree was built by RAxML 7.2.8(24). Sequences accession numbers and other information were listed in Table S2.3.

Efforts to enrich PCE-to-ethene-dechlorinating cultures at low pH were not successful (A total of 16 samples from chlorinated solvent-impacted sites and pristine area tested), which suggested dechlorinators preferred a neutral environment for PCE-to-ethene dechlorination. Although dechlorination of PCE-to-ethene is an acidification process, dechlorinators themselves did not develop strategies to function under low pH conditions, but relied on the buffer capacity from natural environments. By comparison, microorganisms in acid mine drainage, which oxidized pyrite and released sulfate acids, could survive under low pH condition (25).

So far only strains of the species *Dhc mccartyi* are able to dechlorinate the intermediary daughter compound VC to ethene. Since *Dhc* is susceptible to low pH conditions,

dechlorination of cDCE-to-ethene is more affected by low pH (5, 29). By comparison, PCE to cDCE steps seem less affected by low pH, since various dechlorinators (e.g. *Desulfitobacterium*, *Desulfuromonas*, *Sulfurospirillum* and *Geobacter*) could degrade PCE to TCE or *c*DCE and, especially, *Sulfurospirillum* genus was demonstrated to dechlorinate PCE to TCE or cDCE at pH 5.5. Community analysis of this consortium suggested one of the dominated group was *Sulfurospirillum*, which matched the screening efforts. This result expanded the previous reported pH range for *Sulfurospirillum* (30). Since PCE to cDCE dechlorination could occur at low pH conditions, reductive dechlorination of PCE to *c*DCE combined with aerobic oxidation of *c*DCE may solve the pH problem when remediating source zones of chlorinated solvents (31).

pH influences microbial community structure, which is shown by the 16S rRNA amplicon sequencing analysis. Rarefaction curves assessed the OTUs in the pH 5.5 and 7.2 dechlorinating communities, indicating low pH condition reduced the number of OTUs compared with neutral pH condition. Although small amount of methane production was observed in the initial pH 5.5 and pH 7.2 microcosms, methanogens were diluted out from the dechlorinating communities at pH 5.5 and 7.2. Methanogens were also sensitive to acidic conditions (32) and probably out-competed by acetogens under certain environments (e.g. oligotrophic marine and terrestrial deep biosphere) (33). *Acetobacterium* was the most abundant genus in the pH 7.2 enrichment, suggesting reductive acetogenesis as one of the dominant metabolisms in the consortium. *Acetobacterium* may fulfill relevant roles for supporting *Dhc* activity. For example, *Dhc*

is a corrinoid auxotroph and prefers cobalamin for reductive dechlorination, which *Acetobacterium* can *de novo* synthesize (34,35). It is also hypothesized that acetogens can couple with *Dhc* for syntrophic acetate oxidation (36). pH may affect populations that have important supporting roles. Then *Dhc* growth may be restricted because the supporting players cannot grow at low pH. Both phyla *Chloroflexi* and *Spirochaetae* were sensitive to low pH conditions, and their relative abundance decreased at lower pH. Moreover, the coexistence between *Dhc* of *Chloroflexi* and *Sphaerochaeta* have been frequently observed in other dechlorinating communities (37), and it was suggested that *Sphaerochaeta* may provide *Dhc* with substrates (e.g., acetate and $H_2$) or protect *Dhc* from redox stress (38). *Desulfovibrio* was enriched in the pH 5.5 enrichment. *Desulfovibrio* has not been implicated PCE reductive dechlorination but can utilize different other electron acceptors (e.g., sulfate, sulfur, nitrate, and nitrite) (39, 40). Studies on the interaction between *Desulfovibrio* and dechlorinators (e.g. *Desulfitobacterium*, *Dhc*) suggested syntrophic relationships and interspecies hydrogen transfer (41, 42). The functional roles of *Desulfovibrio* in the PCE dechlorinating enrichment at pH 5.5 remains to be identified.

Several *Sulfurospirillum* strains are capable of PCE dechlorination, such as *Sulfurospirillum* sp. strain MV, *Sulfurospirillum multivorans*, *Sulfurospirillum halorespirans* and *Sulfurospirillum* sp. strain JPD-1(23, 30, 43, 44). Their pH ranges and optimal pH were between 5.9 and 8.5 (43, 44). Here two novel strains (PLC-TCE and PLC-DCE) were isolated with 98.6% and 98.4% 16S rRNA gene sequence similarities to

*Sulfurospirillum* sp. strain JPD-1. strains PLC-TCE and PLC-DCE were able to degrade PCE to TCE and *c*DCE at pH 5.5, respectively. A similar microbial consortium SL2-PCEb also possessed two different *Sulfurospirillum* populations (45). Although *Sulfurospirillum* populations were not isolated from consortium SL2-PCEb, two types of reductive dehalogenases responsible for step-wise PCE dechlorination were identified by a terminal restriction fragment length polymorphism (TRFLP), which indicates strains PLC-TCE and PLC-DCE may possess different types of PCE reductive dehalogenases. *Sulfurospirillum multivorans* was demonstrated to be capable of enhancing PCE DNAPL (Dense Non-Aqueous Phase Liquid) dissolution (46). PCE dechlorination at the source zone area will produce large amounts of strong acid HCl, which will reduce pH values of the aquifer. Since *Sulfurospirillum* can deal with low pH better than other PCE dechlorinators, this type of microorganism may be important for achieving enhanced PCE DNAPL dissolution. Moreover, *Sulfurospirillum multivorans* strain PLC-TCE and strain PLC-DCE were capable of PCE dechlorination as low as pH 5.5, suggesting their potentials of wide applications in bioremediation of PCE source zone area.

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# Appendix

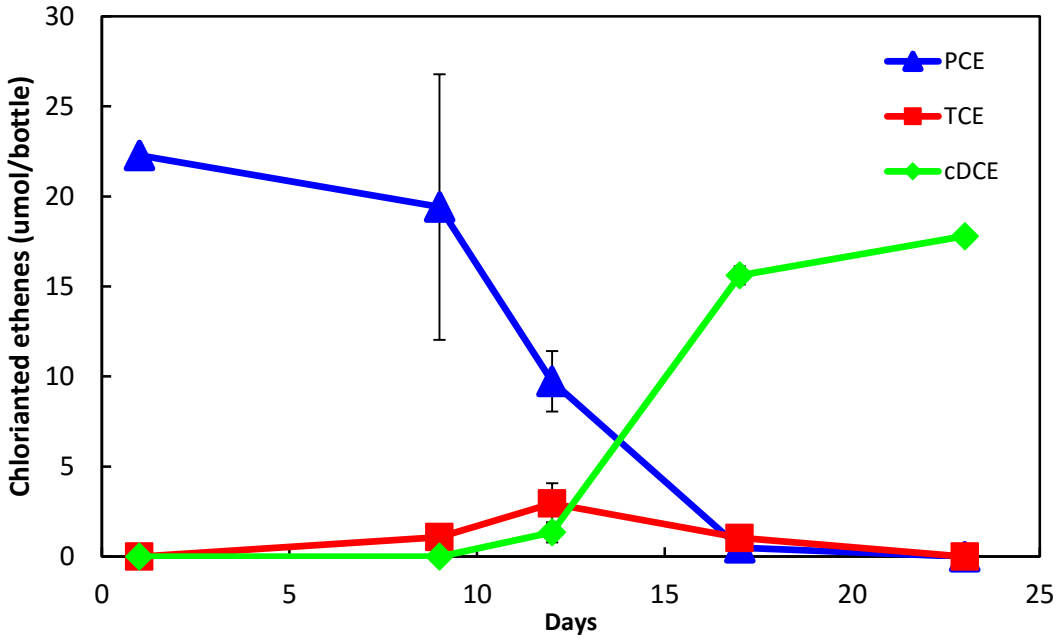


**Figure S2.1** PCE degradation by *Sulfurospirillum multivorans* at pH 5.5. Error bars represent one standard deviation below or above the average of triplicate samples. (Blue Triangle-PCE, Red Square-TCE and Green Diamond-cDCE).

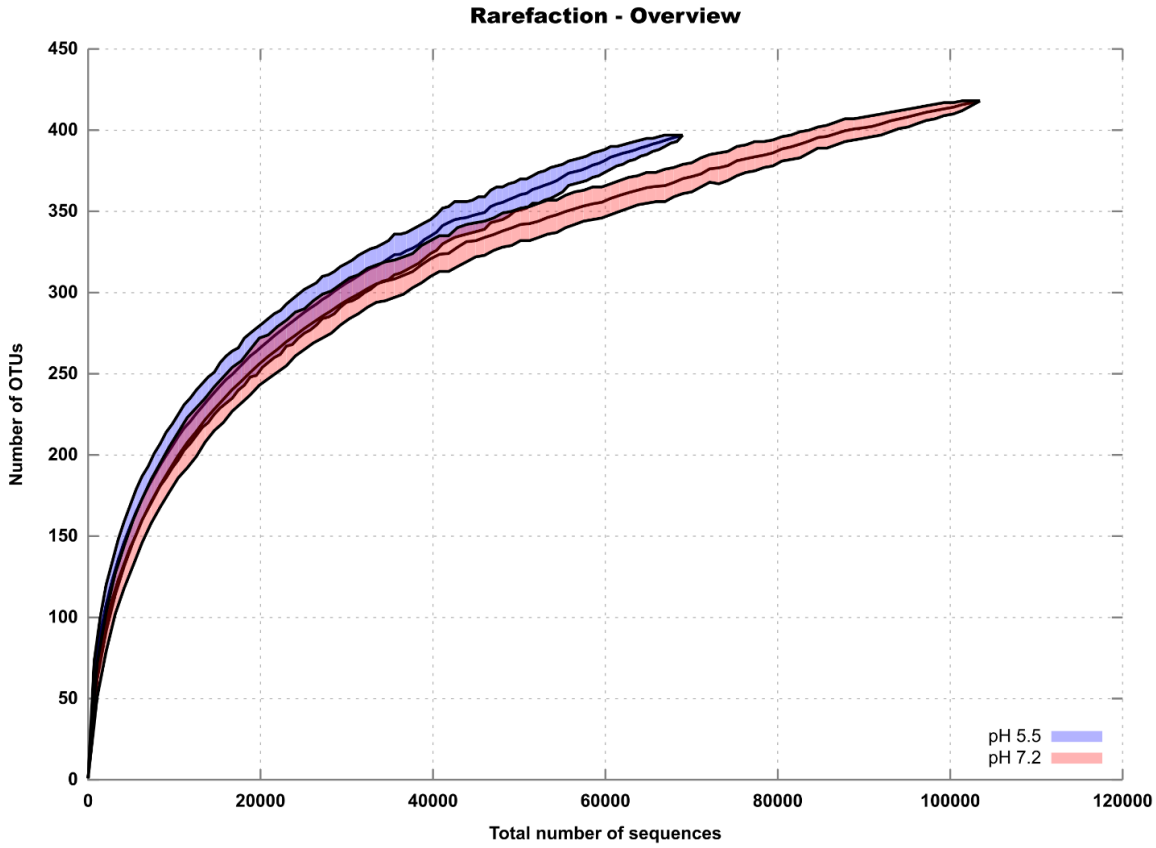


**Figure S2.2.** Rarefaction curve between pH 7.2 (Pink line) and pH 5.5 (Light Purple line) enrichments, indicating pH 7.2 enrichment has more richness than pH 5.5. Acidic conditions limited growth of some microorganisms. Each curve was accompanied by a pair of lines representing the corresponding upper and lower 95% confidence intervals.

**Table S2.1** Soil/groundwater sample list and test conditions

| # | Sample sites ID | Locations | Sample Type | Carbon Source Electron Donor | Electron Acceptor | PCE Degradation End Product | |
|---|---|---|---|---|---|---|---|
| | | | | | | pH 5.5 | pH 7.2 |
| 1 | Ft. Pierce | USA | Soil | Lactate + $H_2$ | PCE | X | X |
| 2 | PNNL | USA | Soil | Lactate + $H_2$ | PCE | X | X |
| 3 | Contaminated Site | CA, USA | Soil | Lactate + $H_2$ | PCE | X | X |
| 4 | - | Brazil | Soil | Lactate + $H_2$ | PCE | X | X |
| 5 | Third Creek | TN, USA | Sediment | Lactate + $H_2$ | PCE | Ethene | Ethene |
| 6 | Neckar River | Germany | Sediment | Lactate + $H_2$ | PCE | Ethene | Ethene |
| 7 | Rotenberg Trester | Germany | Soil | Lactate + $H_2$ | PCE | VC, Ethene | VC, Ethene |
| 8 | Rotenberg Creek | Germany | Soil | Lactate + $H_2$ | PCE | X | X |
| 9 | McGuire AFB | USA | Soil, GW | Lactate + $H_2$ | PCE | X | X |
| 10 | - | USA | Soil, GW | Lactate + $H_2$ | PCE | X | X |
| 11 | - | USA | Soil, GW | Lactate + $H_2$ | PCE | Ethene | Ethene |
| 12 | Shady Valley | TN, USA | Soil, Sediment | Lactate + $H_2$ | PCE | *c*DCE | *c*DCE |
| 13 | Axton Cross | USA | Soil, GW | Lactate + $H_2$ | PCE | VC | Ethene |
| 14 | - | USA | Soil | Lactate + $H_2$ | PCE | X | X |
| 15 | Tidal Flat | Korea | Soil | Lactate + $H_2$ | PCE | X | TCE |
| 16 | Elkhart Rail Yard | USA | Soil, GW | Lactate + $H_2$ | PCE | Ethene | Ethene |

Note: - indicated the contaminated site names were not disclosed. X indicated no PCE dechlorination was detected.

**Table S2.2** 16S rRNA gene amplicon sequencing analysis summary by SILVA-NGS

| **Project Summary** | | |
|---|---|---|
| Sequence Type: | SSU | |
| Number of Samples: | 2 | |
| Number of Sequences: | 172533 | |
| Number of Rejected Sequences: | 83 | -0.05% |
| **Raw Sequence Information** | | |
| Min. Length: | 191 | |
| Avg. Length: | 253 | |
| Max. Length: | 374 | |
| **Aligned Sequence Information** | | |
| Min. Length: | 191 | |
| Avg. Length: | 253 | |
| Max. Length: | 374 | |
| **Clustering Information** | | |
| Number of OTUs: | 815 | -0.47% |
| Number of Clustered Sequences: | 12540 | -7.27% |
| Number of Replicates: | 159095 | -92.21% |
| **Classification Information** | | |
| Number of Classified Sequences: | 172409 | -99.93% |
| Number of No Relative: | 41 | -0.02% |
| **Alignment** | | |
| SILVAngs: | SINA v1.2.10 for ARB SVN (revision 21008) | |
| Min Align. Identity (%): | 50 | |
| Min Align. Score: | 40 | |
| Min Basepair Score (%): | 30 | |

**Table S2.2.** continued**.**

| Project Summary | |
|---|---|
| **Quality Control** | |
| Min. Sequence Quality (%): | 30 |
| Min. Length (aligned nuc.): | 50 |
| Max. Ambiguities (%): | 2 |
| Max. Homopolymers (%): | 2 |
| | |
| **Clustering** | |
| CD-Hit: | 3.1.2 |
| Min. OTU Identity (%): | 98 |
| | |
| **Classification** | |
| BLAST: | 2.2.30+ |
| Reference: | SILVA |
| Reference Version: | 123 |
| Similarity (%): | 93 |

**Table S2.3** Details of 16S rRNA gene sequences used to build the phylogenetic tree

| Name | Accession Number | Sequence Length |
|---|---|---|
| *Desulfitobacterium dehalogenans* strain ATCC 51507 | NR_074128.1 | 1447 |
| *Desulfitobacterium dichloroeliminans* | AJ565938.1 | 1467 |
| *Desulfitobacterium frappieri* | U40078.1 | 1655 |
| *Desulfitobacterium hafniense* strain DCB-2 | NR_122068.1 | 1554 |
| *Escherichia coli* str. K12 | AP009048.1 | 1551 |
| *Sulfurospirillum alkalitolerans* strain HTRB-L1 | GQ863490.1 | 1437 |
| *Sulfurospirillum arcachonense* strain F1F6 | NR_026408.1 | 1433 |
| *Sulfurospirillum arsenophilum* | U85964.1 | 1321 |
| *Sulfurospirillum arsenophilum* strain MIT-13 | NR_044806.1 | 1321 |
| *Sulfurospirillum barnesii* SES-3 | NR_102929.1 | 1497 |
| *Sulfurospirillum carboxydovorans* strain MV | AY740528.1 | 1354 |
| *Sulfurospirillum cavolei* | AB246781.1 | 1336 |
| *Sulfurospirillum cavolei* strain Phe91 | NR_041392.1 | 1336 |
| *Sulfurospirillum deleyianum* strain DSM 6946 | NR_074378.1 | 1497 |
| *Sulfurospirillum deleyianum* strain Spirillum 5175 | NR_026422.1 | 1431 |
| *Sulfurospirillum halorespirans* strain PCE-M2 | AF218076.1 | 1489 |
| *Sulfurospirillum multivorans* strain DSM 12446 | NR_121740.1 | 1498 |
| *Sulfurospirillum multivorans* strain K | NR_044868.1 | 1464 |
| *Sulfurospirillum* sp. C6 | DQ228139.1 | 1201 |
| *Sulfurospirillum* sp. EK7 | AJ535704.1 | 1431 |
| *Sulfurospirillum* sp. JPD-1 | AY189928.1 | 1415 |
| *Sulfurospirillum* sp. NO3A | AY135396.1 | 1300 |
| *Sulfurospirillum* sp. strain PLC-DCE | | 1375 |
| *Sulfurospirillum* sp. strain PLC-TCE | | 1015 |

# CHAPTER III RECOVERY OF *DEHALOCOCCOIDES MCCARTYI* EXPOSED TO LOW PH AND DISTRIBUTION OF *DEHALOCOCCOIDES MCCARTYI* IN GROUNDWATER WITH TWO PH RANGES

A version of this chapter is going to be submitted for publication. Yang, Y., N.L. Cápiro, J. Yan, T.F. Marcet, K.D. Pennell, and F.E. Löffler. Recovery of *Dehalococcoides mccartyi* Exposed to Low pH and Distribution of *Dehalococcoides mccartyi* in Groundwater with Different pH Ranges.

## Abstract

*Dehalococcoides mccartyi* (*Dhc*) is a keystone microorganism for VC-to-ethene detoxification. Although successful bioremediation has been achieved at many sites impacted with chlorinated ethenes, fermentation of electron donor amendments and dechlorination (i.e., release of hydrochloric acid) can cause groundwater pH decreases and impact *Dhc* activity. The goal of this study was to evaluate *Dhc* response to and recovery from low pH conditions. The *Dhc*-containing consortium BDI dechlorinated PCE to ethene within 40 days at pH 7.2, but no PCE dechlorination was observed at pH 5.5. While some reductive dechlorination of PCE to *c*DCE occurred at pH 5.5, the monitoring of *Dhc* biomarker genes (i.e., 16S rRNA, *tceA* and *vcrA* genes) with quantitative PCR (qPCR) demonstrated that *Dhc* cells did not grow at pH 5.5. *Dhc* reductive dechlorination activity and growth recovered in pH 7.2 medium, when the pH 5.5 exposure did not exceed 16 days; however, the cultures performance catalyzing the VC-to-ethene reductive dechlorination step was impaired. qPCR monitoring demonstrated that *Dhc* strain GT carrying the *vcrA* VC reductive dehalogenase genes was more susceptible to low pH-induced stress than *Dhc* strain FL2. *Dhc* cells exposed to pH

5.5 conditions for >40 days did not recover dechlorination activity and did not grow following transfer to pH 7.2 growth medium. Apparently, the duration of pH 5.5 exposure strongly affected the ability of *Dhc* to recover at circumneutral pH and *Dhc* strain-specific responses were observed. *Dhc* strain GT carrying the *vcrA* gene responsible for VC reductive dechlorination to ethene was less tolerant to low pH exposure than *Dhc* strain FL2 carrying the *tceA* gene implicated in TCE-to-VC reductive dechlorination. To further investigate how low pH in situ affected the abundance of *Dhc* at chlorinated ethenes contaminated sites, monitoring data from more than 200 wells were collected and analyzed, indicating the distribution of *Dhc* biomarkers (16S rRNA gene, *tceA* gene and *vcrA* gene) was not determined by pH alone, and *Dhc* was also abundant at low pH wells. These findings together will provide useful information for low pH sites and chlorinated ethenes source zone bioremediation.

## Introduction

Chlorinated solvents remain major hazardous groundwater contaminants as documented in the Substance of Priority List (https://www.atsdr.cdc.gov/spl/). Different technologies have been developed to clean up sites contaminated with chlorinated solvents, such as in situ chemical oxidation, in situ thermal treatment, air sparging and soil vapor extraction (1). One of the promising *in situ* remedial approaches enhanced reductive dechlorination (ERD) that uses anaerobic microorganisms to degrade chlorinated solvents to innocuous end products (2). In the past decades, different dechlorinating microbial isolates (e.g.

*Dehalobacter*, *Dehalococcoides*, *Geobacter*, *Desulfuromonas*, *Desulfitobacterium*) and consortia (e.g. KB-1, SDC-9, ANAS, BDI) have been intensively investigated, which provide useful insights for *in situ* bioremediation of chlorinated ethenes (3). *Dehalococcoides* (*Dhc*) has been well known for its uniqueness since only *Dhc* has been demonstrated to perform VC-to-ethene reductive dechlorination (4). However, the successful application of *Dhc* cultures to clean up chlorinated solvent-contaminated sites is constrained by geochemical factors. In particular, pH affects successful application of bioremediation (5).

Dechlorination releases hydrochloride acid, and this strong acid could result in the groundwater acidification (6). Moreover, fermentation of organic electron donor amendments, which are added with the intention to increase hydrogen flux, will also contribute to pH decreases. Low pH will affect microbial populations and their activities, including *Dhc*. *Dhc* dechlorinates chlorinated ethenes within a fairly narrow pH range of 6.5- 8 (4). Thus successful bioremediation based on *Dhc* activity requires a stable circumneutral pH. When the buffering capacity of contaminated aquifer is sufficient, pH can be maintained within the range suitable for dechlorinators; but at other sites without enough buffering capacity, pH decreases are observed and become detrimental to dechlorinators.(7) Also cleaning contaminated sites with low pH groundwater is still challenging.

To address adverse impacts of low pH on *Dhc in situ*, one of the solutions is to precondition and adjust the pH of contaminated groundwater to neutral and counteract acid production by adding enough buffer solutions, such as carbonate, bicarbonate, formate and other commercial available buffer agents (e.g., AquaBupH and Neutral Zone) (6, 8). Typically, the addition of pH stabilizers is not done proactively, and pH adjustments occur after reductive dechlorination activity slows down due to pH decreases. Thus, dechlorinating populations, both native or bioaugmented, experience low pH conditions. While it is established that *Dhc* perform best at circumneutral pH, information about the effects of low pH exposure on the ability of *Dhc* to recover reductive dechlorination activity is lacking. To address this knowledge gap, experiments were conducted to investigate the growth of *Dhc* under acidic conditions, and to study the recovery of *Dhc* after low pH exposure. To further investigate how low pH of groundwater affects the distribution of *Dhc* at chlorinated ethenes contaminated sites, monitoring data from more than 200 wells were collected and analyzed.

## Materials and Methods

**Chemicals.** PCE and TCE were purchased from Acros Organics (Distributed by VWR international, West Chester, PA, USA). cDCE, VC and ethene were bought from Sigma-Aldrich Chemicals (St. Louis, MO, USA). MES (2-(N-morpholino)ethanesulfonic acid) was purchased from Acros Organics. Sodium bicarbonate was purchased from Fisher Scientific (Pittsburgh, PA, USA).

**Medium preparation and pH measurement.** Reduced mineral salts medium was prepared following established protocols (9). Vitamin stock solution was added by passing through sterile 0.22 µm membranes filters after the medium had been autoclaved. Lactate (5 mM) and hydrogen gas (10 mL) were added into 160 mL serum bottles as carbon source and electron donor, respectively. The pH 7.2 mineral salts medium was buffered with 30 mM bicarbonate. For pH 5.5 mineral salts medium, 30 mM bicarbonate was replaced with 30 mM MES. The pH of bulk liquid phase was measured by transferring 1 mL liquid aliquots from a culturing vessel into a 2-ml plastic tube. After centrifuging the tube for 30 seconds at 14,000 rpm, the pH of the supernatant was measured with Fisher Scientific Accumet Glass AgCl pH electrode (Pittsburgh, PA, USA).

**Quantification of chlorinated ethenes.** Total chlorinated solvent mass or concentrations of chlorinated compounds were measured by analyzing 100 µL headspace gas samples on a gas chromatograph (GC). The concentrations of chlorinated ethenes were calculated by normalizing the peak area values to standard curves generated by adding known amounts of chlorinated ethenes into the bottles with same gas to liquid ratio. The total moles of polychlorinated ethenes per bottle was calculated by the formula: total moles = (volume x density) / molecular weight. Gas samples (100 µL) were removed from the headspace of 160 mL serum bottles using a gastight 250 µL Hamilton SampleLock syringe and then injected into the GC manually. Samples were measured with an Agilent 7890A GC equipped with an Agilent DB624 column (30 m x 0.53 mm I.D., 3 µm.) with a flame

ionization detected (FID). The retention times were determined by injecting neat compounds into the GC. The retention time was used as the identity for the specific chlorinated compounds.

**pH Tolerance and resilience of a PCE-to-ethene-dechlorinating consortium.** To better understand whether dechlorination activity resumes following exposure to low pH conditions after pH adjustment, we conducted a resilience experiment with the a PCE-to-ethene consortium. The consortium biomass grown at pH 7.2 with PCE as electron acceptor was collected and suspended in pH 5.5 medium. Following incubation of 8, 16, and 40 days at pH 5.5, the biomass was collected again and transferred to pH 7.2 medium amended with hydrogen (electron donor), lactate (carbon source) and PCE. Chlorinated ethenes and ethene are monitored to explore if reductive dechlorination activity recovers from the exposure to low pH for 8-40 days. In addition, *Dhc* 16S rRNA gene copies were being enumerated with qPCR to evaluate *Dhc* cell growth. Further, the reductive dehalogenase genes *tceA*, *vcrA* and *bvcA* are monitored to determine of different *Dhc* strains respond differently to low pH (Figure S3.1).

**DNA extraction and PCR.** Microbial biomass was collected from 2-mL liquid culture suspension by vacuum filtration through 0.22 µm membrane filters (Millipore GVWP025000). Filter-trapped microbial cells were suspended in the PowerSoil® bead tubes (Mo Bio Laboratories Inc., Carlsbad, CA) and ruptured with a high efficiency Bead Ruptor Homogenizer (Omni International, Kennesaw, GA, USA) at a speed of 3.25 m/s

for 5 minutes. Genomic DNA was extracted using the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA) following the manufacturer's recommendations. DNA concentrations were quantified with a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE). DNA samples extracted from replicate cultures were pooled and stored at -20°C. Molecular tools, such as quantitative PCR have been used to investigate how low pH condition exposure affected dechlorinators and their functional genes. qPCR assay followed establishes protocols and used the primers and probes in the published paper (10).

**Functional genes and 16S rRNA gene data from contaminated sites.** Functional genes (*bvcA, vcrA* and *tceA*) and 16S rRNA gene of *Dhc* data were kindly provided by Microbial Insights Inc. (Knoxville, TN). Groundwater samples collected from various undisclosed chlorinated solvents contaminated sites were subject to different chemical and microbial tests (e.g. pH and qPCR assays). And groundwater samples with less than 100 functional genes or *Dhc* 16S rRNA gene copies per liter groundwater were excluded from analysis.

**Statistical analysis.** All statistical analyses were performed using R Statistical Software (version 3.2.4., R Foundation for Statistical Computing, Vienna, Austria). Variance homogeneity of two pH intervals was tested by Bartlett test and Fligner-Killeen test with the default parameters. One-way t-test was used to compare the average of two pH intervals with the alternative parameter set as "less".

# Results

***Dhc* growth at pH 5.5 and pH 7.2.** qPCR targeting the *Dhc* 16S rRNA gene and the reductive dehalogenase genes *tceA*, *bvcA* and *vcrA* was applied to investigate the response of *Dhc* strains in a PCE-to-ethene consortium to low pH conditions. In pH 5.5 medium, PCE was not degraded, and the *Dhc* cell numbers did not increase during the incubation period at pH 5.5. Since different steps of PCE-to-ethene were accomplished by different dechlorinators, *Dhc* may also be affected if PCE could not be degraded to TCE or *c*DCE. By comparison, within 2 weeks, about 75% of the initial amount of PCE was transformed to ethene at pH 7.2, and qPCR demonstrated growth of *Dhc* (Figure 3.1). The *Dhc* 16S rRNA gene copies increased from 1.55±0.42 X $10^8$ $mL^{-1}$ (cells introduced with the inoculum) to 6.99±1.99 X $10^8$ $mL^{-1}$. The *vcrA* and *tceA* genes increased from 1.57±0.09 X $10^8$ and 1.29±0.11 X$10^8$ $mL^{-1}$ to 4.92±1.79 X $10^8$ and 2.31±0.47 X $10^8$ $mL^{-1}$, respectively. *Dhc* Strain BAV1 carrying the *bvcA* gene is part of consortium BDI but this strain is not competitive in cultures fed with PCE and was consequently not detected, which was also reported in the previous publication (11).

**pH tolerance and resilience.** To explore the effects of low pH exposure on *Dhc* growth and reductive dechlorination performance, consortium BDI biomass was suspended in pH 5.5 medium for 8, 16, and 40 days. Recovery of growth and dechlorination activity was then tested in pH 7.2 medium. Consortium BDI biomass exposed to pH 5.5 for 8 days recovered activity at pH 7.2 and the cultures dechlorinated PCE to VC. qPCR monitoring demonstrated that the 16S rRNA, *tceA* and *vcrA* genes increased 39.2 ± 9.6-, 50.9 ± 8.9-

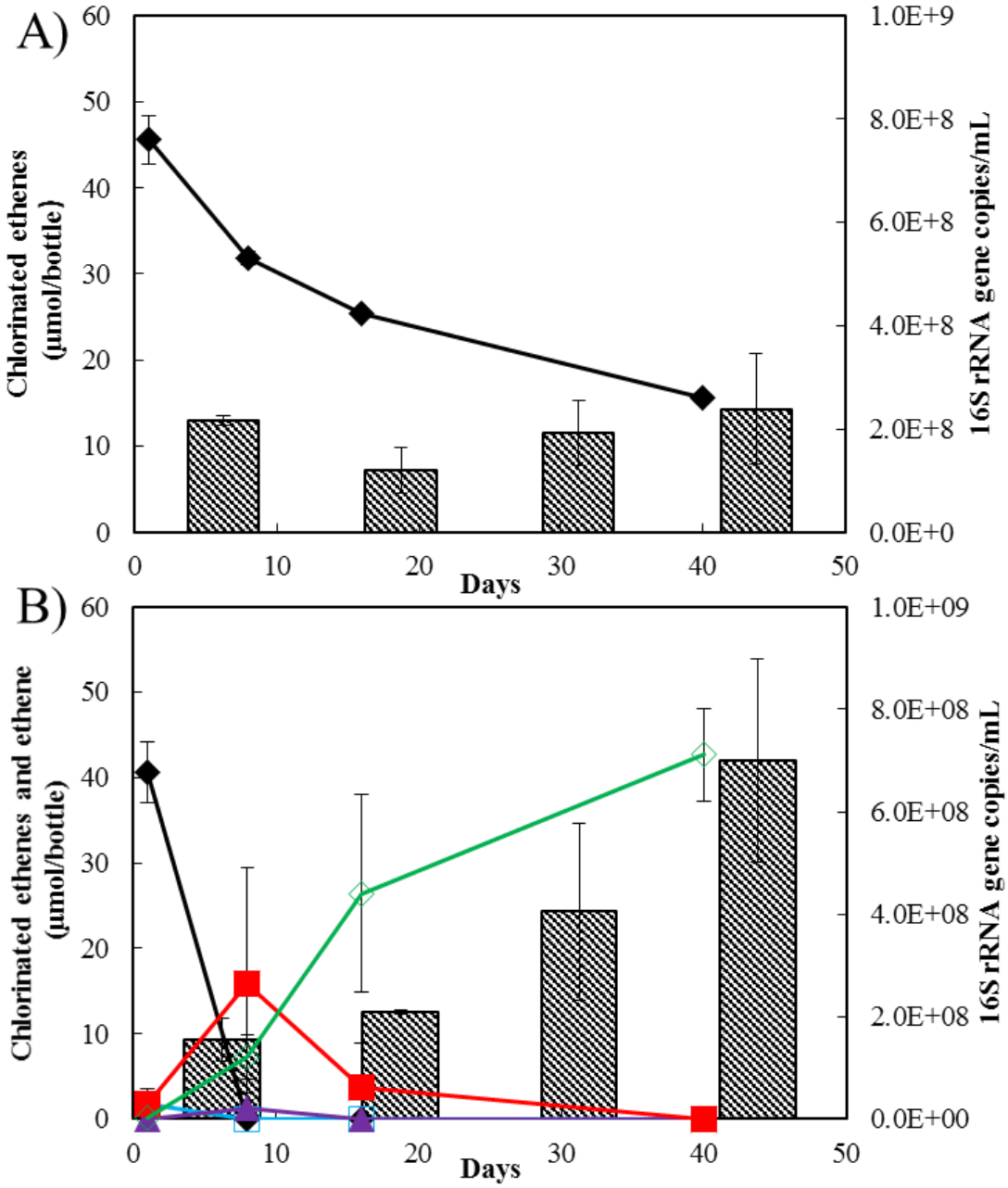


**Figure 3.1** PCE dechlorination at pH 5.5 and 7.2 by a PCE-to-ethene consortium containing *Dhc*. No dechlorination activity and growth of *Dhc* at pH 5.5; while PCE could be degraded to ethene at pH 7.2 coupled with *Dhc* growth. PCE loss at pH 5.5 was mainly due to abiotic loss (e.g. absorption to the stoppers). (Filled black diamond-PCE, empty blue square-TCE, filled purple triangle-*c*DCE, filled red square-VC, empty green diamond-ethene, shaded bar-*Dhc* 16S rRNA gene copy number).

and 14.2 ±7.4-fold, respectively. PCE-to-VC dechlorination was also observed in pH 7.2 cultures initiated with biomass that experienced a 2-week exposure to pH 5.5, and the *Dhc* 16S rRNA, *tceA* and *vcrA* genes increased 11.5 ±3.9-, 41.3 ± 15.6- and 6.3 ± 2.5-fold. Following a 40-day exposure to pH 5.5, the consortium degraded PCE to mainly *c*DCE and some VC when transferred to pH 7.2 medium, and the *Dhc* 16S rRNA, *tceA* and *vcrA* genes increased only 6.0 ± 3.8-, 11.6 ± 8.8- and 2.5 ± 1.1-fold. The recovery experiments showed longer low pH exposure time will result in longer recovery time of dechlorinators. Statistical analysis on average fold increases of 16S rRNA, *tceA* and *vcrA* genes suggested no statistically differences between pH 5.5 and pH 7.2 (control group) after 8 days' incubation (16S rRNA gene: p-value =0.211; *tceA* gene: p-value = 0.567; *vcrA* gene: p-value: 0.242; Table 3.1). There is no statistical difference between pH 5.5 and pH 7.2 groups after 16 days' incubation, indicating up to 16 days' pH 5.5 acid stress did not severely affect *Dhc*'s survival; but there was statistical significance between pH 5.5 and pH 7.2 after 40 days' incubation (16S rRNA gene: *p*-value =0.014; *tceA* gene: *p*-value = 0.034; *vcrA* gene: *p*-value: 0.000; Table 3.1), suggesting *Dhc* was inhibited after extended acid stress.

The VC-to-ethene dechlorination step was most severely inhibited and only the cultures initiated with biomass exposed to pH 5.5 for 8 days produced some ethene. The pH 7.2 control cultures produced ethene demonstrating that the manipulations of the biomass (i.e., centrifugation and resuspension) were not the reason for the limited reductive dechlorination activity (Figure 3.2). These findings suggest that the duration of low pH exposure determines the ability of *Dhc* to recover from low pH-induced stress.

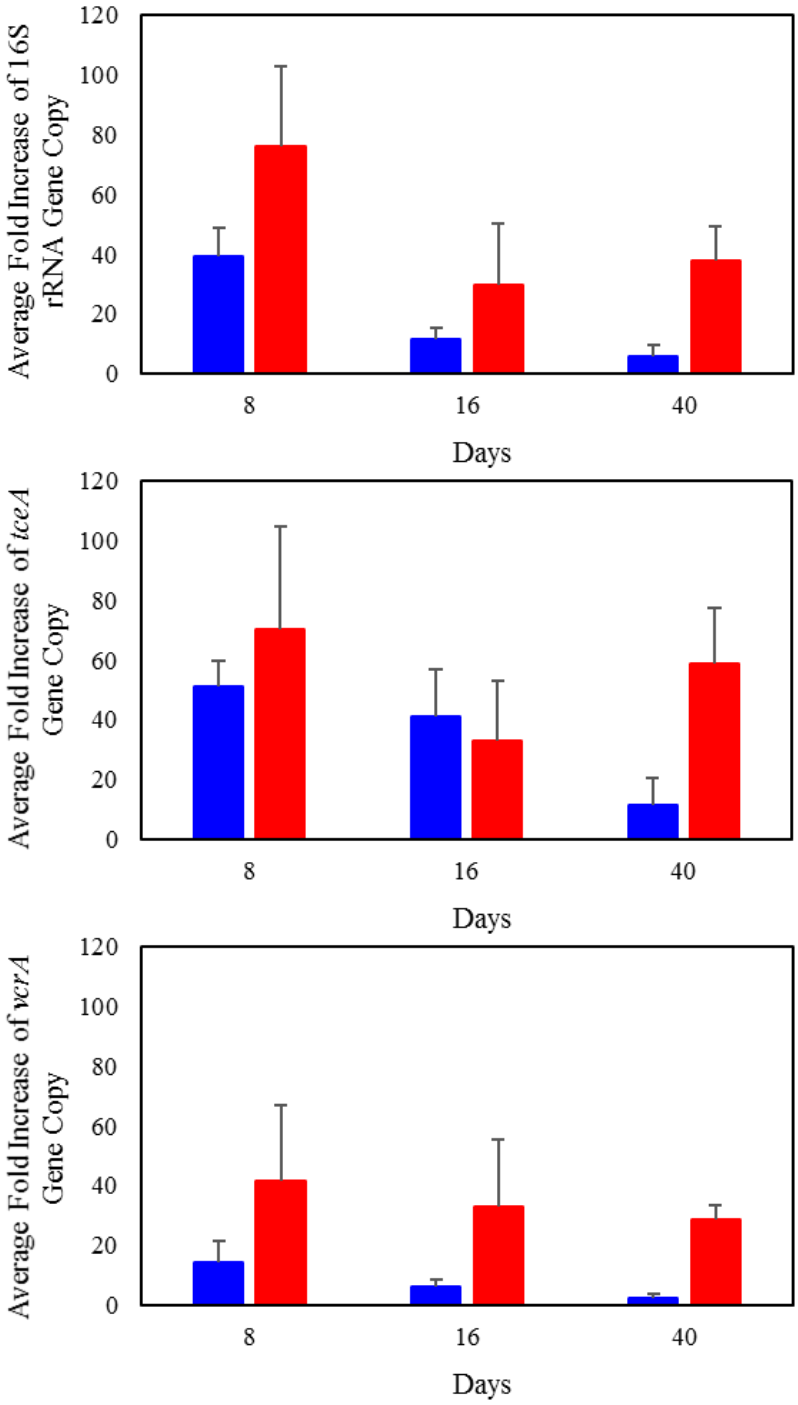


**Figure 3.2** Average fold increase of 16S rRNA gene (A), *tceA* (B) and *vcrA* (C) genes after 8, 16, 40 days' pH 5.5 (blue bar) and pH 7.2 (red bar) incubations. The error bar indicates one standard error (for pH 5.5 n=6; for pH 7.2 n =4.)

Apparently, recovery is *Dhc* strain-specific because the VC-to-ethene step was more affected than the *c*DCE-to-VC reductive dechlorination step suggesting that *Dhc* strain GT carrying the VC-RDase *vcrA* was more susceptible to pH stress.

**Relationship between groundwater pH and *Dhc* abundance.** To further assess whether pH values will affect the abundance of *Dhc* between neutral and acid conditions at chlorinated ethenes contaminated sites, a total number of 221 groundwater wells from 23 chlorinated solvents-contaminated sites were investigated. These groundwater wells were chosen for the availability of both geochemical and biological data. In 50 groundwater wells, *Dhc* 16S rRNA gene were below 100 copies/L; and these 50 wells were not included into further analysis. The pH of the rest 171 groundwater wells ranged from 5.2 to 8.3, with a median number of 6.4. And *Dhc* 16S rRNA gene copy numbers in these 171 wells spread from 100 to 4.0 X $10^6$ copies/L, with a median 6.96 X $10^5$ copies/L. Since the optimal pH for dechlorinating consortia applied for bioaugmentation is 6.0~8.3 (8), pH values were categorized into two intervals: acidic range (4.5~6.0) and circumneutral range (6 ~8.3). Both Bartlett and Fligner-Killeen tests accepted the null hypothesis of variance homogeneity (Bartlett test: k-square =0.49, df =1, *p*-value = 0.4827; Fligner-Killeen test: chi-squared = 0.76, df=1, *p*-value = 0.38). Comparison of the average *Dhc* abundance between acid and circumneutral ranges by one-way t-test suggested statistically significant difference of the average *Dhc* abundance (df=67.4, *p*-value=0.009). The group mean of *Dhc* abundance for pH range 4.5~6.0 was 7.49 X $10^5$ copies/L (Figure 3.3). By comparison, the average *Dhc* abundance for pH range 6.0~8.3

was 1.14 X $10^6$ copies/L (Figure 3.3). The abundances of the functional genes *tceA* and *vcrA* between acid and circumneutral pH ranges followed a similar pattern. Statistical analysis of the average *tceA* and *vcrA* gene copies at two pH ranges indicated statistically significant differences (*vcrA*: *p*-value=0.001; *tceA*: *p*-value=0.015, Figure 3.3, Table S3.2). Yet analysis of total bacterial 16S rRNA gene abundance indicated no statistical significance between 4.5~6.0 pH range and 6.0~8 pH range (*p*-value=0.314, Figure 3.3, Table S3.2).

## Discussion

This study demonstrated that no *Dhc* growth occurred at pH 5.5. Limited reductive dechlorination activity was observed indicating that *Dhc* cells maintained some level of activity but cell division did not occur. Application of *Dhc* to clean up low pH sites or chlorinated solvents source zones will be restricted. To overcome this problem, two possible solutions are suggested: either adjusting in situ pH to neutral or seeking dechlorinators capable of dechlorinating chlorinated ethenes at low pH. One study suggested using economical silicate minerals to adjust and maintain neutral pH, but how silicate minerals affected dechlorinators need further studies (12). Also some cultures were acclimated to low pH conditions by exposure to stepwise decreasing pH environments (e.g. a consortium KB-1 Plus containing *Dhc*, http://www.siremlab.com/products/kb-1); but KB-1 Plus performs dechlorination only at pH 5.8~6.3. Although several studies claimed having enriched consortia capable of dechlorinating PCE to ethene at low pH conditions, further evidences are required to

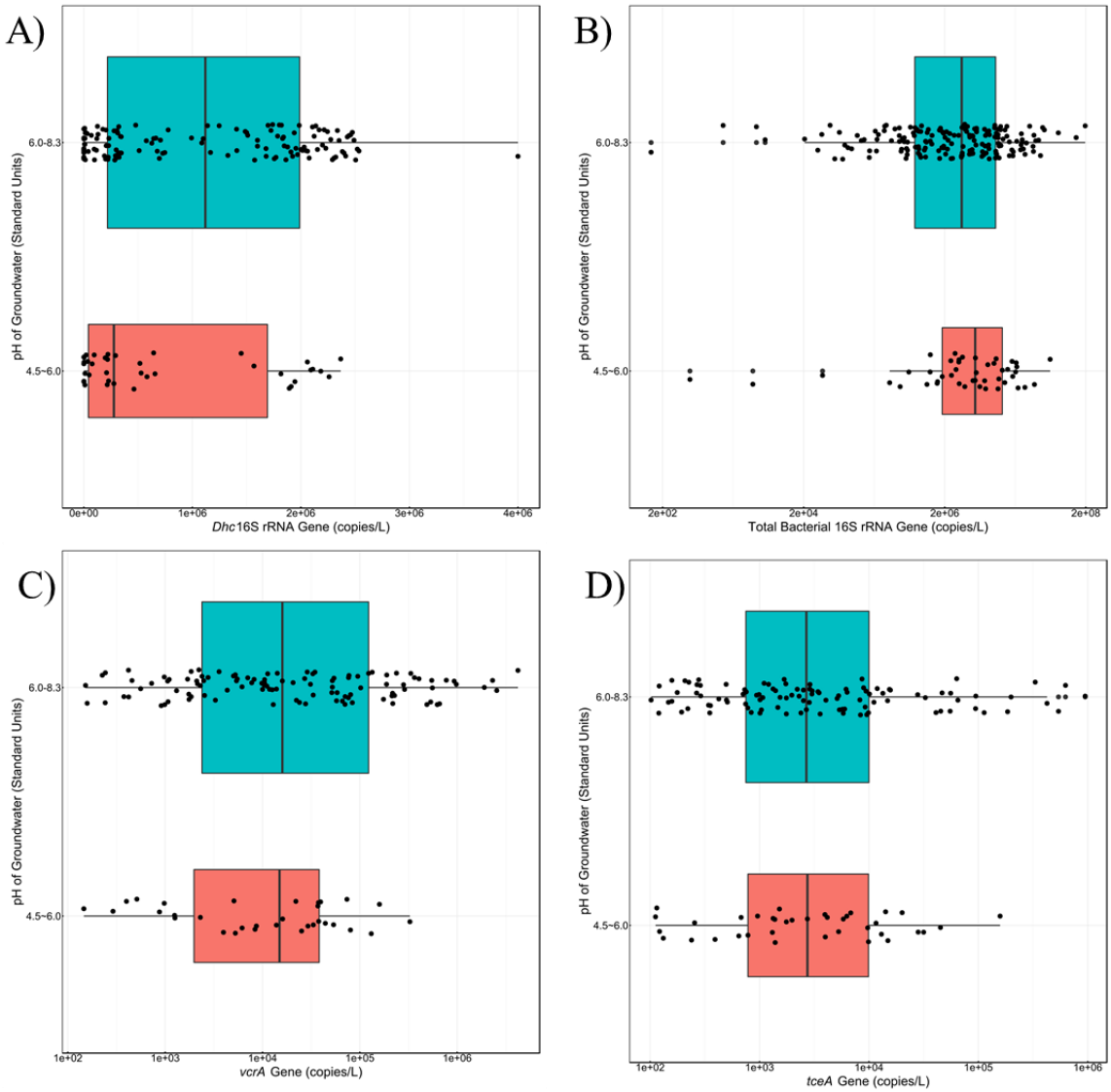


**Figure 3.3** Distribution of *Dhc* 16S rRNA (A), total bacterial 16S rRNA (B) and *vcrA* (C), and *tceA* (D) gene copy numbers in terms of two pH categories (pH 4.5~6.0 and pH 6.0~8.3) from a survey of 221 groundwater wells contaminated with chlorinated ethenes.

prove the cultures' sustainability of dechlorinating chlorinated ethenes at low pH.

One of the key findings in this study is that *Dhc* does not grow at pH 5.5, and that prolonged pH 5.5 exposure reduces the ability of *Dhc* to recover from low pH exposure. These observations have implications for low pH contaminated sites clean-up and DNAPL (Dense Non-Aqueous Phase Liquid) source zone bioremediation. Adjusting and maintaining neutral pH by buffer additions was one of the popular strategies to create suitable pH for bioremediation (13). Also pH decrease from 7.2 to 5.3 was observed after nutrients injection during cleaning up DNAPL area. And pH fluctuations inhibited the initial establishment of dechlorinating microbial activity (14). But no study has been conducted to assess *Dhc*'s recovery after low pH exposure. This study then suggested buffer amendments should be applied in tandem with the evaluation of *Dhc*'s recovery if *Dhc* has been exposed to low pH conditions for extended time. Without proper evaluation of *Dhc*'s viability, dechlorination of *c*DCE or VC cannot be achieved successfully with pH adjustment only. Buffer systems (e.g. bicarbonate, formate) may be consumed by other microorganisms or washed away by groundwater flow long before *Dhc* becomes active. Under such circumstances, bioaugmentation combined with pH adjustment would be a better strategy to clean up DNAPL area and/or low pH contaminated sites.

*Dhc* was reported to have a strain-specific susceptibility towards environment stress (e.g. oxygen, temperature) (11, 15). Of three *Dhc* strains in BDI consortium, only strain FL2 carrying *tceA* gene could survive oxygen exposure or increased temperature, but not

strain GT carrying *vcrA* gene and strain BAV1 carrying *bvcA* gene (11, 15). This study also found *Dhc* carrying *tceA* gene, which cannot dechlorinate VC to ethene, was more tolerant of low pH exposure. This commonality suggested *c*DCE or VC stall observed in the chlorinated ethenes contaminated sites was also due to the vulnerability of *Dhc* strains responsible for VC-to-ethene step.

*Dhc* could recover dechlorination after 16 days' weak acid exposure. But the mechanisms of *Dhc* to tackle acid stress was not well understood yet. To distinguish different mechanisms to cope with different low pH conditions, the acid responses mechanisms were categorized into acid tolerance responses (ATR) for mild acid pH (> pH 3.0) and extreme acid resistance (XAR) for extreme acid pH (< pH 2.0) (16). Microorganisms apply different mechanisms to deal with low pH stress, such as proton exchange/consumption system (e.g. $F_1F_0$-ATPase, amino-acid dependent decarboxylase/antiporter systems), buffer production system (e.g. deiminase and deaminase, or urease enzymes to produce ammonia), and cell modification/repair (e.g. changing the composition of cell membrane) (16). Although *Dhc* could not perform dechlorination at pH 5.5, *Dhc* may possess ATR systems to survive at mildly acidic pH for extended period. But the mechanisms of *Dhc* surviving under low pH are still not well understood. *Escherichia coli* (*E. coli*) has been a model microorganism to study the mechanisms of both ATR and XAR, which may offer insights to figure out *Dhc*'s acid resistance mechanisms. For example, *E. coli* could survive at pH 2.5 or lower, when the cultivating media rich with amino acids, but was quickly killed in minimal glucose

medium lack of amino acids (17). Another mechanism to resist extreme acid is involved with ubiquitous chloride channels (ClC) possessed by *E. coli*. Two homologues genes (*eriC* and *mriT*) of ClC channels were annotated from *E. coli* genome, and deletion of both genes severely reduced their ability to survive the extreme acid condition (18). BLASTing these two ClC genes against publicly available *Dhc* genomes rendered no hits, suggesting different types of ClC possessed by *Dhc*, or absence of ClC in *Dhc*. If *Dhc* does not possess ClC, it may indicate that *Dhc* cannot survive extreme acidic pH. Last but not least, periplasmic carbonic anhydrase, which can convert carbon dioxide into bicarbonate, was suggested to help the Gram-negative bacterium *Helicobacter pylori* to survive in the acid environment of the stomach (16). Genomes of *Dhc* strains also possess carbonic anhydrase genes, indicating *Dhc* may be capable of using carbonic anhydrase to keep periplasmic pH above 6.0 when the environmental pH is below pH 6. But some questions are still open to answers, such as the location of carbonic anhydrase in *Dhc*, whether the pH in the periplasm is directly affected by the groundwater pH or dechlorinators have mechanisms to adjust the periplasmic pH.

A data mining approach was to investigate the factors that can be used to predict *in situ* dechlorination; but pH failed to be incorporated into the modeling process possibly due to the lack of input sites with pH < 6.0 (19). In this study, we demonstrated that the average *Dhc* abundance in the pH range 6.0~8.3 was much higher than that in the pH range 4.5~6.0, suggesting pH affects the abundance of *Dhc* in situ. *Dhc* was not commonly detected below 4.5 or above 8.5, suggesting their neutrophile lifestyle. To improve the

modeling process, pH parameters may be treated as a category variable (pH<6.0 and pH >6.0) rather than a numeric variable.

This study demonstrated that *Dhc* can survive under mildly acid stress and recover dechlorination capability but the duration to low pH exposure also matters. Besides, *Dhc* strains have different tolerance towards low pH, and the strain carrying *tceA* gene is more tolerant. Although the average bacterial abundances were similar between pH range 4.5~6.0 and pH range 6~8.3, *Dhc* and functional genes were more abundant in the pH range 6~8.3. *Dhc* could be detected at contaminated sites with pH from 4.5 to 8.5.

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# Appendix

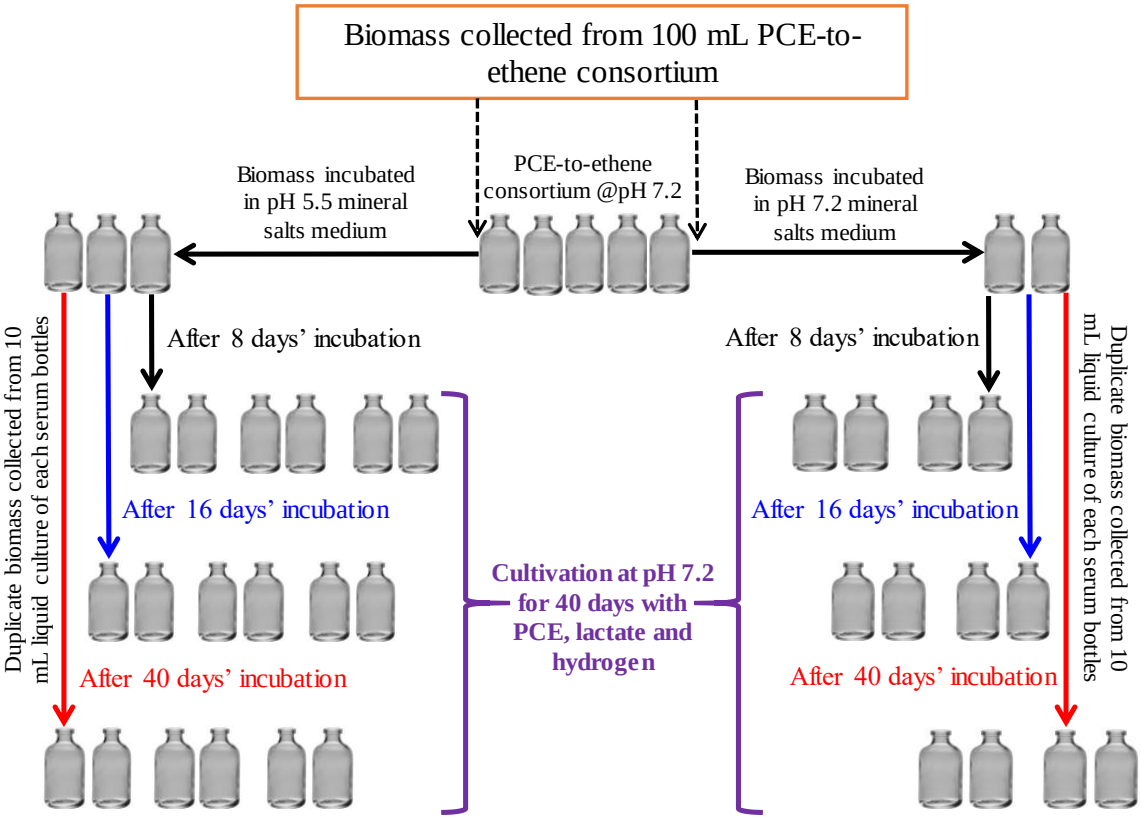


**Figure S3.1.** Experiment scheme of recovery of a PCE-to-ethene consortium exposed to low pH stress. pH 7.2 incubation was set as the control group.

**Table S3.1** Statistical analysis of average fold increase between pH 5.5 and pH 7.2 incubation.

| Exposure Time (Days) | Genes | Average Fold Increase | | *p*-Value of t-Test |
|---|---|---|---|---|
| | | pH 5.5 (n=6) | pH 7.2 (n=4) | |
| 8 | 16S | 39.2 | 76.2 | 0.211 |
| | *tceA* | 50.9 | 70.2 | 0.567 |
| | *vcrA* | 14.2 | 41.7 | 0.242 |
| 16 | 16S | 11.5 | 29.9 | 0.304 |
| | *tceA* | 41.3 | 32.8 | 0.743 |
| | *vcrA* | 6.3 | 33.1 | 0.174 |
| 40 | 16S | 6.0 | 38.0 | 0.014* |
| | *tceA* | 11.6 | 58.7 | 0.034* |
| | *vcrA* | 2.6 | 28.7 | 0.000* |

**Table S3.2.** One-way t-test of different gene abundances between acid and circumneutral pH ranges (df: degree of freedom; significance level: ** 0.01, * 0.05)

| **Gene** | **Average copies/L** | | **t value** | **df** | ***p*-value** |
|---|---|---|---|---|---|
| | pH 4.5~6.0 | pH 6.0~8.3 | | | |
| ***Dhc* 16S rRNA** | $7.49 \times 10^5$ | $1.14 \times 10^6$ | -2.44 | 67.4 | 0.009** |
| ***tceA*** | $1.11 \times 10^4$ | $4.15 \times 10^4$ | -2.19 | 121.8 | 0.015* |
| ***vcrA*** | $3.77 \times 10^4$ | $2.05 \times 10^5$ | -3.04 | 117.2 | 0.001** |
| **Total bacteria 16S rRNA** | $1.06 \times 10^7$ | $1.20 \times 10^7$ | -0.49 | 126.1 | 0.314 |

# CHAPTER IV GRAPE POMACE COMPOST AS A HABITAT FOR STRICTLY ORGANOHALIDE-RESPIRING *DEHALOGENIMONAS* SPECIES HARBORING NOVEL REDUCTIVE DEHALOGENASE GENES

A version of this chapter is going to be submitted for publication. Yang Y., S. Higgins, J. Yan, B. Şimşir, K. Chourey, R.L. Hettich, B. Baldwin, D.M. Ogles, F.E. Löffler. Grape Skin Compost as a Habitat for Strictly Organohalide-Respiring *Dehalogenimonas* Species Harboring Novel Reductive Dehalogenase Genes. In preparation.

## Abstract

Organohalide-respiring bacteria play key roles in the natural chlorine cycle; however, most of the current knowledge has been obtained from cultures derived from contaminated environments. We demonstrate that grape pomace, without prior exposure to chlorinated solvents, harbors a *Dehalogenimonas* (*Dhgm*) species capable of respiring chlorinated ethenes, including the human carcinogen and common groundwater pollutant vinyl chloride (VC). Grape pomace microcosms amended with lactate and tetrachloroethene (PCE) produced trichloroethene (TCE), *cis*-1,2-dichloroethene (*c*DCE), 1,1-dichloroethene (1,1-DCE), VC and ethene. Solid-free enrichment cultures dechlorinated TCE to ethene and 16S rRNA gene amplicon sequencing linked this activity to the presence of *Dhgm*. The enumeration of *Dhgm* 16S rRNA genes demonstrated VC-dependent growth, and $4.4 \pm 0.2 \times 10^8$ cells were produced per µmole of chloride released. Metagenome sequencing enabled the assembly of a *Dhgm* draft genome, and 52 putative reductive dehalogenase (RDase) genes were identified. Proteomics applied to biomass grown with TCE, *c*DCE, 1,1-DCE or VC as electron acceptors identified an RDase with 49% (34.9%) and 56.1% (42.1%) amino acid similarity (identity) to the known VC RDases VcrA and BvcA, respectively. A survey of 1,237 groundwater samples collected from 111 chlorinated solvent-contaminated sites

revealed quantifiable *Dhgm* and *Dehalococcoides mccartyi* (*Dhc*) 16S rRNA genes in 812 samples with a median *Dhgm*-to-*Dhc* ratio of 3.83. These findings demonstrate that non-polluted environments are a source of strictly organohalide-respiring bacteria with novel RDase genes, and that *Dhgm* are relevant contributors to chlorinated solvent reductive dechlorination in contaminated aquifers.

## Significance

Most of the current understanding about organohalide-respiring bacteria reflects cultures derived from environments impacted with anthropogenically-released chloroorganic compounds. We demonstrate that grape pomace never exposed to chlorinated solvents harbors strictly organohalide-respiring bacteria and is a reservoir for novel RDases, including an RDase that detoxifies the priority pollutant VC. To date, respiratory VC reductive dechlorination has been exclusively attributed to *Dehalococcoides* (*Dhc*) bacteria, and the finding that a broader bacterial diversity shares this phenotype has implications for environmental monitoring regimes and predictions about the fate of VC in contaminated aquifers. The discovery demonstrates that highly specialized organohalide-respiring bacteria contribute to the natural terrestrial chlorine cycle and emphasize their contributions to nutrient turnover.

## Introduction

Chlorinated hydrocarbons have been widely used in different areas of modern societies, such as cleaning of machinery, manufacturing, and agrochemicals (e.g. pesticides) (1).

Widespread usage and uncontrolled disposal of chlorinated hydrocarbons has caused environmental and human health concerns. For example, the widely used chlorinated solvent trichloroethene (TCE) has been implicated in increased risk of cancer (2) and Parkinson's disease (3). Vinyl chloride (VC), a TCE transformation product, is a notorious groundwater contaminant and a proven human carcinogen (4). TCE and VC are ranked #16 and #4 on the Substance Priority List (SPL) and have been detected in 1,153 and 593 superfund sites, respectively (www.atsdr.cdc.gov/spl/resources/index.html).

A landmark achievement was the discovery of organohalide-respiring bacteria, laying the foundation for *in situ* bioremediation (5). Diverse microorganisms, including members of the genera *Desulfitobacterium* (6), *Sulfurospirillum* (7), *Dehalobacter* (8), *Desulfuromonas* (9), *Geobacter* (10), and *Dehalococcoides* (11, 12), were isolated and demonstrated the ability to degrade PCE and TCE. Interestingly, the reductive dechlorination of chlorinated ethenes to non-toxic ethene has been attributed exclusively to *Dehalococcoides mccartyi* (*Dhc*) strains (13) and a few reductive dehalogenase (RDase) genes implicated in the detoxification of chlorinated ethenes have been identified (14). Consequently, contaminated site characterization, bioremediation monitoring, and decision-making rely on the quantitative assessment of *Dhc* biomarker genes in groundwater or aquifer solids. Although correlations between the presence and abundance of *Dhc* with the detoxification of chlorinated ethenes have been established, VC disappearance at sites lacking *Dhc* biomarkers has been observed (15). Moreover, the

presence/absence of *Dhc* biomarker genes does not always explain dechlorination activity and ethene formation (16, 17).

Information regarding microbial degradation of chlorinated ethenes has been almost exclusively derived from organisms obtained from environments impacted with contaminants. For obvious reasons, this approach was justified to derive process-relevant understanding; however, more recent discoveries demonstrated that chlorinated hydrocarbons, including priority pollutants, also have natural origins (18). For instance, even the human carcinogen VC can be generated abiotically in the soil environment, a process likely occurring since the first soils formed on Earth some 400 million years ago (19). Apparently, VC had been part of the biosphere long before human activities affected environmental concentrations of this carcinogen. A recent study correlated the abundance of *Dhc*-like *Chloroflexi* with the quantity of natural organohalogens in soils, supporting the notion that the organohalide-respiring phenotype is not merely a consequence of anthropogenic activities (20).

We observed PCE reductive dechlorination and ethene formation in microcosms established with grape pomace (GP) compost never exposed to chlorinated solvents. Characterization of the microcosm-derived enrichment culture GP demonstrated that the ability to grow with VC as electron acceptor is not limited to members of the *Dhc* genus. Thus, our study expands the current understanding of the diversity of bacteria capable of metabolizing VC under anoxic conditions, provides an explanation for ethene formation

in chlorinated solvent contaminated aquifers in the absence of *Dhc*, and demonstrates that agroecosystems harbor strictly organohalide-respiring bacteria, which use priority pollutants as electron acceptors and likely contribute to the chlorine-cycle in soil (21).

## Materials and Methods

**Chemicals.** PCE and TCE (both 99+% purity) were both purchased from ACROS ORGANICS (Distributed by VWR International, Inc., West Chester, PA, USA). *c*DCE (>96.0% purity), VC (≥99.5%) and ethene (≥99.5%) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). All other chemicals were of scientific grade or better and purchased from Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA).

**Microcosms and transfer cultures.** Reduced, bicarbonate-buffered mineral salts medium with 5 mM lactate was prepared following established protocols (61). Anoxic, filter-sterilized (0.22 µm) Wolin vitamin solution (62) was added after the medium had been autoclaved. Microcosms were established with grape pomace compost collected in the wine-growing area of Rotenberg near Stuttgart, Germany (Stuttgart Rotenberg). Following opening 20 mL vials containing 10 mL of medium inside the glove box (filled with $N_2$ and 3% $H_2$), samples (1 gram of wet solids) were quickly transferred to the vials using autoclaved stainless steel spatulas. The vials were sealed with black stoppers and crimped with aluminum caps. After removing the vials from the glove box, 1 µL neat

PCE was added to each vial with a Hamilton micro-syringe to reach an aqueous concentration of approximately 575 µM. Duplicate microcosms were incubated statically at room temperature in the dark. After the formation of VC and ethene, the vials were shaken by hand, and about 3 mL suspension was transferred with nitrogen-flushed 3-mL plastic syringes to 160 mL serum bottles containing 100 mL of fresh mineral salts medium amended with 5 mM sodium lactate, 10 mL hydrogen gas and 5 µL neat PCE (360 µM). The bottles were incubated at 30°C in the dark without agitation. Subsequent transfers yielded solid-free enrichment cultures, and in replicate bottles 2 mL VC (0.53 mM) replaced PCE as electron acceptor. To inhibit methanogenesis, 1.2 mM 2-bromoethanesulfonate (BES) was added. Prior to use, all plastic syringes were flushed with sterile, oxygen-free nitrogen to remove any residual air. Microcosms and transfer cultures were all set up in at least duplicate. Autoclaved microcosms and transfer cultures served as negative controls.

**DNA extraction and PCR procedures.** Microbial cells were collected from 2-mL culture suspensions by vacuum filtration onto 0.22 µm membrane filters (Millipore GVWP025000, EMD Millipore Corp., Billerica, Mass., USA). Trapped cells were broken up by bead beating at a speed of 3.25 m/s for 5 minutes at room temperature (Omni Bead Ruptor Homogenizer, Kennesaw, GA). Genomic DNA was extracted with the PowerSoil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA) following the manufacturer's manual. DNA quantity and purity were estimated with a NanoDrop 1000

(Thermo Fisher Scientific, NanoDrop Wilmington, DE). Genomic DNA samples from two replicate cultures were pooled for Illumina sequencing.

PCR assays to detect *Dhc* and *Dhgm* 16S rRNA genes, as well as the *tceA*, *bvcA* and *vcrA* genes, were performed following established procedures (63). DNA-free water and DNA samples from a PCE-to-*c*DCE-dechlorinating enrichment not containing *Dhc* and *Dhgm* populations served as negative controls. *Dhc* strain BAV1 genomic DNA and DNA samples from the PCE-to-ethene-dechlorinating consortium containing multiple *Dhc* strains (64) were used as positive controls.

Quantitative real-time PCR (qPCR) assays were performed using an Applied Biosystems ViiA™ 7 Real-Time PCR system. Assays targeting *Dhgm* and *Dhc* 16S rRNA genes enumerated *Dhgm* and *Dhc* cell numbers based on the observation that the known genomes harbor single copy 16S rRNA genes. Calibration curves (log-transformed gene copy numbers versus cycle threshold values) were obtained using 10-fold serial dilutions of plasmid DNA carrying either a cloned *Dhc* or *Dhgm* 16S rRNA gene. qPCR assay efficiency was calculated using the formula $E=10^{(-1/slope)}$. The efficiencies for all *Dhc*- and *Dhgm*-targeted qPCR assays were in the 90-110% range. The quantification limits for *Dhc* and *Dhgm* 16S rRNA genes were in the range of 30 gene copies per assay volume. The primers and probe for quantifying *Dhc* 16S rRNA genes were previously described (65). Quantification of *Dhgm* 16S rRNA genes used forward primer 5'-AGCAGCCGCGGTAATACG (Dhgm478F), reverse primer 5'-

CCACTTTACGCCCAATAAATCC (Dhgm536R), and probe 5'-AGGCGAGCGTTAT (Dhgm500Probe). Primers and probe were designed with the Realtime PCR tool of IDT and specificity was verified using NCBI primer design tool.

**16S rRNA amplicon sequencing and analysis**. DNA extracted from PCE-fed and VC-fed culture GP biomass was cleaned and concentrated using the Genomic DNA Clean and Concentrator Kit (Zymo Research, Irvine, CA, USA). Purified DNA samples were PCR-amplified using barcoded-primers F515/R806 targeting the V4 region. The amplicon sequencing approach followed established protocols (66, 67). Raw sequences were paired and analyzed using the mothur software package (http://www.mothur.org/) following MiSeq standard operating procedures (68). Paired sequences trimmed and filtered by mothur were uploaded to SILVA (https://www.arb-silva.de/ngs/) for verification and comparison.

**Metagenomic sequencing and analyses.** Shotgun sequencing was performed to investigate the genetic content of the VC-grown enrichment culture GP. The resultant sequencing run produced 15,059,934 paired-end reads (150 bp length), which were filtered using the software NGS QC Toolkit v2.3.3 (69) with a minimum phred score of 30 and a read cutoff length of 70%. The resulting 13,667,850 (90.7%) paired-end reads were then assembled following established procedures (70). Briefly, paired-end reads were assembled with De Bruijn graph assembler velvet v1.2.07 (71) and RAY v2.2.0 (72). Contigs representing the three best assemblies from each program (assembly quality

assessed by N50 value, maximum number of reads retained, and fewest number of contigs produced) were then fragmented by in-house scripts to 1,500 nucleotides in length and subjected to a final round of assembly with Newbler v2.6 (Roche Applied Science, Penzberg, Germany). The resulting assembly produced 4,925 contigs (≥500 nt). Average coverage of each contig was calculated by aligning quality-filtered reads to assembled contigs with Bowtie2 v2.1.0 (73) and multiplying the number of reads aligned to each contig by the average length of aligned reads and dividing by contig length. The assembled contigs were BLASTXed against non-redundant database of NCBI by DIAMOND (74) with a maximum e-value cutoff of $10^{-3}$. MEGAN (75) was used to analyze the functional assignment from the BLASTX output compared with the SEED database (76). To understand the differences between the *Dhgm*-dominant culture GP and *Dhc*-dominant dechlorinating cultures, comparative metagenomic analyses was conducted using MG-RAST pipelines following published methods (22, 77). 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 the 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 datasets from an acid mine drainage site (Richmond Mine, Iron Mountain, CA; MG-RAST ID: 4441137.3 and 4441138.3) and a pristine freshwater in Antarctica (Ace Lake; MG-RAST ID: 4443683.3) were chosen as non-dechlorinating communities for comparison. These two metagenomes were chosen for their well-documented meta information, good-quality reads, and their distinct environment sources. Metagenomic raw sequences were

classified into SEED categories using a maximum e-value of 1e-5, a minimum identity of 60%, and a minimum alignment length of 50 measured in aa for protein and bp for RNA by MR-RAST. Annotation results were then imported into STAMP for principle component analysis (PCA) and visualization (78).

**Genome binning and annotation.** Binning of metagenomic contigs was conducted with MetaWatt v1.7 (79) and VizBin (80), using GC content, tetranucleotide frequency, and coverage as quality metrics to assess consistency of contigs within the genomic bin (80). Contigs belonging to the *Dhgm* bin were further assessed with CheckM (23) using default settings to further assess genome bin completeness, contamination, and taxonomic affiliation. The draft genome bin was uploaded to RAST (Rapid Annotation using Subsystem Technology) (81) for annotation (Access ID: 1536648.4). RAST annotation results were validated by using additional annotation pipelines including Prokka (82). Sequence similarity and identity of different RDase genes identified within the coding sequences were calculated by EBI EMBOSS Needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/).

**Phylogenetic analyses.** Additional rRNA sequences from representative bacteria of the phylum *Chloroflexi* were retrieved from NCBI's RefSeq database (83) and rRNA genes were extracted with RNAmmer. The 5S, 16S, and 23S rRNA genes from each organism were individually aligned using mafft v7.130b (84) and subjected to optimal model estimation with jmodeltest v2.1.5 (85). Maximum likelihood tree estimation was

performed using PhyML v3.0 (86) on individual and concatenated rRNA gene alignments with 100 bootstrap replicates using the TN93 model (selected from jModelTest) (87) with estimations of the proportion of invariable sites and rate heterogeneity among sites (8 substitution rate categories). A phylogenetic tree including all RDase sequences annotated from the draft genome of *Dhgm* sp. strain GP and other *Dhc* and *Dhgm* genomes was built (88). Other RDase A protein sequences were searched and downloaded from the UniProt database. The phylogenetic tree was then imported into Interactive Tree of Life web browser (itol.embl.de) for enhancement and beautification (89).

**Proteomics analysis**. The microbial cells were harvested by passing the culture suspension through 0.22 µm membranes filters (Millipore GVWP025000, EMD Millipore Corp., Billerica, Mass., USA). The filters were cut into small pieces ~ 1 cm in size) and proteins were extracted following established procedures (90, 91). Amounts of extracted protein was calculated using the RC/DC protein estimation kit (Bio-Rad Laboratories, Hercules, CA, USA) as per the manufacturer's instructions. Bovine serum albumin (supplied with the kit) was used as standard for the assay. Protein digestion was initiated by the addition of trypsin to the sample (40 µg trypsin/ 1-3 mg protein), resulting peptides desalted and solvent exchanged as described (92). The peptides were stored at -80 until MS analysis. Peptides (~75 µg) were loaded onto an in-house prepared resin packed SCX (Luna, Phenomenex, Torrance, CA) and C18 (Aqua, Phenomenex, Torrance, CA) columns and subjected to an offline wash as described (93). The biphasic

column was connected to a 10 cm C18-packed nanospray tip (New Objective, Woburn, MA) aligned to an Proxeon (Odense, Denmark) nanospray source (93). Peptides were subjected to 24-hour, 11-step chromatographic separation and measurements using the Multi-Dimensional Protein Identification Technology (MuDPIT) approach (92-94). Measurements were carried out using LTQ Velos mass spectrometer (Thermo Fisher Scientific, Germany) coupled to the Ultimate 3000 HPLC system (Dionex, USA) and operated in data dependent mode regimented by Thermo Xcalibur software V2.1.0. Each full scan was followed by fragmentation via collision-activated dissociation (CID) using 35% collision energy of 20 most abundant parent ions with a mass exclusion width of 0.2 m/z and dynamic exclusion duration of 60 seconds. For protein identifications, the raw spectra were searched against selected databases (CDs annotated from draft *Dhgm* genome) via Myrimatch v2.1 algorithm (95) set to parameters described by (96) with minor modifications. Static cysteine and dynamic oxidation modifications were not considered and identification of at least two peptides per protein (one unique and one non-unique) sequence was a prerequisite for protein identifications. Common contaminant peptide sequences from trypsin and keratin were concatenated to the database. Spectral counts of identified peptides were normalized as described (97) to obtain the normalized spectral abundance factor (NSAF), also referred to as normalized spectral counts (nSpc). Average nSpc values from duplicate runs were used to get the final proteome profile of the sample.

**Analytical methods**. Chlorinated solvents were measured in 100 µL headspace gas samples on a gas chromatograph (GC) (Agilent Technologies, Santa Clara, CA, USA).

The concentrations of chlorinated ethenes were calculated by normalizing the peak areas to standard curves generated by adding known amounts of chlorinated ethenes and ethene into the bottles with the same gas to liquid ratios. Gas samples (100 µL) were removed from the headspace using a gastight 250 µL Hamilton SampleLock syringe and then manually injected into the GC. Samples were measured with an Agilent 7890A GC equipped with an Agilent DB624 column with a flame ionization detector (FID). The retention times were determined by injecting neat compounds into the GC. The total amounts of PCE, TCE, *c*DCE and 1,1-DCE were calculated using the equation: Mole mass of chlorinated solvent = (volume of chlorinated solvent) x (density of chlorinated solvent) / (molecular weight of chlorinated solvent). The total moles of VC and ethene were calculated by applying the ideal gas law (PV=nRT). The concentrations of chlorinated compounds and ethene in the aqueous phase were calculated using the equation: $C_{liquid} = \frac{total\ molar\ mass}{V_{liquid}+H_{cc}\times V_{gas}}$. The dimensionless Henry's constants for PCE, TCE, cDCE, 1,1-DCE, VC and ethene at 21°C were 0.576, 0.308, 0.133, 0.922, 0.933 and 9.222, respectively (https://www3.epa.gov/ceampubl/learn2model/part-two/onsite/esthenry.html).

## Results

**Reductive dechlorination of chlorinated ethenes in grape pomace microcosms and transfer cultures.** In anoxic grape pomace microcosms, PCE was reductively dechlorinated to ethene via TCE, *c*DCE, 1,1-DCE and VC as intermediates after a 300-day incubation period. Transfer cultures also produced TCE, *c*DCE, 1,1-DCE, and VC as

dechlorination daughter products and ethene as end product (Figure 4.1). Following the addition of BES, an inhibitor of methanogenesis, *c*DCE was the dechlorination end product and VC and ethene were not formed (data not shown). Without BES addition, the transfer cultures maintained the ability to produce ethene in completely synthetic, defined medium.

**Community structure of ethene-producing enrichment cultures.** To identify the population(s) responsible for the observed dechlorination activity, DNA was extracted from ethene-producing PCE- and VC-fed cultures for 16S rRNA gene amplicon profiling. No evidence for the presence of *Dhc* 16S rRNA gene sequences was obtained, a finding supported by PCR-based analyses, which failed to detect *Dhc* 16S rRNA genes and the *tceA*, *bvcA*, and *vcrA* reductive dehalogenase genes implicated in dechlorination of chlorinated ethenes. Instead, *Dhgm* 16S rRNA gene amplicons 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 4.2). Also detected were sequences of not-yet-cultured bacteria of the WWE1 and WPS-2 candidate divisions, which contributed 3.6% and 8.3%, respectively, in the PCE-fed cultures and 2.6% and 9.4%, respectively, in the VC-fed cultures.

**Growth of *Dehalogenimonas* coupled with VC to ethene reductive dechlorination.** VC dechlorination to ethene commenced after a lag phase of about 20 days, and transfer cultures provided with VC (83.3 µmol/bottle) as electron acceptor produced

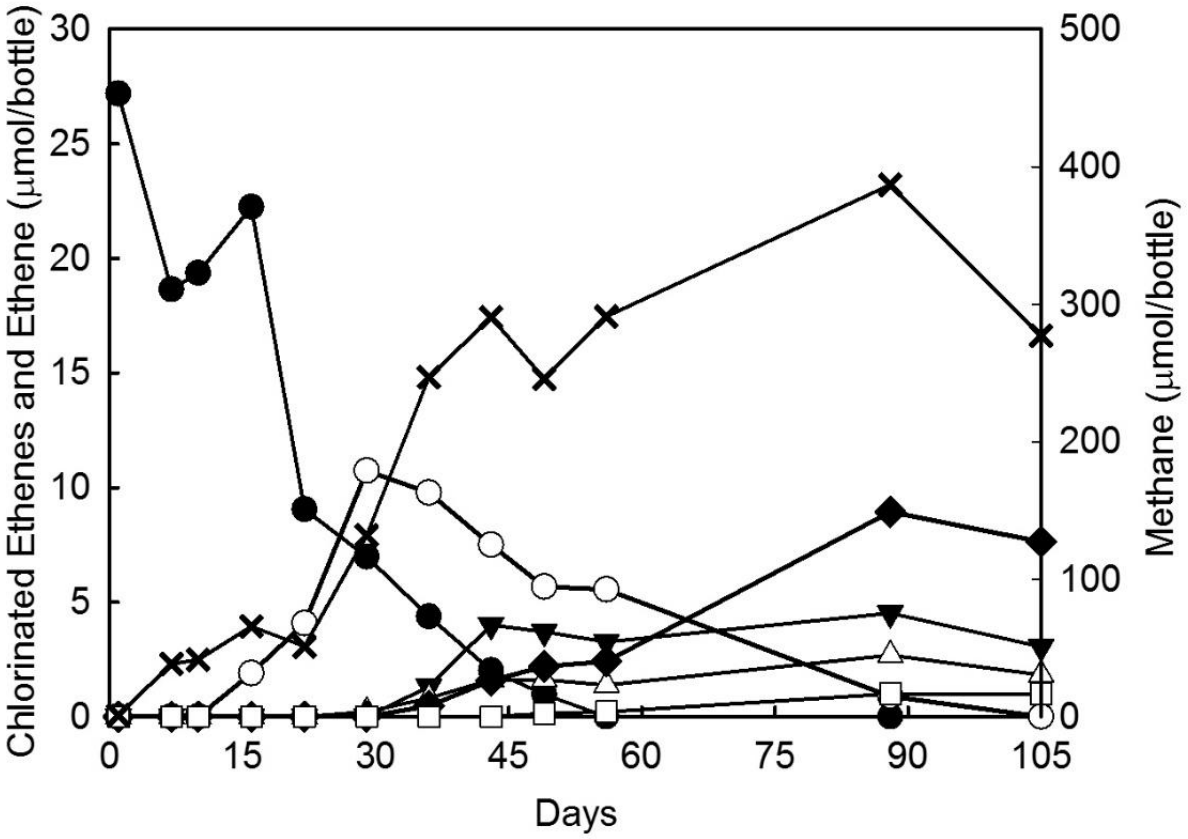


**Figure 4.1** PCE enrichment from Grape Pomace without inhibiting methanogenesis. Dechlorination activity was not optimized under current cultivating condition. The culture demonstrated the potential of PCE degradation to innocuous ethene. (Filled circle-PCE; Open circle-TCE; Filled inverse triangle-cDCE; Open triangle -11DCE; Filled diamond-VC; Open square-ethene; Cross-Methane). Data points represent one of the duplicate dechlorinating cultures; both cultures followed the same dechlorination pattern with time difference.

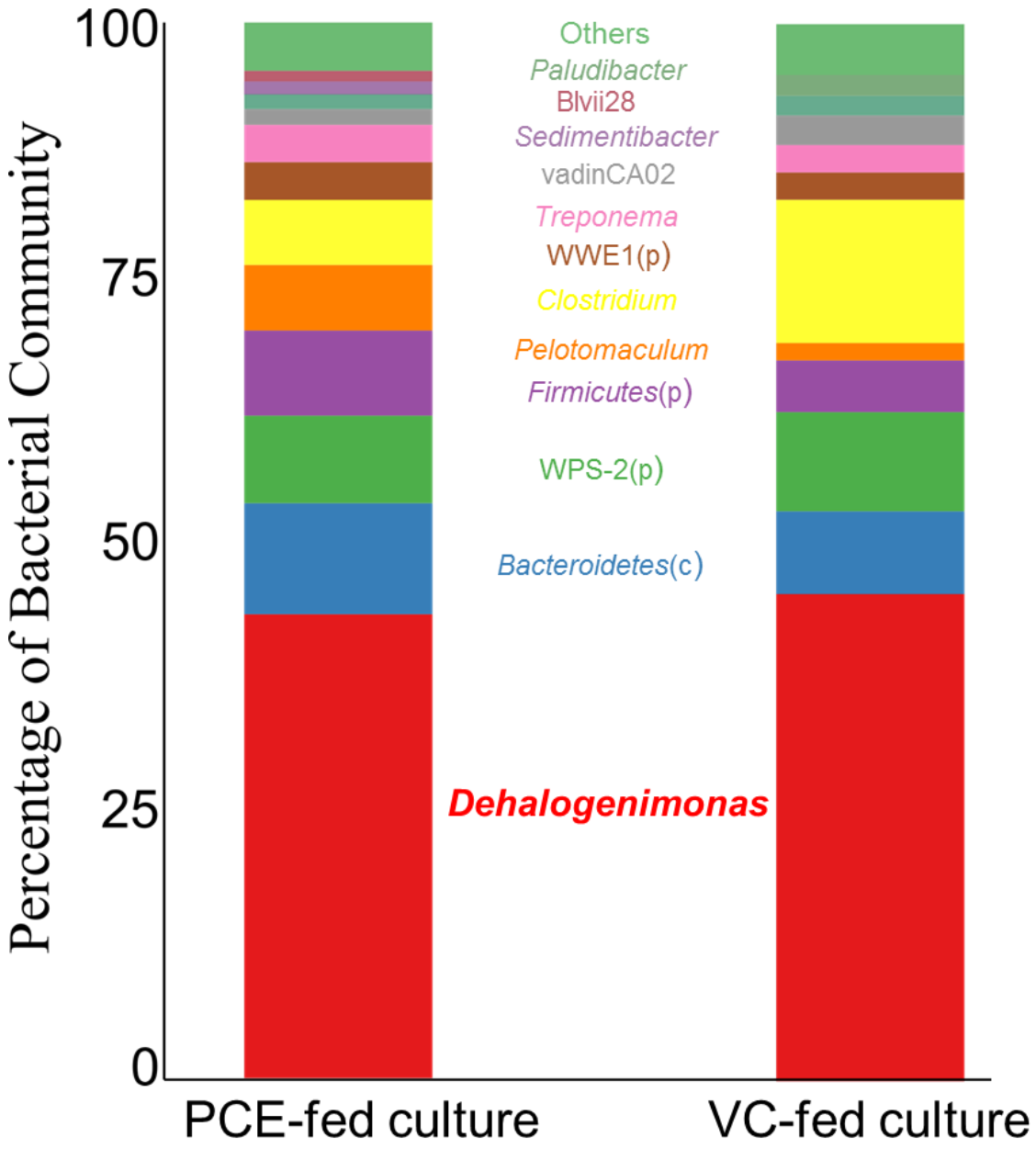


**Figure 4.2** Relative abundance of genera in PCE-fed and VC-fed cultures GP as revealed by 16S rRNA gene amplicon sequencing. Rare groups (less than 1% of total community) were classified into "Others". The Bacteroidetes (uncl) and Firmicutes (uncl) represented the phylum level (uncl stands for unclassified).

stoichiometric amounts of ethene within a 50-day incubation period (Figure 4.3). Quantitative real-time PCR (qPCR) results demonstrated that the *Dhgm* 16S rRNA gene copy numbers per mL increased from 1.02 ±0.12 x $10^7$ (cells transferred with the inoculum) to 3.76 ±0.14 x $10^8$ (a 37-fold increase) following complete VC degradation.

Following seven repeated transfers with VC as electron acceptor, culture GP maintained the ability to dechlorinate TCE, 1,1-DCE and *c*DCE, but failed to dechlorinate PCE, suggesting that the VC-dechlorinating population can also dechlorinate polychlorinated ethenes but not PCE (Figure S4.2). The growth yields of *Dhgm* strain GP with TCE, 1,1-DCE, *c*DCE, or VC provided as electron acceptor ranged from 5.9 ±1.5 to 8.6 ±0.1 x$10^8$ per µmol of $Cl^-$ released, which were up to 2 orders of magnitude higher compared to the growth yields reported for *Dhgm lykanthroporepellens* strain BL-DC-9 and in the range reported for *Dhc* strains (Table 4.1). Culture GP could not dechlorinate carbon tetrachloride, 1,2-dichloroethane (1,2-DCA), 1,2,3,-trichloropropane (1,2,3-TCP) and 1,2-dichloropropane(1,2-DCP).

**Comparative metagenomic analysis.** To further characterize the dechlorinating culture, metagenome sequencing of DNA derived from VC-grown biomass was performed. More than 50% of the coding sequences from assembled contigs could not be assigned to a SEED (www.theseed.org) functional group, indicating the presence of many genes with unknown functions in dechlorinating culture GP. Among assigned SEED functional categories, genes encoding the metabolisms of carbohydrates, amino acids and

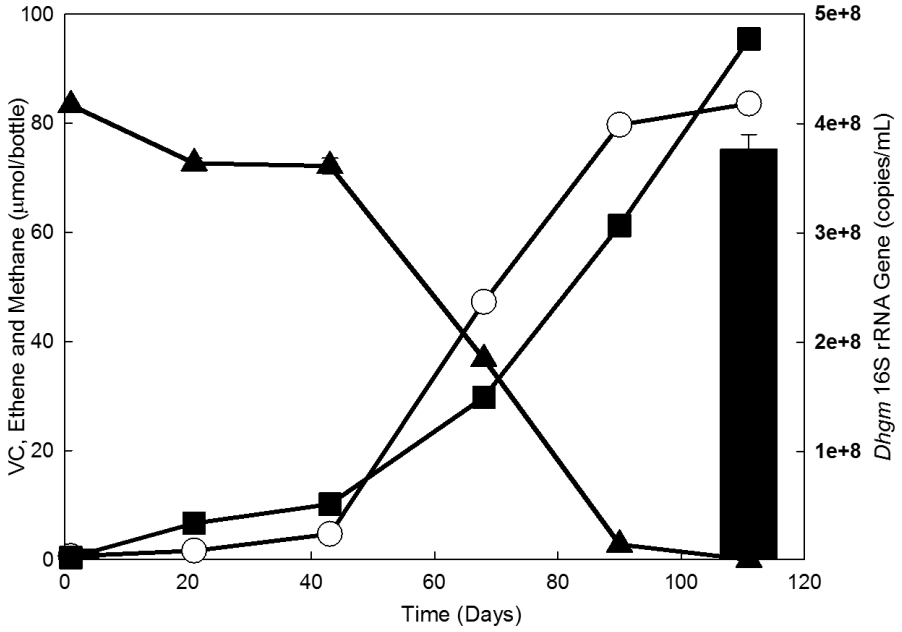


**Figure 4.3** VC degradation by culture GP (Fill triangle-VC; open circle-ethene; filled square-methane; filled bar- *Dhgm* 16S rRNA gene copy number). Data points are average of duplicate cultures; the error bars are one standard deviation, which are masked by the data symbols.

**Table 4.1** Comparison of doubling times and growth yields between *Dhc* and *Dhgm* strains and culture GP

| Genus | Strain | Electron Acceptor | Doubling Time (Days) | Yield per µmol $Cl^-$ released |
|---|---|---|---|---|
| *Dehalococcoides* | BAV1 | VC | 2.2 | 6.30E+07 |
| | GT | VC | 2-2.5 | 2.50E+08 |
| | VS | VC | 1.7‡ | 5.20E+08 |
| *Dehalogenimonas* | BL-DC-9 | 1,2,3-TCP | 4.1 | 2.90E+06 |
| | | 1,2-DCP | ND | 1.50E+07 |
| | GP | TCE | 6.1 | 5.55E+08 |
| | | 1,1-DCE | 5.9 | 8.62E+08 |
| | | *c*DCE | 10.0 | 3.61E+08 |
| | | VC | 8.4 | 6.55E+08 |

‡Determined in a highly enriched mixed culture. ◊Data from Löffler *et al.*, 2013

derivatives, proteins, DNA, and cofactors/vitamins/prosthetic groups/pigments were highly represented in the assembled reads (Table S4.2). Tetrapyrrole, folate and pterine biosynthesis were dominant subgroups in the SEED cofactors/vitamins category. Aside from reductive dehalogenase genes, functional genes related to electron-accepting processes (e.g., tetrathionate respiration, trimethylamine-N-oxide (TMAO) reductase, dimethyl sulfoxide (DMSO) reductase, sulfite reductase, arsenate reductase) were present in the VC-dechlorinating culture GP (Table S4.2). Genes encoding dehydrogenases (e.g., formate dehydrogenase, NADH dehydrogenase, L-lactate dehydrogenase, succinate dehydrogenase, and carbon monoxide dehydrogenase) and hydrogenases (e.g. [Ni/Fe] hydrogenase, periplasmic [Fe] hydrogenase), which associated with electron transport systems, were abundant in culture GP. Metagenome sequence information is available for three *Dhc*-containing consortia capable of dechlorinating chlorinated ethenes to ethene (ANAS, KB-1 and Donna ll) (22), and comparative analysis focused on taxonomic and functional genes was conducted. Included in the analysis were two metagenomes representing non-dechlorinating communities (i.e., acid mine drainage and Antarctic freshwater sample). The taxonomic comparison between dechlorinating and non-dechlorinating communities suggested differences at the phylum level among six communities were distinct (Figure 4.4A); but functional analysis indicated dechlorinating communities were more similar to each other at functional levels than to non-dechlorinating communities (Figure 4.4B).

**Draft genome of the *Dhgm* strain GP.** Binning of the metagenome sequences allowed the assembly of 16 contigs ranging in size between 1.0 kbp and 6.7 kbp (N50 = 2.3 kbp),

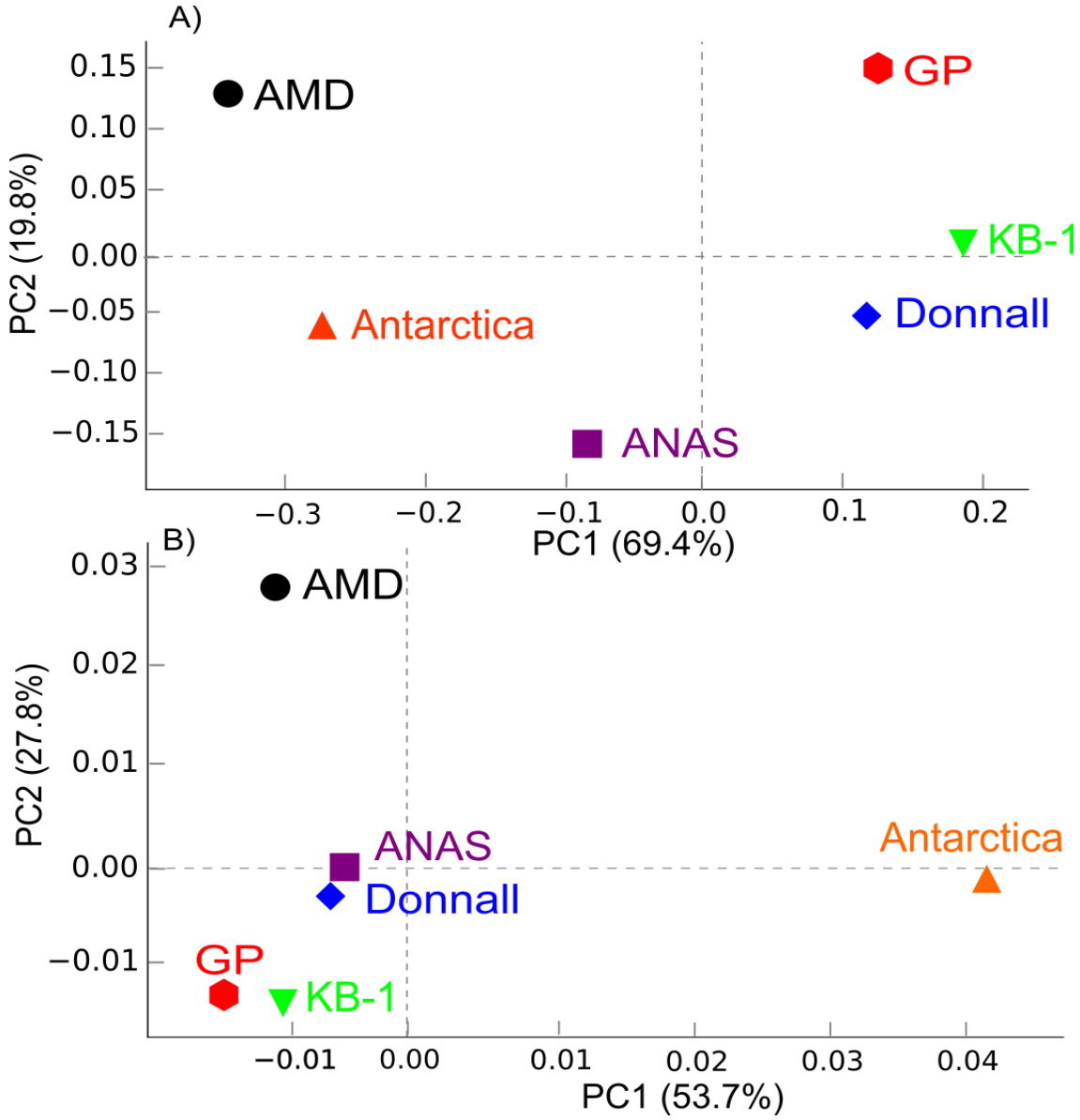


**Figure 4.4** Principal component analysis of taxonomic (A) and functional (B) profiles of six metagenomes. Dechlorinating (ANAS, DonnaII, KB-1 and GP) and non-dechlorinating (AMD and Antarctica) communities were compared at phylum level (A). Metagenomic sequences of previous six communities were classified into SEED categories, and the distribution of SEED categories were compared (B).

and a draft genome of the organohalide-respiring *Dhgm* strain GP was obtained. The draft genome had a size is 2.02 Mbp with a G+C content of 52%. CheckM analysis (23) 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 averagegenome 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 *Dhgm* strain GP shares 96.0% and 95.3% sequence identities with *Dhgm* sp. strain WBC-2 and *Dhgm lykanthroporepellens* strain BL-DC-9, respectively. Phylogenetic analysis based on concatenated 5S-16S-23S rRNA gene alignments supported affiliation with the *Dhgm* genus (Figure 4.5). A characteristic feature of obligate organohalide-respiring bacteria is the presence of multiple hydrogenase genes, and gene clusters encoding a [Ni/Fe] hydrogenase complex ( EC 1.12.2.1), an 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 sequenced *Dhgm* genomes, three genes encoding the major subunits of formate dehydrogenase (EC 1.2.1.2) were identified, whereas *Dhc* genomes harbor only one copy of the respective gene. On the contrary, *Dhc* and *Dhgm* could not utilize formate (13, 24), and it was speculated that formate dehydrogenase(s) in *Dhgm* and *Dhc* may in fact function as hydrogenase(s). Phylogenetic analysis of putative

formate dehydrogenases annotated from *Dhgm* and *Dhc* genomes and several characterized formate dehydrogenases genes suggested *Dhgm* may possess two types of formate hydrogenases, while only one type of formate hydrogenase possessed by *Dhc* (Figure S4.2).

A total of 52 putative RDase genes were identified, 10 of which were associated with B genes, which encode B proteins with 1 to 4 trans-membrane spanning helices (Table S3). All putative RDase protein sequences had either TAT signal peptide or sec signal peptide predicted by PRED-TAT (Table S3) (25). 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 (26), suggesting the *Dhgm* strain GP has the potential to dechlorinate polychlorinated biphenyls (PCBs). Although *c*-type cytochrome genes were not found, two *c*-type cytochrome biogenesis genes, *ccsA* and *ccsB*, were present. Both *c*-type cytochrome biogenesis genes were also present on other sequenced *Dhgm* and *Dhc* genomes but their functions remain unclear (27). Genes for *de novo* corrin ring biosynthesis were absent, 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 possessed genes encoding two distinct cobinamide (Cbi-)-salvaging pathways: the bacterial pathway relying on *cobU/cobP* genes and the archaeal pathway with the *cbiZ* gene. Moreover, genes coding for the vitamin $B_{12}$ ABC transporter *BtuFCD* and the dual-functional cobalt/nickel transporter system *cbiMNQO* were also present. Heterodisulfide reductase (HdrABC) was proposed to be involved in different electron bifurcation systems (e.g., HdrABC-

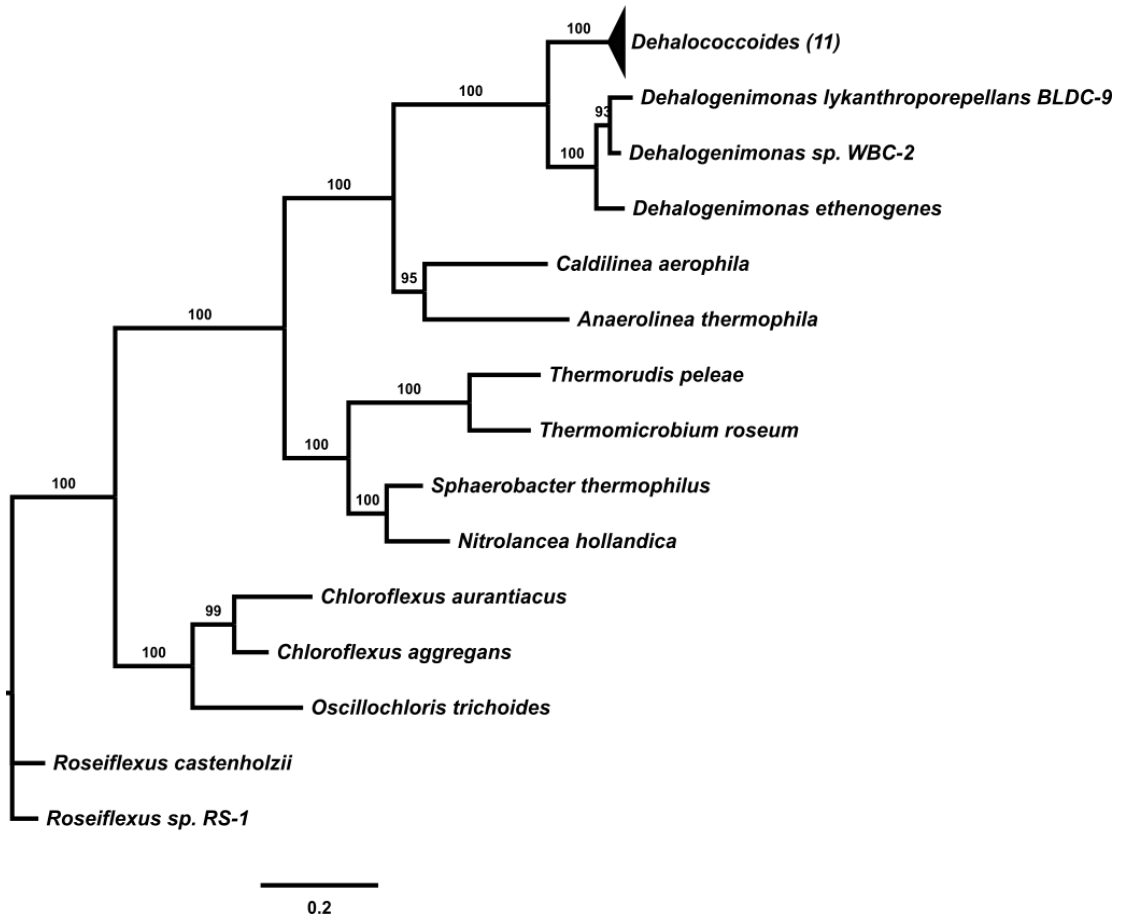


**Figure 4.5** Phylogenetic tree based on concatenated 5S-16S-23S rRNA genes. "*Dehalogenimonas ethenogenes*" was clustered with *Dhgm* strain BL-DC-9 and WBC-2.

MvhADG, HdrABC-FlxABCD). In these electron bifurcation systems, HdrABC complex was responsible for splitting electrons (from hydrogen or NADH) to oxidized ferredoxin and CoM-S-S-CoB heterodisulfide (28).Genes encoding subunits of the heterodisulfide reductase (HdrABC) were annotated in the strain GP draft genome, which were also present in the other two available *Dhgm* genomes (strain WBC-2 and strain BL-DC-9) but absent in all sequenced *Dhc* genomes, suggesting *Dhgm* and *Dhc* may employ different electron transfer proteins. It is also worth mentioning that the *Dhgm* draft genome encodes the arsenic resistance genes *arsA*, *arsD* and *arc3* in a single operon

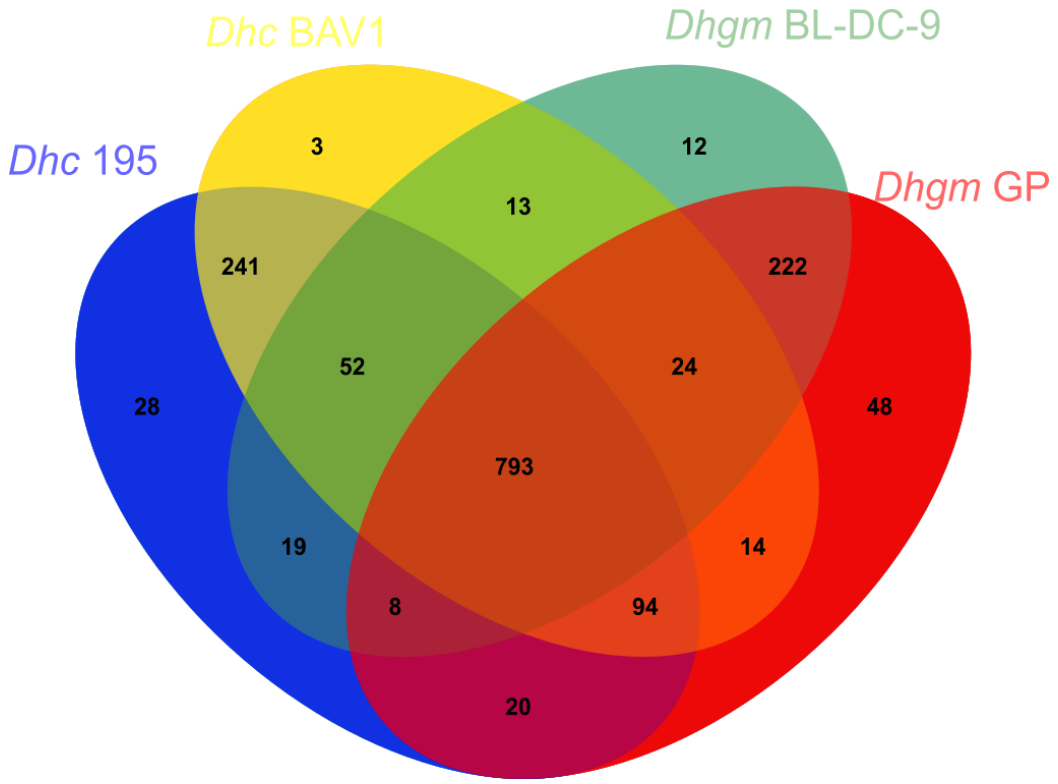


**Figure 4.6** Orthologous clusters from two *Dhc* genomes (strains 195, BAV1) and two *Dhgm* genomes (strains BL-DC-9, GP). From the genomes of 195, BAV1, BL-DC-9 and GP, a total of 1580, 1371, 1659 and 2036 coding sequences were annotated and used in this comparison, respectively. These sequences were compared and clustered using markov cluster algorithm with e-value1e-5 and inflation value 1.5.

suggesting *Dhgm* GP was under arsenic selection pressure and is capable of detoxifying arsenicals. The comparative analysis of whole genome coding sequences (CDs) between the *Dhgm* strain BL-DC-9 and strain GP genomes and the genomes of *Dhc* strains 195 and BAV1 identified a total of 1591 orthologous gene clusters, and 1500 gene clusters were shared by at least by two genomes. *Dhgm* strain GP shared 222, 20 and 14 orthologous clusters with strains BL-DC-9, 195, and BAV1, respectively (Figure 4.6).

**Protein profiling and identification of a novel putative VC RDase.** *Dhgm* strain GP grew with VC as electron acceptor but, consistent with the draft genome sequence, qPCR

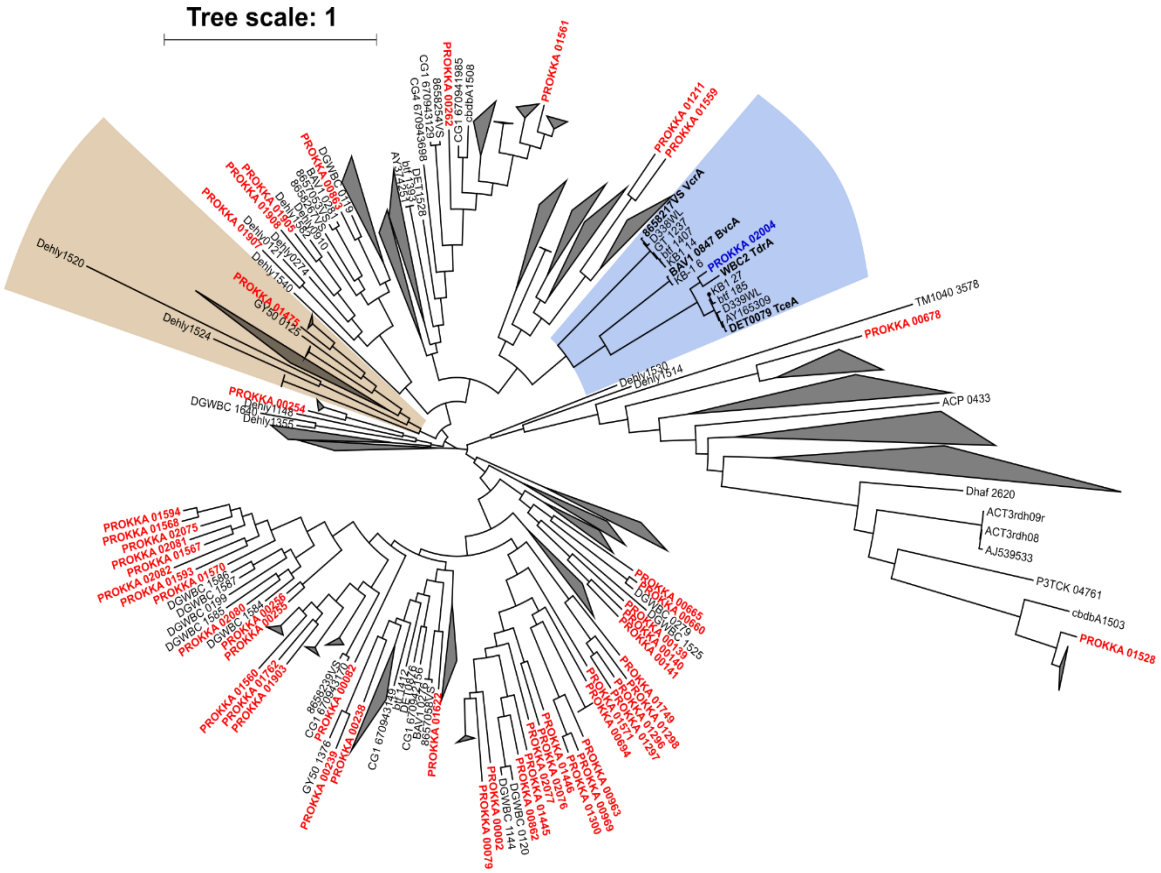


**Figure 4.7** Phylogenetic tree of total 528 reductive dehalogenases (355 sequences were collected by Hug *et. al*. (58); the rest were annotated and collected from genomes of *Dhc* strains CG1, CG4, CG5 and *Dhgm* strains WBC-2, GP, SG1). The phylogenetic tree was built by Geneious software using default settings of MAFFT Alignment and Geneious Tree Builder. (yellowish brown-"PcbA" group; light blue-"Bvc/Vcr/TceA" group; 51 RDase of *Dhgm* strain GP were labeled in red; CerA RDase of *Dhgm* was in blue).

assays failed to detect the known VC RDase genes *tceA*, *vcrA* and *bvcA* suggesting strain GP harbors a different (novel) VC RDase gene. One of the RDases encoded on the *Dhgm* genome (prokka_02004) grouped in a cluster comprising the characterized RDases TceA, BvcA and VcrA (Figure 4.7), suggesting the prokka_02004 RDase may have activity towards chlorinated ethenes. This *Dhgm* RDase shared 83.3%, 56.1% and 49% amino acid similarity (76.2%, 42.1, and 34.9% amino acid identity) with TceA, BvcA and VcrA, respectively. To verify if the prokka_02004 RDase is responsible for the observed VC-to-

ethene reductive dechlorination activity, proteins extracted from culture GP biomass grown with TCE, 1,1-DCE, *c*DCE, and VC as electron acceptor were analyzed using proteomic workflows. Normalized spectral counts indicated high expression of the prokka_02004 RDase in cells collected from 1,1-DCE-, cDCE-, and VC-grown GP cultures (Table S4.1). Based on the phylogenetic and proteomics analysis, the prokka_02004 RDase gene is a likely candidate encoding the VC RDase and was designated *cerA* (i.e., chloroethene reductase gene). In addition to CerA, the proteomics analysis identified three additional RDases (PROKKA 01300, PROKKA 01297 and PROKKA 00862), albeit in relatively lower abundance (Figure 4.8, Table S4.3). The examination of expressed *Dhgm* proteins during active dechlorination of different chlorinated ethenes revealed high abundance of chaperonin proteins GroES, GroEL and Hsp20 (Table S4.1). High expression levels of rubrerythrin and thioredoxin suggested that the cells had to cope with oxidative stress either caused by their own metabolic activity or caused by the cultivation conditions. Glyoxalase, responsible for detoxifying reactive aldehydes, was expressed in strain GP (Table S4.1). Although formate is not known to be metabolized by the available *Dhgm* isolates, it was observed that formate dehydrogenase were expressed abundantly by *Dhgm* strain GP.

**Detection and abundance of *Dhgm* at sites impacted with chlorinated solvents.** For contaminated site assessment, monitoring, and treatment decision-making, the quantitative measurement of *Dhc* biomarker genes has become routine practice. A survey of samples collected from 1,237 groundwater wells from 111 sites impacted with

chlorinated solvents revealed *Dhc* and *Dhgm* 16S rRNA genes in 957 (77%) and 964 (78%) locations, respectively. At 954 wells, *Dhgm* 16S rRNA gene copy number exceeded $1x10^4$ $L^{-1}$, and in these wells, the average *Dhgm*-to-*Dhc* ratio was 3.8 (Table 4.2). These findings strongly suggest that *Dhgm* contribute to reductive dechlorination activity in contaminated aquifers.

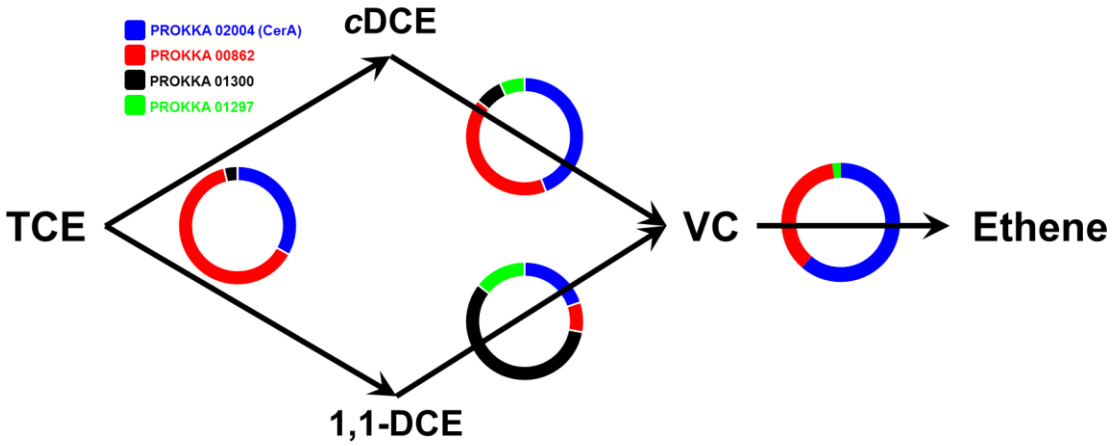


**Figure 4.8** Relative abundances (based on normalized spectral counts) of RDase-A proteins detected in GP cultures grown with TCE, cDCE, 1,1-DCE, and VC as electron acceptors.

**Description of *Dehalogenimonas ethenoformans* sp. nov.** *Dehalogenimonas ethenoformans* (e.the.no.for'mans. N.L. n. ethenum, ethene; N.L. pref. *etheno*-, pertaining to ethene; L.v. formo to establish, produce; N.L. gen. masc. n. *ethenoformans*, ethene-producing bacteria), named to emphasize the organism's ability to form ethene form chlorinated ethenes. *Dehalogenimonas ethenoformans* utilizes TCE, *c*DCE, 1,1-DCE, and VC as respiratory electron acceptors. The organism uses hydrogen as electron donor, and, acetate and/or lactate as carbon sources. Growth occurs at 20-30°C and pH 7.2. The G +

C content of strain GP is 52 mol%. Strain GP was present in a mixed culture derived from non-contaminated grape pomace collected from the wine-growing region of Rotenberg near Stuttgart, Germany. Phylogenetic, genotypic and phenotypic characteristics place strain GP in the *Dehalogenimonas* genus within the organohalide-respiring *Chloroflexi*, and warrant classifying strain GP as the type strain of a new species, *Dehalogenimonas ethenoformans* sp. nov..

**Table 4.2** *Dhc* and *Dhgm* 16S rRNA genes detected in groundwater collected wells at sites impacted with chlorinated solvents.

| **16S rRNA Gene Copies per Liter Groundwater (cells/L)** | **# of Wells with** | | ***Dhgm*-to-*Dhc* Detection Frequency Ratio**[◊] |
|---|---|---|---|
| | ***Dhc*** | ***Dhgm*** | |
| $>10^3$ | 21 | 10 | 0.48 |
| $>10^4$ | 17 | 51 | 3.0 |
| $>10^5$ | 18 | 110 | 6.1 |
| $>10^6$ | 21 | 239 | 11.38 |
| $>10^7$ | 880 | 554 | 0.63 |

[◊]**The *Dhgm*-to-*Dhc* Detection Frequency Ratio** was calculated according to

$$\frac{\#\text{ of } Dhgm\text{−positive wells with } Dhgm}{\#\text{ of Dhc−positive wells with } Dhc}$$

## Discussion

To date, metabolic VC-to-ethene reductive dechlorination has been exclusively linked to the presence and activity of *Dhc* strains carrying the VC RDase genes *vcrA* or *bvcA*. Here

we report metabolic VC reductive dechlorination in the absence of *Dhc* and the known VC RDase genes *vcrA* or *bvcA* in enrichment culture GP. The dechlorinating organism, strain GP, affiliated with the genus *Dehalogenimonas* and coupled the reductive dechlorination of TCE, *c*DCE, 1,1-DCE and VC with growth, producing biomass and environmentally benign ethene as products. Culture GP was obtained from grape pomace composted in the wine-growing area of Rotenberg near Stuttgart, Germany. Fungicides containing halogenated hydrocarbons are applied to the grape vine foliage during the growing season (April-August); however, none of these compounds are associated with the grapes at the time of harvest (September-October) and therefore not present in grape pomace. This raises the question why an organism whose energy metabolism hinges on the presence of certain organohalogens is found in grape pomace that has never encountered chlorinated solvents. There is ample evidence for the natural formation of organohalogens in soil, including the human carcinogen VC (19). A plausible explanation is active production of organohalogens in soils, possibly including grape pomace, which support organohalide-respiring *Chloroflexi* such as *Dhgm* strain GP. The presence of 16S rRNA gene sequences associated with the organohalide-respiring *Chloroflexi* in pristine grassland and forest soils has been linked to the soils' organochlorine content (20). Our findings provide additional support that pristine environments harbor specialized organohalide-respiring bacteria that use priority contaminants, including chlorinated ethenes, as electron acceptors. Further, strain GP harbors a novel VC RDase gene (*cerA*) that has so far not been reported at contaminated sites, demonstrating that a broader

diversity of VC RDases exists. Thus, the search for organisms and genes with potential applications in bioremediation should not be limited to contaminated environments. *Dhgm* and *Dhc* are both obligate organohalide-respiring bacteria that characteristically carry multiple RDase genes on their genomes. For example, 19, 32, 36, 11 and 19 RDase genes were identified on the genomes of *Dhc* strains 195, CBDB1, VS, BAV1 and *Dhgm* strain BL-DC-9, respectively (29), suggesting that the utilization of a broader suite of organohalogens as electron acceptors is a common feature. *Dhgm* strain GP possesses an RDase (prokka_01475), which shared more than 65% amino acids identity with PCB RDases PcbA-CG4 and PcbA-CG5 of *Dhc* strains CG4 and CG5, respectively. *Dhgm* 16S rRNA gene sequences have been detected in PCB-dechlorinating enrichment cultures (30) and PCB-impacted marine sediment (31), and it is likely that prokka_01475 represent a novel PCB RDase. Strain GP carries a staggering number of 52 RDase genes on its genome, a possible adaptation to the non-contaminated soil environment, from where the culture was obtained. For survival in pristine environments, obligate organohalide-respiring bacteria must rely on naturally produced organohalogens, and very likely must use a diversity of halogenated compounds to derive sufficient energy for cell maintenance and growth. Evidence is accumulating that chlorinated hydrocarbons, including priority pollutants such as VC, are produced naturally in many environments, including soils (19, 32). This is an important observation suggesting that RDases that use priority pollutants (e.g., VC) as substrates evolve in environments without anthropogenic chlorinated solvent contamination. A survey detected *Dhc*-like *Chloroflexi* 16S rRNA gene fragments in nearly 90% of the investigated 116 soil samples collected from

locations not impacted by anthropogenic chlorinated hydrocarbons (20). These findings are consistent with the hypothesis that organohalide-respiring bacteria evolved long before human activities released chlorinated chemicals into the environment. It is plausible that this bacterial metabolism evolved in response to soil processes generating organohalogens, which could have started when soils first formed in the late Silurian to Early Devonian some 400 million years ago (19, 33, 34). Microorganisms control the turnover of chlorine from organic compounds to inorganic chloride in environmental systems, and thus affect estimates of the chlorine budget (35). Efforts to enrich and isolate organohalide-respiring bacteria from pristine environments can help elucidating the biogeochemical cycling and turnover of organochlorine, and also be a source of novel organisms and RDase genes with value for biotechnological applications.

The characterized *Dhgm* cultures show preference for chlorinated ethanes as electron acceptors (36, 37). Enrichment efforts with chlorinated ethenes from contaminated aquifer materials generally yield *Dhc*- rather than *Dhgm*-containing cultures. A possible reason is the slower growth of *Dhgm* compared to *Dhc* (Table 1) and *Dhc* out-compete *Dhgm* strains. The growth conditions (e.g., medium composition) have not been refined to meet the nutritional requirements of chlorinated ethene-dechlorinating *Dhgm*, an issue that has also limited the initial experimental efforts with *Dhc* cultures (13). For example, it was recently demonstrated that the lower base of the essential RDase corrinoid prosthetic group can affect reductive dechlorination rates and extents (45, 46), and the

exact cobamide requirement to support efficient CerA maturation and maximum catalytic activity has not been elucidated.

BES, a competitive inhibitor of coenzyme M (2-mercaptoethanesulfonate), a key cofactor in the final step of methane formation, inhibited *c*DCE and VC reductive dechlorination in culture GP. This is not unprecedented and BES has been demonstrated to inhibit organohalide-respiring *Chloroflexi* and reductive dechlorination beyond *c*DCE (38). The mechanistic underpinning of this inhibition is not understood but could have a nutritional basis. For example, methanogens in culture GP (i.e., *Methanocorpusculum* spp.) could be required to supply an essential cobamide that corrinoid auxotrophic *Dhgm* require to assemble functional RDases. BES did not affect PCE-to-*c*DCE dechlorination indicating that the observed inhibitory effect had some specificity towards the *Dhgm* population.

Attempts to assemble nucleotide sequences derived from mixed communities can produce artifacts, but improved binning methodologies identify chimeric sequences and robustly delineate distinct microbial populations from metagenomes (23, 39). Previous *in silico* investigations of genome binning from community metagenomes suggested that a coverage of at least 20X is required for binning and draft genome assembly (40). Coverages of contigs from *Dhgm* strain GP were on average an order of magnitude above this 20X cutoff. Only the shortest contig possessed a lower coverage, but BLASTn alignment of this 3810-bp contig to NCBI's nt database revealed 96% nucleotide identity to the genome of *Dhgm lykanthroporepellens* strain BL-DC-9, which suggested this

contig belonged to the *Dhgm* genome bin, and was not an artifact of the binning methodology. Furthermore, the apparent lack of strain heterogeneity and contaminating sequences in the genomic bin supports the classification of the *Dhgm* genomic bin as a single species. It is generally challenging to determine the strain diversity within cultures that were not derived from single colonies. For instance, a *Dhc* culture that contained a single 16S rRNA gene sequence harbored multiple *Dhc* strains (41). Genome sequencing using the current technologies will not settle this issue, especially in the case for the organohalide-respiring *Chloroflexi* with streamlined and similar genomes, and strain-level resolution cannot be attained. Characterization of *Dhc* genomes showed that the majority of RDase A genes encoding the catalytically active A unit are associated with B genes encoding membrane-anchor proteins (e.g., *Dhc* strain CBDB1 had 32 pairs of RDase A and B genes) (42). By comparison, *Dhgm lykanthroporepellens* strain BL-DC-9 possessed 17 putative RDase A genes, of which only six had cognate RDase B genes (43). Similar observations of missing RDase B genes were made for *Dhc* strain 11a and strain MB, and 3 out of 11 and 9 out of 38 putative RDase A genes, respectively, do not have accompanying B genes (44).

An interesting observation was the persistence of 16S rRNA genes of the bacterial phylum WWE1 during the enrichment process. Phylum WWE1 was first identified in a municipal anaerobic sludge digester (45). To date, no stable enrichment cultures or isolates representing this phylum have been obtained, likely due to their symbiotic relationships with hydrogenotrophic microorganisms (46). Cultivation-independent

metagenomic analysis of a municipal anaerobic sludge digester lead to the assembly of the genome of "*Candidatus Cloacimonas acidaminovorans*", a member of phylum WWE1. Annotation of the genome suggested amino acid fermentation as the organism's main metabolism (47). In meromictic Sakinaw Lake, the depth-dependent co-occurrence of *Chloroflexi*, Candidate divisions WWE1, OP9/JS1, OP8 and OD1, and methanogens suggested syntrophic interactions between these groups (48). The findings reported in several metagenomic studies support a coexistence pattern between *Chlorofexi*, candidate phylum WWE1 and methanogens (49-51). Dechlorinating culture GP harbored *Dhgm*-type *Chloroflexi*, phylum WWE1, and hydrogenotrophic methanogens, and this community could be maintained in defined, bicarbonate-buffered medium amended with lactate and VC. Thus, culture GP is a potential source for isolating representative culture from bacterial phylum WWE1, which has been proposed as candidate phylum Cloacimonetes (52).

Among the organohalide-respiring *Chloroflexi*, *Dhc* have received most attention because of their ability to detoxify priority pollutants (12, 26), their demonstrated relevance for *in situ* bioremediation (5), and the availability of representative isolates (11, 12, 53) and bioaugmentation consortia (54). The presence and abundance of *Dhc* has been linked to ethene formation 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 (55). 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 (56, 57) or aerobic microbial VC oxidation (58-60). The discovery of non-*Dhc* populations carrying novel VC RDase genes indicates that a broader diversity of microorganisms contributes to anaerobic VC 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. A survey of 1,237 groundwater wells from chlorinated solvent-impacted sites demonstrated that *Dhgm* were as equally distributed as *Dhc,* and in fact more abundant than *Dhc* at 77% of the wells examined. 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 are metabolically active. Thus, the contribution of this organismal group to attenuation of chlorinated solvent contaminant plumes is probably far greater than is currently realized. Apparently, both *Dhgm* and *Dhc* are distributed in contaminated aquifers, and at the majority of sites not impacted by bioaugmentation with *Dhc*-containing consortia, *Dhgm* outnumber *Dhc* cells.

Collectively, these results demonstrate that pristine environments (e.g., grape pomace compost) harbor strictly organohalide-respiring bacteria and can be a source of novel RDases, such as CerA, involved in detoxification of the priority pollutant VC. *Dhgm* bacteria are commonly present in contaminated aquifers, and evidence that this bacterial group contributes to VC detoxification has implication for contaminated site assessment and monitoring, and thus will affect decision-making. The findings emphasize that

organohalide-respiring *Chloroflexi* participate in the natural cycling of chlorine, and also highlight that the global biogeochemical cycle of halogens is currently poorly understood.

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# Appendix

**Figure S4.1** Grape pomace samples used for establishing microcosms

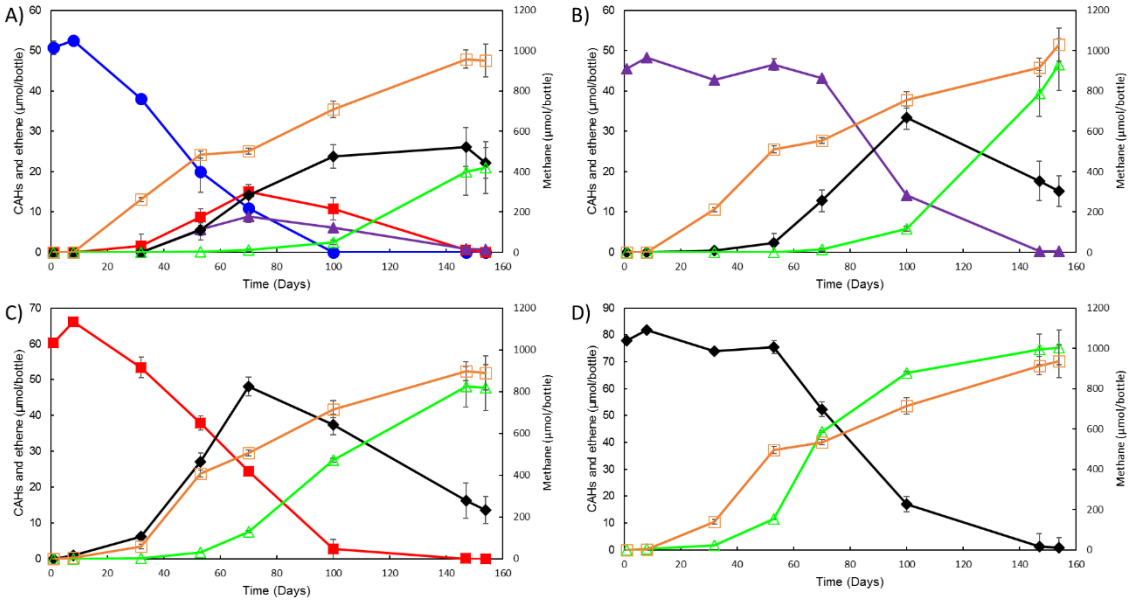


**Figure S4.2** Dechlorination of a) TCE, b) 1, 1-DCE c) cDCE, and d) VC by VC-enriched culture GP. (Blue filled circle-TCE, purple filled triange-1,1-DCE, red filled square-cDCE, black filled diamond-VC, green open triangle-ethene and orange open square-methane). The data points represent the average of triplicate cultures and the error bars are one standard deviation.

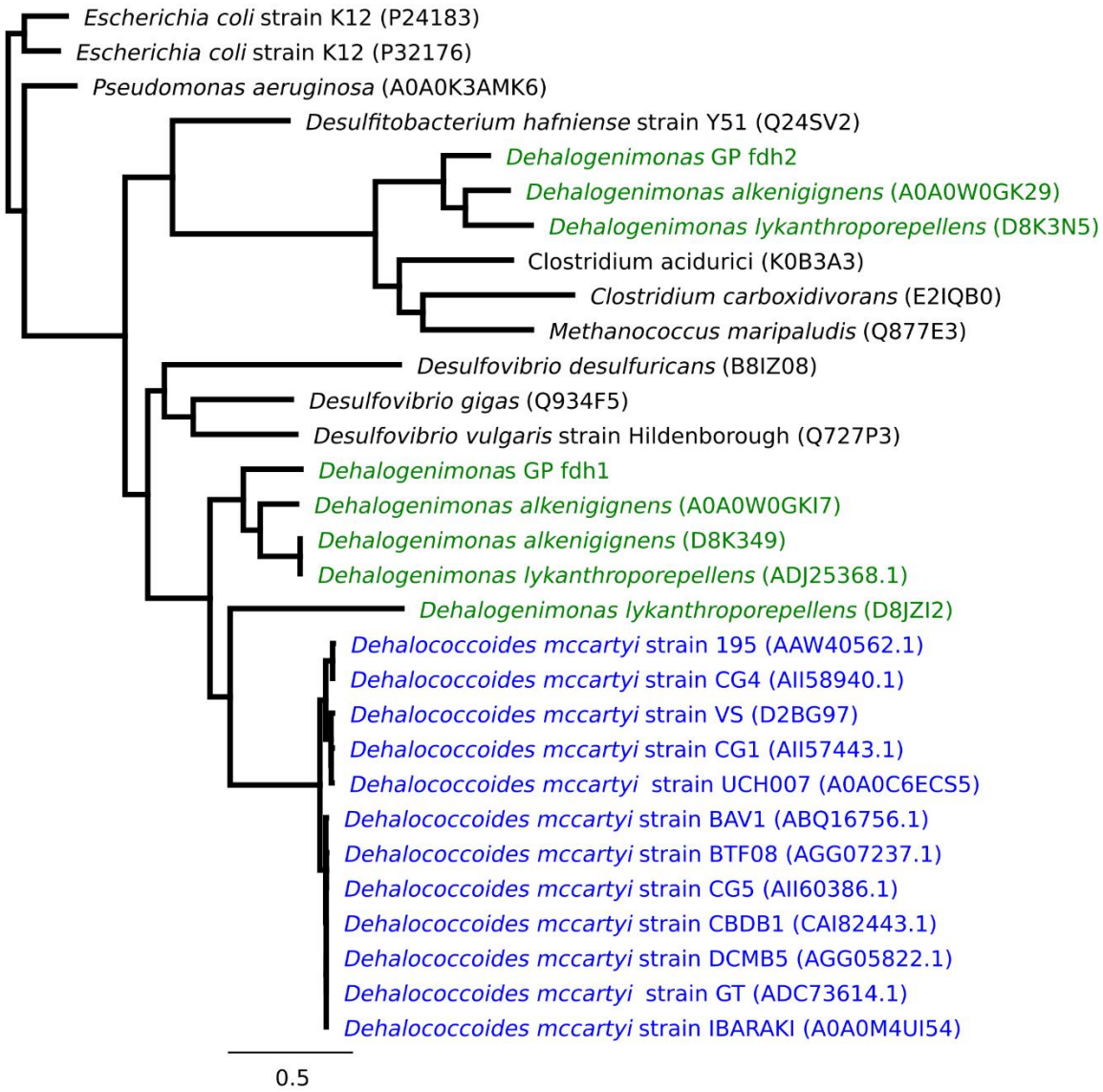


**Figure S4.3** Phylogenetic analysis of formate dehydrogenases among putative and characterized formate dehydrogenases. All sequences were aligned by MUSCLE in Geneious software; and the tree was built based on previous alignment by FastTree in Geneious. Scale bar indicated 0.5 amino acid substitutions per site.

**Table S4.1** Highly expressed proteins detected by proteomics.

| Protein | Average of normalized spectral counts (nSpc) | | | | | Protein description |
|---|---|---|---|---|---|---|
| | Starved VC enrichment culture | VC-fed culture | TCE-fed culture | *c*DCE-fed culture | 1,1-DCE-fed culture | |
| PROKKA_01118 | 7515.55 | 13733.03 | 15065.80 | 13557.64 | 16707.29 | Co-chaperonin GroES |
| PROKKA_01119 | 4991.84 | 10481.78 | 9835.54 | 9180.91 | 8770.31 | Chaperone GroEL |
| PROKKA_01145 | 2247.41 | 1921.01 | 1497.33 | 987.19 | 1015.84 | Hypothetical protein |
| PROKKA_01650 | 2228.71 | 3630.08 | 3473.64 | 5100.72 | 3397.74 | Heat shock protein Hsp20 |
| PROKKA_02004 | 2152.66 | 1168.84 | 473.61 | 914.34 | 280.03 | CerA vinyl chloride reductase |
| PROKKA_01029 | 2039.75 | 727.68 | 475.09 | 663.57 | 695.65 | DNA repair and recombination protein RadA |
| PROKKA_00277 | 1650.88 | 2905.44 | 2484.33 | 2241.27 | 1902.19 | Elongation factor Tu |
| PROKKA_01300 | 1514.07 | 0.00 | 52.97 | 159.44 | 815.50 | Reductive dehalogenase |
| PROKKA_00800 | 1437.57 | 1732.79 | 1759.87 | 1258.86 | 1154.16 | Hypothetical protein |
| PROKKA_00285 | 1325.31 | 866.77 | 870.07 | 966.88 | 806.69 | 50S ribosomal protein L7/L12 |
| PROKKA_01751 | 1188.78 | 1555.33 | 1890.23 | 1024.40 | 836.32 | Rubrerythrin |

# CHAPTER V DEVELOPMENT OF DECISION-MAKING TOOL: BIOLOGICAL PATHWAY IDENTIFICATION CRITERIA (BIOPIC)

## Abstract

Monitored natural attenuation (MNA) is a remedy harnessing naturally occurring biological and abiotic processes to clean up sites impacted by chlorinated solvents. At contaminated sites where MNA alone cannot meet remediation goals, engineering strategies including bioaugmentation and biostimulation are introduced to clean up the contaminations. Several guidance and protocols on implementing in situ bioremediation of chlorinated solvents have been published by government agencies, such as ESTCP (Environmental Security Technology Certification Program) and AFCEE (Air Force Center for Environmental Excellence). A quantitative framework to direct the remedial practitioners to select the appropriate bioremediation strategy is lacking in these government guidance and recommendations. This research is aimed to develop a quantitative framework by correlating site-specific biogeochemical and aquifer matrix parameters with pseudo first order rate constants to estimate in situ degradation rates of chlorinated contaminants. In addition, a user-friendly, Excel-based screening tool named BioPIC (Biological Pathway Identification Criteria) was developed. Together, the quantitative framework and BioPIC are intended to allow the remedial practitioners to select the most efficient remedial strategies in order to minimize detrimental environmental impacts and reduce cleanup costs.


## Introduction

Analytical and numerical modeling has become a valuable tool for remediation project managers to design appropriate remediation plans based on cost-effectiveness analysis.

Due to the complexity of contaminated sites, some key challenges, such as the incorporation of biological reactions terms into the models, needs to be addressed. Researchers have improved the methodology by developing different analytical and numerical models to more accurately simulate the fate and transport of chlorinated solvents in the subsurface environment (1). Commonly used analytical models include Biochlor, REMChlor, ART3D, Natural Attenuation software (NAS), MNAtoolbox and BioBalance Toolkit, while numerical models include SEAM3D, BioRedox, RT3D, MISER and PHT3D. These modeling tools have been applied to different sites based on site-specific aims and requirements. Among these tools, the updated Biochlor version 2.2 can be used to estimate biotransformation rate constants (2).

Briefly, Biochlor is a screening model that simulates remediation by natural attenuation of dissolved solvents at chlorinated solvent release sites (http://www.epa.gov/ada/csmos/models/biochlor.html). This tool has three types of models: 1) solute transport without decay; 2) solute transport with biotransformation modeled as a sequential first-order decay process; 3) solute transport with biotransformation modeled as a sequential first-order decay process with two different reaction zones (i.e., each zone has a different set of rate coefficient values). Figure 5.1 shows the input screen of Biochlor and detailed explanations of the input parameters can be found in the manual (3). Biochlor output includes plume centerline graphs (Figure 5.2), three-dimensional color plots of plume concentrations and mass balance data showing the contaminant mass removal by each chlorinated solvent (Figure 5.3) which

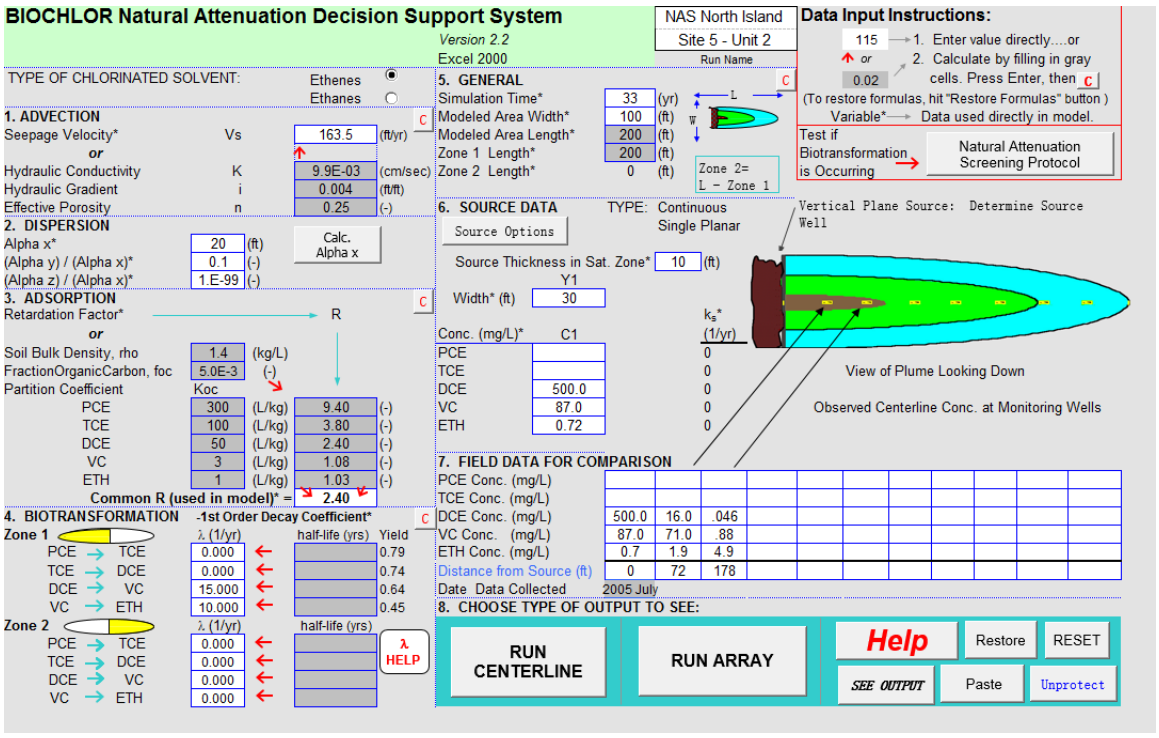


**Figure 5.1** Input screen of Biochlor. NAS North Island Site 5 Unite 2, CA.

can be obtained by clicking the corresponding buttons using the input interface. Following the protocol developed by Wiedemeier *et al*. (4, 5), Clement *et al.* conducted a case study on a chlorinated solvent Superfund site located in Louisiana, USA. The site data combined with Biochlor modeling results indicated that the chlorinated solvents plume could be naturally attenuated within 1,000 feet down gradient from the source zone before reaching the exposure point; therefore, monitored natural attenuation could be considered as one of the feasible remediation options for the site (6). Aziz *et al.* compiled a Biochlor chlorinated solvent plume database to aid site managers to estimate potential effectiveness of natural attenuation for plume management by offering them general plume length and to estimate field-scale biodegradation rate constants (https://clu-in.org/download/contaminantfocus/tce/BIOCHLOR-plume-database.pdf).

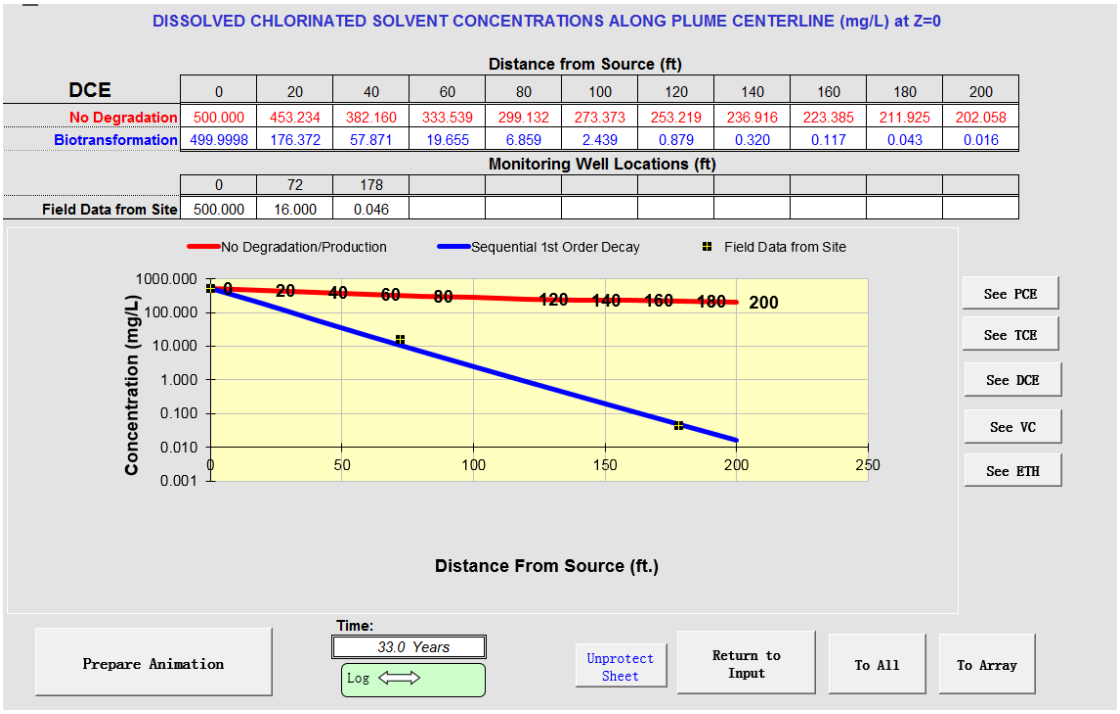


**Figure 5.2** Individual centerline output for VC. NAS North Island Site 5 Unite 2, CA

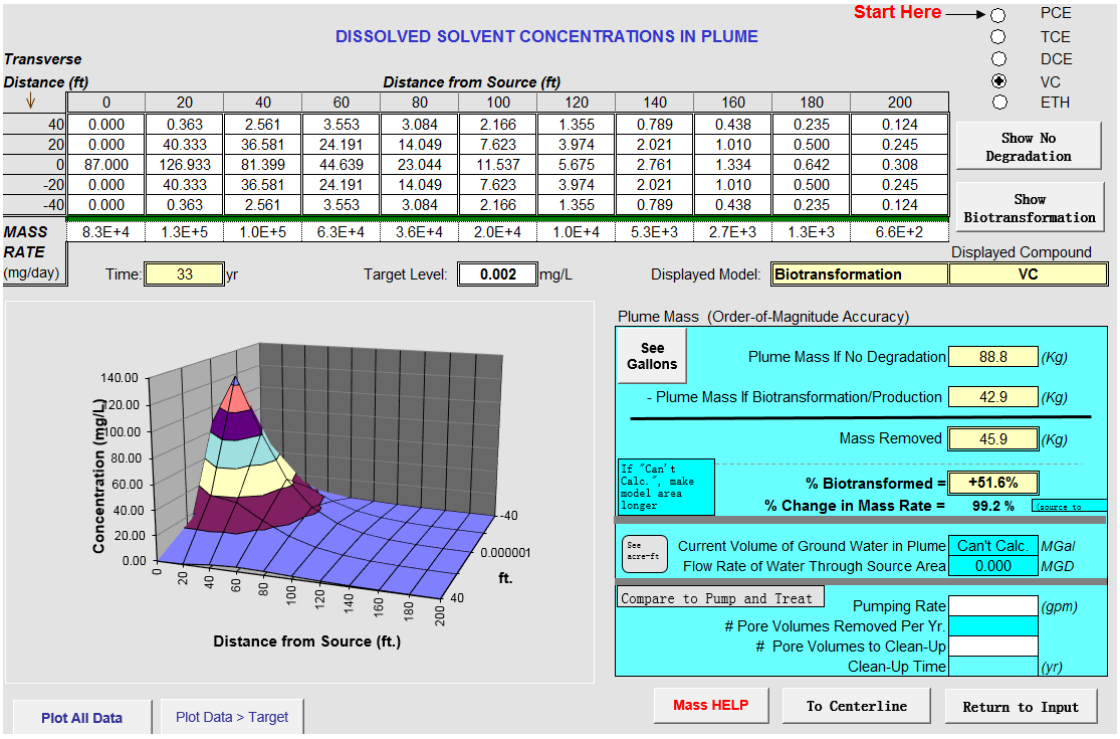


**Figure 5.3** Array concentration output for VC. NAS North Island Site 5 Unite 2, CA.

The previous researches validated the application of Biochlor tool to investigate *in situ* bioremediation.

The protocol developed by Wiedemeier *et al*. was published in 1996 (4,5), and new results and findings since then make revising the protocol a necessity. Based on the previous protocol, a quantitative framework has been developed, which will help remediation project managers to select the most appropriate bioremediation strategy for a given chlorinated solvent site (http://www.serdp.org/Program-Areas/Environmental-Restoration/Contaminated-Groundwater/Persistent-Contamination/ER-201129).

This research project consists of the following sequential tasks:

- **Developing a database including more than 100 different geophysical, geochemical and biological parameters, such as hydraulic conductivity, porosity, seepage velocity, temperature, pH, concentrations of sulfate, nitrate, and chlorinated solvents, bacterial 16S rRNA gene copies. This database will be used to model biodegradation rate constants and conduct statistical modeling analysis.**
- **Using the Biochlor software to model and estimate biotransformation rate constants of selected contaminated sites.**
- **Developing a decision support tool (programmed in a Microsoft Excel spreadsheet environment) that can be used to link quantifiable biogeochemical parameters with remediation of chlorinated ethenes.**

# Materials and Methods

**Methods for estimating pseudo first order biotransformation rate by Biochlor**. A database derived from several site remedial investigation reports and site monitoring data was compiled before estimating the field-scale biodegradation rate constants. Rate constants were estimated by calibrating the Biochlor model to *in situ* chlorinated solvents concentrations along the selected plume centerline. This centerline was identified in two steps: 1) finding the potentiometric surface map and identify the flow path, and 2) choosing the monitoring wells from source zone to the downstream along the flow path (Note: only wells with a D.O. (Dissolved Oxygen) concentration of less than 1 mg/L were chosen indicating anoxic conditions along the plume). Seepage velocity, one of the most important parameters in the Biochlor model, could be either calculated from the mean hydraulic conductivity, hydraulic gradient and porosity, or extracted from the database. Parameters, such as longitudinal dispersivity, transverse dispersivity and vertical dispersivity were set as default values in accordance with the User's Manual. The simulation time was estimated and adjusted to the respective contaminated sites. The concentrations of chlorinated solvents in the source zone area were treated as initial concentration data of the plume. Retardation factors, although considered as "less important and did not impact the magnitude of the rate constant" (7), were calculated in the Biochlor model or assumed from experiences. The hydrogeological data and monitoring well data that were entered into the Biochlor model were extracted from the previously compiled database. The biodegradation rate constants were estimated by

adjusting the rate constants until the Biochlor modeling fitted the concentrations of chlorinated solvents *in situ*.

## Results and Discussion

**Database development**. A database (Microsoft Excel) with more than 100 parameters (see Appendix) was extracted from remedial investigation documents from 21 contaminated sites (Figure 5.4). This database will be utilized to estimate biodegradation rate constants of chlorinated solvents.

**Estimation of rate constants**. The Biochlor modeling tool was applied to estimate the biotransformation rates for different chlorinated solvents at 21 contaminated sites. Table 5.1 shows a summary of pseudo-first order rate constants for selected chlorinated ethenes.

**Development of decision-making tool (BioPIC).** A quantitative framework that will aid remediation project managers in evaluating and selecting the most appropriate biologically-mediated remediation strategy for a given chlorinated solvent site is updated and developed (Figure 5.5). This quantitative framework will then be developed into a decision support tool (BioPIC: Biological Pathway Identification Criteria) based on the Microsoft Excel platform. BioPIC, following the USEPA lines of evidence for Monitored Natural Attenuation (MNA), is intended to support and facilitate the remedial investigation process to determine if MNA would be an effective remediation strategy at a specific contaminated sites. If MNA is not appropriate, BioPIC also provides guidance on selecting bioaugmentation or biostimulation during *in situ* bioremediation. BioPIC and

users' manual can be accessed at https://www.serdp-estcp.org/Program-Areas/Environmental-Restoration/Contaminated-Groundwater/Persistent-Contamination/ER-201129/ER-201129.

| Site name | Site location (State) | Well ID | Internal ID | Sample collected (mm/dd/yyyy) | Matrix (type of sample material provided) | Easting coordinates of well | Northing coordinates of well | Ground surface elevation (altitude) [ft above mean sea level] | Datum Elevation (i.e., top of well casing) [ft above mean sea level] | Depth to top of screen [ft bgs] | Depth to bottom of screen [ft bgs] | Elevation top of Screen [feet msl] | Elevation bottom of Screen [feet msl] | Depth to water [ft below datum (usually top of casing)] | Groundwater elevation [ft msl] | Groundwater temperature [°C] | Electrical conductivity [µS/cm] | Salinity (Percent) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NASNI Site 5 Unit 2 | California | 10-001 | | | | | | | | | | | | | | | | |
| NASNI Site 5 Unit 2 | California | EB-052313 | EB-052313 | 5/20/2013 | | | | | | | | | | | | | | |
| NASNI Site 5 Unit 2 | California | FB-052313 | FB-052313 | 5/20/2013 | | | | | | | | | | | | | | |
| NASNI Site 5 Unit 2 | California | S5-MW-15 | MW-15-001 | 5/20/2013 | | | | | | | | | | | | 21.32 | 6248 | |
| NASNI Site 5 Unit 2 | California | S5-MW-18 | MW-18-001 | 5/20/2013 | | | | | | | | | | | | 18.24 | 2119 | |
| NASNI Site 5 Unit 2 | California | S5-MW-02 | | NA | | 6269606.17 | 1832708.01 | NA | 15.47 | NA | NA | NA | NA | | | | | |
| NASNI Site 5 Unit 2 | California | S5-MW-07 | | NA | | 6270157.38 | 1832240.32 | NA | 13.73 | NA | NA | NA | NA | | | | | |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 3/12/1992 | | 6269694.15 | 1832302.41 | | | | | | | | | | | |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 10/8/1993 | | 6269694.15 | 1832302.41 | | | | | | | | | 6.88 | 6 | NA |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 1/6/1994 | | 6269694.15 | 1832302.41 | | | | | | | | | 5.97 | 7 | NA |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 4/15/1994 | | 6269694.15 | 1832302.41 | | | | | | | | | 6.62 | 6 | NA |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 7/8/1994 | | 6269694.15 | 1832302.41 | | | | | | | | | 6.68 | 6 | NA |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 3/4/1996 | | 6269694.15 | 1832302.41 | 11.27 | 13.02 | 5 | 10 | 6.27 | 1.27 | | | | | |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 3/13/1996 | | 6269694.15 | 1832302.41 | | | | | | | | | | | |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 1/3/1997 | | 6269694.15 | 1832302.41 | | | | | | | | | NA | NA | NA |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 10/9/1997 | | 6269694.15 | 1832302.41 | | | | | | | | | | | |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 1/7/1998 | | 6269694.15 | 1832302.41 | | | | | | | | | | | |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 4/16/1998 | | 6269694.15 | 1832302.41 | | | | | | | | | | | |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 5/21/1998 | | 6269694.15 | 1832302.41 | | | | | | | | | NA | NA | NA |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 7/9/1998 | | 6269694.15 | 1832302.41 | | | | | | | | | | | |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 8/12/1998 | | 6269694.15 | 1832302.41 | | | | | | | | | | | |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 4/24/1999 | | 6269694.15 | 1832302.41 | | | | | | | | | NA | NA | NA |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 7/7/1999 | | 6269694.15 | 1832302.41 | | | | | | | | | NA | NA | NA |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 1/4/2001 | | 6269694.15 | 1832302.41 | | | | | | | | | | | |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 1/17/2001 | | 6269694.15 | 1832302.41 | | | | | | | | | 7.01 | 6,210 | NA |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 4/5/2001 | | 6269694.15 | 1832302.41 | | | | | | | | | 7.10 | 9,340 | 0.50 |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 7/24/2001 | | 6269694.15 | 1832302.41 | | | | | | | | | 7.49 | 9,632 | NA |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 10/4/2001 | | 6269694.15 | 1832302.41 | | | | | | | | | 7.04 | 9,210 | 5.16 |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 5/22/2002 | | 6269694.15 | 1832302.41 | | | | | | | | | | | |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 8/13/2002 | | 6269694.15 | 1832302.41 | | | | | | | | | | | |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 4/21/2003 | | 6269694.15 | 1832302.41 | 11.27 | 13.02 | | | | | 4.90 | 8.12 | | | |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 4/25/2003 | | 6269694.15 | 1832302.41 | | | | | | | | | | | |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 7/8/2003 | | 6269694.15 | 1832302.41 | | | | | | | | | | | |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 1/11/2005 | | 6269694.15 | 1832302.41 | 11.27 | 13.02 | | | | | 2.57 | 10.45 | | | |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 1/18/2005 | | 6269694.15 | 1832302.41 | | | | | | | | | 7.09 | 6200.00 | |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 1/18/2005 | | 6269694.15 | 1832302.41 | | | | | | | | | 7.01 | 6200.00 | |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 4/4/2005 | | 6269694.15 | 1832302.41 | 11.27 | 13.02 | | | | | 4.68 | 8.34 | | | |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 4/6/2005 | | 6269694.15 | 1832302.41 | | | | | | | | | | | |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 7/18/2005 | | 6269694.15 | 1832302.41 | 11.27 | 13.02 | | | | | 5.91 | 7.11 | | | |
| NASNI Site 5 Unit 2 | California | S5-MW-10 | | 7/25/2005 | | 6269694.15 | 1832302.41 | | | | | | | | | | | |

**Figure 5.4** Screenshot of the excel-based database

**Table 5.1** Summary of source zone concentrations and pseudo first order rate constants of chlorinated ethenes at various contaminated sites

| Facility/ Location | Date | PCE | TCE | *c*DCE | VC | PCE | TCE | *c*DCE | VC |
|---|---|---|---|---|---|---|---|---|---|
| | | Concentration near source (µg/L) | | | | Pseudo-first order rate constant for dechlorination (per year) | | | |
| Cecil OU9 Site 59-Northern Plume | | 1.1 | 0.006 | 0 | 0 | | 0.277 | 3.3 | 2.567 |
| Cecil OU9 Site 59-Southern Plume | | 0.477 | | | | | 0.277 | 3.3 | 2.567 |
| NASNI-OU-11 | 2000 | | 310000 | 14000 | 0 | | 0.7 | 1.2 | 5 |
| Parris Island-Site45-Lower | 2005 | 11 | 3.3 | 0.23 | 0.02 | 3.5 | 0.9 | 0.8 | 3 |
| Parris Island-Site45-Upper | 2005 | 0.7 | 35 | 110 | 3 | 1 | 10 | 6 | 60 |
| Parris Island-Site45-RFI | 2004 | 18.8 | 3.15 | 0.825 | 0 | 1.12 | 0.77 | 3.47 | 2.77 |
| Parris Island-Site45-RI | 2004 | 18.8 | 3.15 | 0.825 | 0 | 0.28 | 0.07 | 0.23 | 1.39 |
| NAS-Whiting-Site 3-Shallow | 1994 | | 0.55 | 0 | 0 | | 0.2 | | |
| NAS-Whiting-Site 3-Intermediate | 2011 | | | | | | 0.38 | | |
| Plasttsburgh AFB | 1996 | | 562 | 12602 | 0 | | 0.7 | 0.9 | 0.5 |
| NCBC Gulfport, Mississippi-Site 4 | 2004 | | 0.12 | 1.77 | 1.6 | | 18 | 11 | 25 |
| KingsBay-Site 11 | 1997 | 4500 | | | | 0.8 | 1.6 | 2.5 | 3 |
| NSA-MidSouth | 1997 | | 1.16 | 0.212 | | | 0.35 | 8.5 | |
| Charleston SWMU 12 | 1999 | 6.15 | 12.8 | 1.32 | 0.7 | 0.1 | 0.2 | 0.15 | 10 |
| Charleston SWMU 17 | 2004 | | 31 | 0.354 | | | 2.2 | 3.7 | |
| NASNI Site 5 OU 2 | 2005.July | | | 500 | 87 | | | 15 | 10 |
| NASNI Site 5 OU 2 | 2005.July | 25 | 120 | 525 | 87 | 6.5 | 7.5 | 15 | 6 |
| NASNI Site 5 OU 2 | 2007.Sept | 0.1 | 0.16 | 322.3 | 18 | 9 | 11 | 16 | 15 |
| NASNI Site 5 OU 2 | 2008.Mar | | | 125.6 | 18 | | | 2 | 0.1 |
| Aniston-Landfill Area | 2002 | 2.2 | 19 | 13.061 | 0.1 | 1.1 | 0.6 | 0.45 | 60 |
| Aniston-Trench Area | 1995 | 2.2 | 15 | 2 | 0.8 | 1.5 | 3.2 | 4 | 30 |
| Aniston-North East Area | 2003.Mar | 3 | 23 | | | 0.27 | 0.3 | 0.65 | 1 |
| Aniston-Industrial Area | 2003.Mar | | 2.8 | 2.3 | | | 1.1 | 2 | |
| Hill AFB-O10-Shallow Plume | 2006 Winter | | | | | | 0.021 | | |
| Hill AFB-O10-Shallow Plume | 2006 Summer | | | | | | 0.039 | | |
| Hill AFB-O10-Shallow Plume | 2005 Summer | | | | | | 0.034 | | |
| Hill AFB-O10-Shallow Plume | 2004 Winter | | | | | | 0.079 | | |
| Hill AFB-O10-Shallow Plume | 2004 Summer | | | | | | 0.056 | | |
| Hill AFB-O10-Deep Northen Plume | 2007 Summer | | | | | | 0.116(0.07-.021) | 0.10(0.08-0.13) | |
| Hill AFB-O10-Deep Southern Plume | 2006 Summer | | | | | | 0.188 | 0.36 | |
| Hill AFB-O10-Deep Southern Plume | 2006 Summer | | | | | | 0.212 | 0.338 | |
| Hill AFB-O10-Deep Southern Plume | 2005 Winter | | | | | | 0.279 | 0.472 | |
| Hill AFB-O10-Deep Southern Plume | 2005 Summer | | | | | | 0.243 | | |
| Hill AFB-O10-Deep Southern Plume | 2004 Winter | | | | | | 0.258 | 0.357 | |

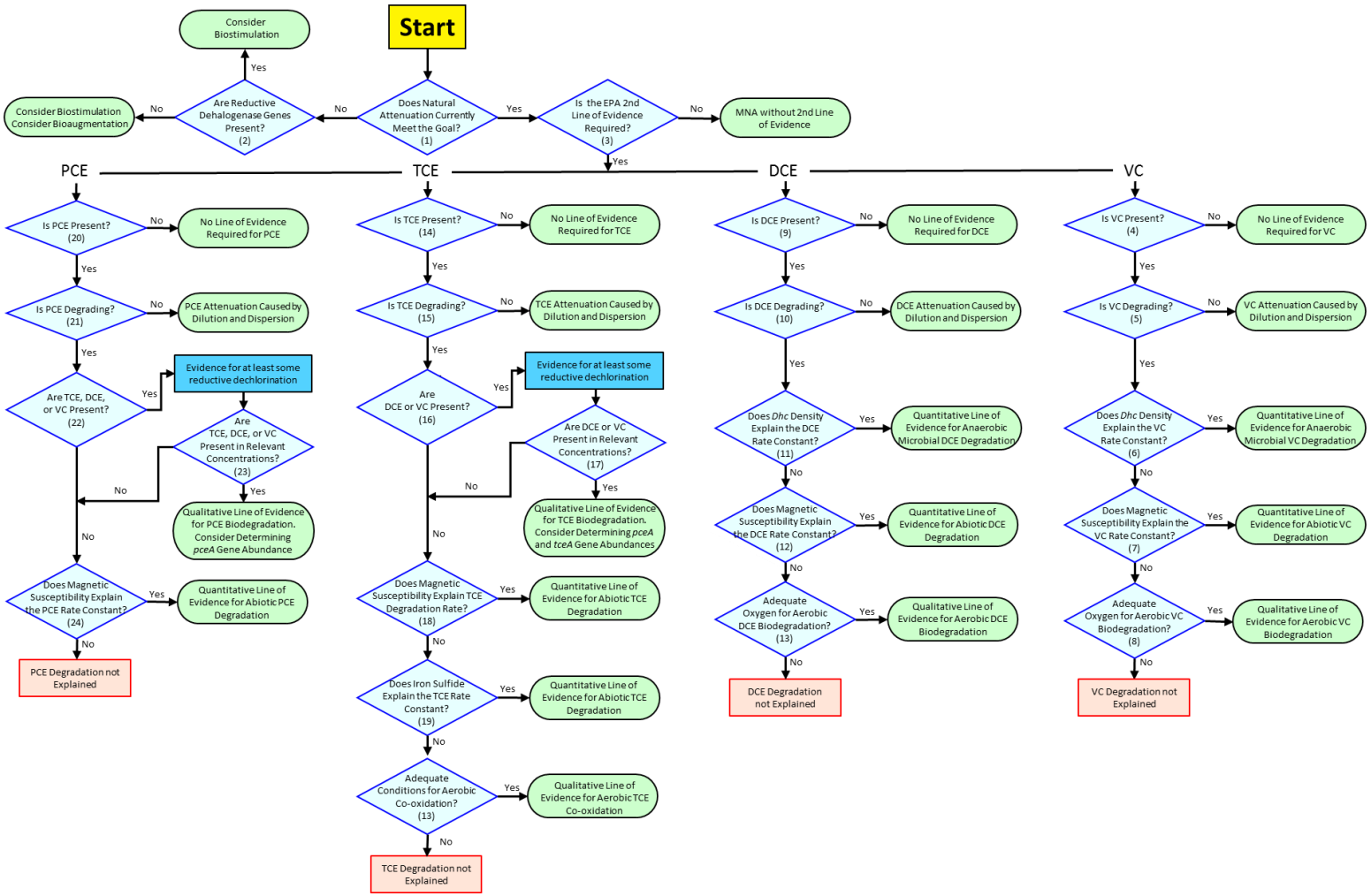


**Figure 5.5** Framework for decision-making tool BioPIC

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# Appendix

**Table S5.1** Complete list of parameters in the database

| Parameter names | | |
|---|---|---|
| Site name | Methane dissolved in groundwater [mg/L] | *cis*-1,2-Dichloroethene [µg/L] |
| Site location (State) | Methane dissolved in groundwater Flag | *cis*-1,2-Dichloroethene Flag |
| Well ID | Butyrate [mg/L] | *cis*-1,2-Dichloroethene $\delta^{13}C$ (‰) |
| Internal ID | Butyrate Flag | *cis*-1,2-Dichloroethene $\delta^{37}Cl$ (‰) |
| Sample collected (mm/dd/yyyy) | Propionate [mg/L] | *cis*-1,2-Dichloroethene $\delta^{2}H$ (‰) |
| Matrix (type of sample material provided) | Propionate Flag | *trans*-1,2-Dichloroethene [µg/L] |
| Easting coordinates of well | Lactate [mg/L] | *trans*-1,2-Dichloroethene Flag |
| Northing coordinates of well | Lactate Flag | *trans*-1,2-Dichloroethene $\delta^{13}C$ (‰) |
| Ground surface elevation (altitude) [ft above mean sea level] | Acetate [mg/L] | *trans*-1,2-Dichloroethene $\delta^{37}Cl$ (‰) |
| Datum Elevation (i.e., top of well casing) [ft above mean sea level] | Acetate Flag | *trans*-1,2-Dichloroethene $\delta^{2}H$ (‰) |
| Depth to top of screen [ft bgs] | Carbon tetrachloride [µg/L] | Vinyl chloride [µg/L] |
| Depth to bottom of screen [ft bgs] | Carbon tetrachloride Flag | Vinyl chloride Flag |
| Elevation top of Screen [feet msl] | Carbon tetrachloride $\delta^{13}C$ (‰) | Vinyl chloride $\delta^{13}C$ (‰) |
| Elevation bottom of Screen [feet msl] | Carbon tetrachloride $\delta^{37}Cl$ (‰) | Vinyl chloride $\delta^{37}Cl$ (‰) |
| Depth to water [ft below datum (usually top of casing)] | Chloroform [µg/L] | Vinyl chloride $\delta^{2}H$ (‰) |
| Groundwater elevation [ft msl] | Chloroform Flag | Ethene [µg/L] |
| Sampling method: Low flow sampling | Chloroform $\delta^{13}C$ (‰) | Ethene Flag |
| Sampling method: Bailer | Chloroform $\delta^{37}Cl$ (‰) | Ethene $\delta^{13}C$ (‰) |
| Sampling method: Hydropunch | Chloroform $\delta^{2}H$ (‰) | Ethene $\delta^{2}H$ (‰) |
| Sampling method: Positive displacement pump | Dichloromethane [µg/L] | Total BTEX+TMB+Naphthalene [µg/L] |
| Sampling method: Peristaltic pump | Dichloromethane Flag | Total BTEX+TMB+Naphthalene Flag |
| Sampling method: Diffusion sampler | Dichloromethane $\delta^{13}C$ (‰) | 1,4-Dioxane [µg/L] |
| Sampling method: Biotrap or similar | Dichloromethane $\delta^{37}Cl$ (‰) | 1,4-Dioxane Flag |
| Sampling method: Other | Dichloromethane $\delta^{2}H$(‰) | First order degradation rate along the flowpath (PCE-$yr^{-1}$) |
| Hydraulic conductivity [ft/day] | Chloromethane [µg/L] | First order degradation rate along the flowpath (TCE-$yr^{-1}$) |
| Hydraulic conductivity measurement technique [ft/day] (i.e., slug test, pumping test, literature value, etc.) | Chloromethane Flag | First order degradation rate along the flowpath (*c*DCE/$yr^{-1}$) |
| Most transmissive material encountered across screen interval (sand/silt/clay/fractures) | 1,1,1-Trichloroethane [µg/L] | First order degradation rate along the flowpath (*t*DCE/$yr^{-1}$) |

**Table S5.1** Continued.

| Parameter names | | |
|---|---|---|
| How was effective porosity determined (measured or literature value) | 1,1,1-Trichloroethane $\delta^{2}H$ (‰) | Point rate decay constant (kpoint) TCE |
| Porosity | 1,1,1-Trichloroethane Flag | First order degradation rate along the flowpath (1,1-DCE-$yr^{-1}$) |
| How was porosity determined (measured or literature value) | 1,1,1-Trichloroethane $\delta^{13}C$ (‰) | First order degradation rate along the flowpath (VC-$yr^{-1}$) |
| Effective porosity | 1,1,1-Trichloroethane $\delta^{37}Cl$ (‰) | Point rate decay constant (kpoint) PCE |
| Seepage velocity [ft/ day] | 1,2-Dichloroethane [µg/L] | Point rate decay constant (kpoint) *c*DCE |
| Treatment (implemented) | 1,2-Dichloroethane Flag | Point rate decay constant (kpoint) *t*DCE |
| Treatment details | 1,2-Dichloroethane $\delta^{13}C$ (‰) | Point rate decay constant (kpoint) 1,1-DCE |
| Groundwater pH | 1,2-Dichloroethane $\delta^{37}Cl$ (‰) | Point rate decay constant (kpoint) VC |
| Groundwater temperature [˚C] | 1,2-Dichloroethane $\delta^{2}H$ (‰) | Biodegradation rate constant (λ) for PCE |
| Electrical conductivity [µS/cm] | 1,1-Dichloroethane [µg/L] | Biodegradation rate constant (λ) for TCE |
| Salinity (Percent) | 1,1-Dichloroethane Flag | Biodegradation rate constant (λ) for *c*DCE |
| Salinity Flag | 1,1-Dichloroethane $\delta^{13}C$ (‰) | Biodegradation rate constant (λ) for *t*DCE |
| Dissolved oxygen (DO)[mg/L] | 1,1-Dichloroethane $\delta^{37}Cl$ (‰) | Biodegradation rate constant (λ) for 1,1-CE |
| Dissolved oxygen (DO) Flag | 1,1-Dichloroethane $\delta^{2}H$ (‰) | Biodegradation rate constant (λ) for VC |
| ORP measured against a silver chloride reference electrode [mV] | Chloroethane [µg/L] | Comments on degradation rates |
| ORP measured against a silver chloride reference electrode Flag | Chloroethane Flag | Volume of groundwater provided for Microbial Analyses [mL] |
| Total organic carbon (TOC) in groundwater [mg/L] | Chloroethane $\delta^{13}C$ (‰) | Bacterial 16S rRNA genes [gene copies per L] |
| Total organic carbon (TOC) in groundwater Flag | Chloroethane $\delta^{37}Cl$ (‰) | *Dhc* 16S rRNA gene-targeted primers [gene copies per L] |
| Total organic carbon (TOC) associated with solids [mg/kg] | Chloroethane $\delta^{2}H$ (‰) | Ratio *Dhc*/Bac 16S rRNA gene copy numbers |
| Total organic carbon (TOC) associated with solids Flag | Ethane [µg/L] | *Geobacter lovleyi* 16S rRNA gene-targeted primers [gene copies per L] |
| Dissolved organic carbon (DOC)[mg C/L] | Ethane Flag | *Dhgm* 16S rRNA gene-targeted primers [gene copies per L] |
| Dissolved organic carbon (DOC) Flag | Ethane δ13C (‰) | *Dhb restrictus* 16S rRNA gene-targeted primers [gene copies per L] |
| Solids-associated organic carbon (Munsell color system) | Ethane δ2H(‰) | *Dhb* CF50 16S rRNA gene-targeted primers [gene copies per L] |
| Solids-associated organic carbon (Munsell color system) Flag | 1,2,3-Trichloropropane [µg/L] | *Dehalobacterium formicoaceticum* 16S rRNA gene-targeted primers [gene copies per L] |
| Hydrogen in groundwater [nM] | 1,2,3-Trichloropropane Flag | *Dhb* RM1 16S rRNA gene-targeted primers [gene copies per L] |
| Hydrogen in groundwater Flag | 1,2,3-Trichloropropane $\delta^{13}C$ (‰) | *Dsf* BB1 16S rRNA gene-targeted primers [gene copies per L] |

**Table S5.1** Continued.

| Parameter names | | |
|---|---|---|
| Sulfate in groundwater Flag | 1,2-Dichloropropane $\delta^{37}Cl$ (‰) | *pceA1* (Dhc) [gene copies per L] |
| Chloride in groundwater [mg/L] | 1,2,3-Trichloropropane $\delta^{37}Cl$ (‰) | *bvcA* [gene copies per L] |
| Chloride in groundwater Flag | 1,2,3-Trichloropropane $\delta^{2}H$ (‰) | *vcrA* [gene copies per L] |
| Nitrate-N in groundwater [mg/L] | 1,2-Dichloropropane [µg/L] | Ratio *bvcA+vcrA*/*Dhc* gene copy numbers |
| Nitrate-N in groundwater Flag | 1,2-Dichloropropane Flag | *tceA* [gene copies per L] |
| Sulfate in groundwater [mg/L] | 1,2-Dichloropropane $\delta^{13}C$ (‰) | *pceA1* (Dhc) [gene copies per L] |
| Total Fe dissolved or suspended in groundwater [mg/L] | 1,2-Dichloropropane $\delta^{2}H$ (‰) | *pceA* (*Geo*) [gene copies per L] |
| Total Fe dissolved or suspended in groundwater Flag | Propene [µg/L] | *pceA* (*Dhb*) [gene copies per L] |
| Total Fe associated with solids [mg/kg] | Propene Flag | *dcpA* [gene copies per L] |
| Total Fe associated with solids Flag | Propene $\delta^{13}C$ (‰) | *mbrA* [gene copies per L] |
| $Fe^{2+}$ in groundwater [mg/L] | Propene $\delta^{2}H$ (‰) | *cbrA* [gene copies per L] |
| $Fe^{2+}$ in groundwater Flag | Tetrachloroethene [µg/L] | *cfrA* [gene copies per L] |
| $Fe^{2+}$ associated with solids [mg/kg] | Tetrachloroethene Flag | *dcrA* [gene copies per L] |
| $Fe^{2+}$ associated with solids Flag | Tetrachloroethene $\delta^{13}C$ (‰) | aprA [gene copies per L] |
| $Mn^{2+}$ in groundwater[mg/L] | Tetrachloroethene $\delta^{37}Cl$ (‰) | *dsrA* [gene copies per L] |
| $Mn^{2+}$ in groundwater Flag | Tetrachloroethene $\delta^{2}H$ (‰) | *etnC* [gene copies per L] |
| Carbonate alkalinity [mg/L] | Trichloroethene [µg/L] | *etnE* [gene copies per L] |
| Carbonate alkalinity Flag | Trichloroethene Flag | *etnE2* [gene copies per L] |
| Magnetic susceptibility (meter set to SI units) [$m^3$/kg] | Trichloroethene $\delta^{13}C$ (‰) | *mcrA* [gene copies per L] |
| Magnetic susceptibility (meter set to SI units) Flag | Trichloroethene $\delta^{37}Cl$ (‰) | General Comments |
| Acid-volatile sulfide (FeS) [mg/kg] | Trichloroethene $\delta^{2}H$ (‰) | Client or site owner |
| Acid-volatile sulfide (FeS) Flag | 1,1-Dichloroethene [µg/L] | Contact phone # (area-xxx-xxxx) |
| Sulfide (as S) [mg/L] | 1,1-Dichloroethene Flag | Contact email address |
| Sulfide (as S) Flag | 1,1-Dichloroethene $\delta^{13}C$ (‰) | |
| Chromium-reducible sulfur [mg/kg] | 1,1-Dichloroethene $\delta^{37}Cl$ (‰) | |
| Chromium-reducible sulfur Flag | 1,1-Dichloroethene $\delta^{2}H$ (‰) | |

# CHAPTER VI CONCLUSIONS

Various biogeochemical factors (e.g., pH, microorganisms and nutrients) affect successfully applying *in situ* bioremediation to clean up chlorinated ethenes contaminated sites. This dissertation work is aimed at further developing our understanding about *in situ* bioremediation of chlorinated ethenes. Based on experimental results in previous chapters, conclusions and recommendations for *in situ* bioremediation are discussed.

Various dechlorinating pure cultures and enrichments showed highest dechlorination activities at circumneutral pH. Only *Sulfurospirillum multivorans* dechlorinated PCE to *c*DCE at pH 5.5. The screening efforts suggest that dechlorinators capable of degrading chlorinated ethenes below pH 6.0 are not common. An enrichment culture was obtained that degraded PCE to *c*DCE at pH 5.5, from which two PCE dechlorinating isolates were obtained. One isolate dechlorinated PCE to TCE (strain PLC-TCE), and the other isolate degraded PCE to *c*DCE (strain PLC-DCE). Both isolates were identified as members of *Sulfurospirillum.* This finding suggested *Sulfurospirillum* may play a significant role in *in situ* bioremediation of chlorinated ethenes under low pH conditions. Also dechlorinating microbial community structure was affected by pH values. *Dehalococcoides* and its potential supporters (e.g. acetogens) were phased out from pH 5.5 environments after continuous transfers at pH 5.5, but dominated at pH 7.2. These findings suggested pH control was critical for applying *Dhc* to *in situ* bioremediation.

Longer low pH exposure would take *Dhc* strains longer time to recover dechlorination activities. *Dhc* could tolerate up to 40 days' low pH exposure, but *Dhc* was severely inhibited by low pH after 40 days' low pH exposure, suggesting pH adjustment at low pH sites may be required in tandem with *Dhc* bioaugmentation. Furthermore, *Dhc* strains harboring *tceA* gene and *Dhc* strains with *vcrA* gene have different resistance to low pH condition, indicating dehalogenase may be susceptible to low pH differently. Although *Dhc* could not perform dechlorination at pH 5.5, *Dhc* may possess ATR (Acid Tolerant

Resistance) systems to survive at mildly acidic pH for extended period. But the mechanisms of *Dhc* surviving under low pH are still not well understood.

It is also demonstrated that pristine environments (e.g., grape pomace compost) harbor strictly organohalide-respiring bacteria and can be a source of novel RDases, such as CerA, involved in detoxification of the priority pollutant VC. *Dhgm* bacterium is commonly present in contaminated aquifers, and evidence that this bacterial group contributes to VC detoxification has implication for contaminated site assessment and monitoring, and thus will affect decision-making. The findings further suggest that organohalide-respiring bacteria participate in chlorine cycling in pristine environments, and emphasize that the global biogeochemical cycle of halogens is currently poorly understood.

# VITA

Yi Yang is born on January 1st 1986, in Hubei Province, China. He got his double bachelor degrees (Environmental engineering and law, 2007) and master of engineering degree (2009) from Wuhan University. Then he has been studying in the Department of Civil and Environmental Engineering with a concentration in environmental biotechnology at the University of Tennessee, Knoxville, supervised by Dr. Frank Löffler. His main interests are biotic and abiotic processes that contribute to contaminant degradation in anoxic environments (e.g. reductive dechlorination). His recent work has focused on assessing pH effects of organohalide-respiring bacteria. These efforts included screening environmental samples for reductive dechlorination activity, and enriching cultures that dechlorinated PCE at low pH. Remarkably, one enrichment culture that dechlorinates TCE to ethene at circumneutral pH does not contain *Dehalococcoides mccartyi* and his current work focuses on identifying biomarkers of novel organohalide-respiring bacteria that use chlorinated ethenes as electron acceptor and produce environmentally benign ethene. In addition, he is contributing to the development of new tools that assist in remediation project managers to select the most efficient remedy at a given site.