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Research Article

RESEARCH ARTICLE

Resilience and recovery of *Dehalococcoides mccartyi* following low pH exposure

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One sentence summary: *Dehalococcoides* can survive low pH stress and recover at least partial dechlorination activity at circumneutral pH, although the duration of low pH exposure affects detoxification potential.

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ABSTRACT

Bioremediation treatment (e.g. biostimulation) can decrease groundwater pH with consequences for *Dehalococcoides mccartyi* (*Dhc*) reductive dechlorination activity. To explore the pH resilience of *Dhc*, the *Dhc*-containing consortium BDI was exposed to pH 5.5 for up to 40 days. Following 8- and 16-day exposure periods to pH 5.5, dechlorination activity and growth recovered when returned to pH 7.2; however, the ability of the culture to dechlorinate vinyl chloride (VC) to ethene was impaired (i.e. decreased rate of VC transformation). *Dhc* cells exposed to pH 5.5 for 40 days did not recover the ethene-producing phenotype upon transfer to pH 7.2 even after 200 days of incubation. When returned to pH 7.2 conditions after an 8-, a 16- and a 40-day low pH exposure, *tceA* and *vcrA* genes showed distinct fold increases, suggesting *Dhc* strain-specific responses to low pH exposure. Furthermore, a survey of *Dhc* biomarker genes in groundwater samples revealed the average abundances of *Dhc* 16S rRNA, *tceA* and *vcrA* genes in pH 4.5–6 groundwater were significantly lower (P-value < 0.05) than in pH 6–8.3 groundwater. Overall, the results of the laboratory study and the assessment of field data demonstrate that sustained *Dhc* activity should not be expected in low pH groundwater, and the duration of low pH exposure affects the ability of *Dhc* to recover activity at circumneutral pH.

Keywords: low pH; *Dehalococcoides*; recovery; strain-specific responses

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INTRODUCTION

Chlorinated solvents are pervasive environmental contaminants that are found at more than 60% of National Priorities List (NPL) sites (www.atsdr.cdc.gov/spl/resources/index.html). Tetrachloroethene (PCE), trichloroethene (TCE), dichloroethene (DCE) isomers and vinyl chloride (VC) are highly ranked (top 30%) on the priority list for hazardous substances based on their ubiquity, toxicity and potential for human exposure at NPL sites (www.atsdr.cdc.gov/spl). Numerous *in situ* technologies have been developed to treat sites contaminated with chlorinated solvents, including chemical oxidation, thermal treatment, air sparging and soil vapor extraction (Kueper *et al.* 2014). A widely employed *in situ* remedial approach is enhanced reductive dechlorination, which relies on anaerobic microorganisms to degrade chlorinated solvents to innocuous end products (Löffler and Edwards 2006; Löffler *et al.* 2013). Over the past two decades, several dechlorinating bacterial isolates (e.g. *Dehalobacter*, *Dehalococcoides*, *Dehalogenimonas*, *Desulfitobacterium*, *Geobacter* and *Sulfurospirillum*) and consortia (e.g. KB-1 (Duhamel *et al.* 2002), SDC-9 (Vainberg, Condee and Steffan 2009), BDI (Ritalahti *et al.* 2005), ANAS (Richardson *et al.* 2002), Donna (Rowe *et al.* 2008) and EV (Yu and Semprini 2004)) have been intensively investigated, which has provided useful insights into the bioremediation of chlorinated ethenes (Stroo and Ward 2010; Atashgahi, Lu and Smidt 2016; Steffan and Schaefer 2016). *Dehalococcoides mccartyi* (*Dhc*) strains with specific reductive dehalogenases (RDases) have emerged as key players based on their unique ability to couple VC-to-ethene reductive dechlorination with energy conservation and growth (Löffler *et al.* 2013). For instance, the PCE-to-ethene dechlorinating consortium BDI contains *Dhc* strains FL2, GT and BAV1, and these strains can be quantified and monitored via quantitative real-time PCR (qPCR) by targeting specific genes that encode RDases unique to individual *Dhc* strains: TceA of strain FL2 involved in TCE to VC dechlorination and cometabolical conversion of VC to ethene, VcrA of strain GT involved in DCE and VC dechlorination to ethene and BvcA of strain BAV1 catalyzing the dechlorination of all DCE isomers and VC to ethene (Ritalahti *et al.* 2006; Amos *et al.* 2008; Löffler *et al.* 2013).

The successful application of *Dhc* cultures to achieve detoxification of chlorinated ethenes is constrained by various geochemical factors, including electron donor (i.e. hydrogen) availability and pH. Biostimulation with fermentable substrates aimed at increasing the hydrogen flux has emerged as a routine treatment; however, decreases in pH due to fermentation (e.g. organic acid formation) and dechlorination (i.e. release of HCl) can negatively impact the success of enhanced reductive dechlorination at chlorinated solvent sites (Robinson *et al.* 2009; Lacroix *et al.* 2014b). Low pH conditions will affect microorganisms and their activities, including *Dhc*, which dechlorinates chlorinated ethenes within a fairly narrow pH range of 6.5–8 (Löffler *et al.* 2013; Yang *et al.* 2017). Thus, successful bioremediation based on *Dhc* activity requires a stable circumneutral pH environment.

When the buffering capacity of the contaminated aquifer is sufficient, pH can be maintained within the range suitable for dechlorinators; however, at sites with low buffering capacity, pH decreases can occur and become detrimental to dechlorinators (Lacroix *et al.* 2014c). The addition of buffer, such as carbonate, bicarbonate or other commercially available buffering agents (e.g. AquaBupH and Neutral Zone; Robinson *et al.* 2009; Stroo, Leeson and Ward 2012) has been employed to address low pH impacts on *Dhc* activity. Typically, the addition of pH stabilizers is not done proactively, and pH adjustments occur after reductive dechlorination activity has stalled due to a decrease in pH. Thus, dechlorinating populations, both native or bioaugmented, will experience low pH stress. While it is well understood that *Dhc* performs best at circumneutral pH, information related to the ability of *Dhc* to recover reductive dechlorination activity following low pH exposure is lacking. To address this knowledge gap, batch culture experiments were conducted to investigate the activity and growth of *Dhc* in consortium BDI at pH 5.5, and to evaluate the recovery of *Dhc* strains (i.e. FL2, GT and BAV1) in consortium BDI after low pH exposure. In addition, quantitative *Dhc* biomarker data from 221 groundwater samples with pH values ranging from 4.5–8.3 were analyzed to explore possible correlations between groundwater pH and the distribution of *Dhc* at sites impacted with chlorinated ethenes.

MATERIALS AND METHODS

Chemicals

PCE (≥99%) and TCE (≥99.5%) were purchased from Acros Organics (distributed by VWR international, West Chester, PA, USA). 1,2-*cis*-dichloroethene (cDCE) (≥99.5%), VC (≥99.5%) and ethene (≥99.9%) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Hydrogen gas (≥99.999%) was obtained from Airgas Inc. (Radnor, PA, USA). 2-(N-morpholino)ethanesulfonic acid (MES) was purchased from Acros Organics. Sodium bicarbonate was purchased from Fisher Scientific (Pittsburgh, PA, USA).

Medium preparation and pH measurement

Reduced, bicarbonate-buffered (30 mM, pH 7.2) mineral salts medium with 5 mM lactate was prepared following established protocols, and 100 mL were dispensed into 160-mL glass serum bottles (Löffler, Sanford and Ritalahti 2005). The vitamin stock solution (Wolin, Wolin and Wolfe 1963) was filter-sterilized (Nalgene 13 mm syringe filters, 0.2 μm polyethersulfone membrane, Fisher Scientific) and added to the medium after autoclaving. Hydrogen gas (10 mL) was added as electron donor from a sterilized hydrogen stock using a plastic syringe. For pH 5.5 medium, 30 mM bicarbonate was replaced with 30 mM M 2-(*N*-morpholino)ethanesulfonic acid (MES). The pH of the bulk liquid phase was measured by collecting 1-mL liquid aliquots from each vessel and placing it into a 2-mL plastic microcentrifuge tube (Eppendorf, distributed by Fisher Scientific). After centrifuging the tube for 30 s at 14 000 *g*, the pH of the supernatant was measured with an Accumet Glass AgCl pH electrode (Fisher Scientific), which was calibrated following standard procedures.

pH tolerance and resilience of a PCE-to-ethene-dechlorinating consortium

To determine if dechlorination activity resumes at pH 7.2 following exposure to low pH (i.e. pH 5.5) conditions, a resilience experiment with the PCE-to-ethene-dechlorinating consortium BDI was conducted. The BDI consortium was grown in replicate 160-mL serum bottles at pH 7.2 with 0.37 mM (aqueous phase) PCE as the electron acceptor. The biomass was collected (14 000 *g* for 15 min at room temperature) from individual pH 7.2 cultures and suspended in pH 5.5 (triplicate 160-mL serum bottles) or pH 7.2 (duplicate 160-mL serum bottles, control group) medium amended with 0.37 mM (aqueous phase) PCE, 5 mM lactate and

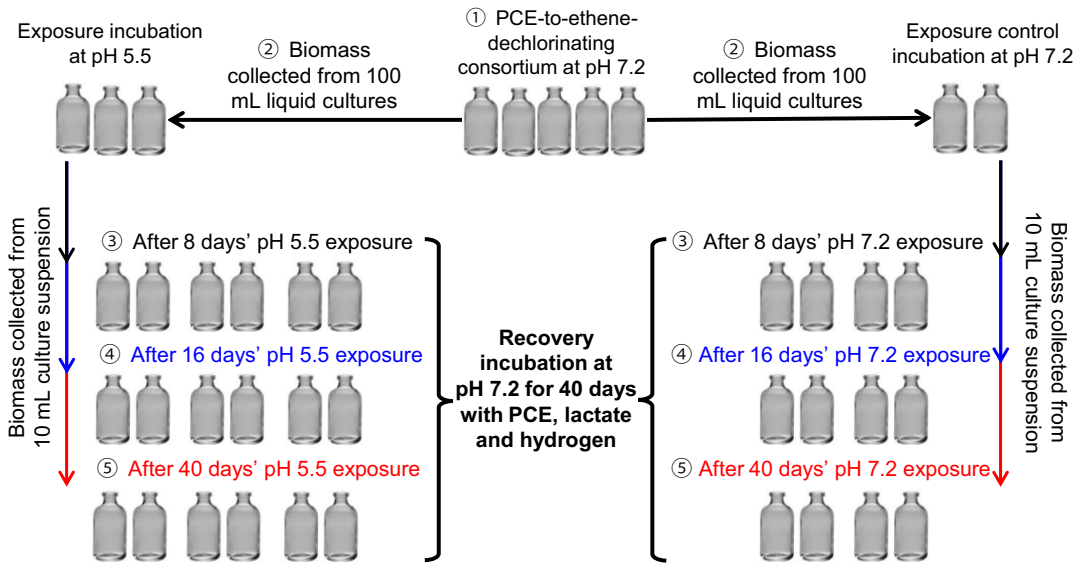

Figure 1. Experimental scheme to test the recovery of reductive dechlorination activity of the PCE-to-ethene-dechlorinating bacterial consortium BDI exposed to pH 5.5 stress, with the pH 7.2 incubation set serving as the control group.

hydrogen (10 mL). Following incubations periods of 8, 16 and 40 days, duplicate 10 mL of culture suspension from each pH 5.5 and pH 7.2 exposure vessel were collected again by centrifugation (14 000 *g* for 15 min), suspended in 2 mL of pH 7.2 medium and transferred to new pH 7.2 medium bottles amended with hydrogen, lactate and PCE. The transfers from pH 7.2 exposure to pH 7.2 recovery medium served as controls to evaluate the effects of the procedure (e.g. centrifugation and suspension) on *Dhc* growth and dechlorination activity. Chlorinated ethenes and ethene were monitored to determine if, and to what extent, reductive dechlorination activity recovered following low pH incubation. *Dhc* 16S rRNA gene copies were enumerated with qPCR to determine *Dhc* cell numbers and to evaluate *Dhc* growth. To assess strain-specific responses to low pH exposure, the RDase genes *tceA* (*Dhc* strain FL2), *vcrA* (*Dhc* strain GT) and *bvcA* (*Dhc* strain BAV1) were quantified. Figure 1 summarizes the experimental setup used to explore *Dhc* resilience in response to low pH exposure.

Quantification of chlorinated ethenes

Concentrations of chlorinated ethenes and ethene were determined by analyzing 100-μL headspace gas samples on an Agilent 7890A gas chromatograph (GC) equipped with a flame ionization detector that was connected to an Agilent DB624 column (30 m $\times$ 0.53 mm I.D., 3 μm; Agilent Technologies, Santa Clara, CA). Gas samples were removed from the headspace of the 160-mL serum bottles using a gas-tight 250 μL Hamilton SampleLock syringe (Hamilton, Reno, NV) and manually injected into the GC. The concentrations of chlorinated ethenes were calculated by normalizing the peak areas to standard curves generated by adding known amounts (i.e. total moles) of chlorinated ethenes or ethene into 160-mL serum bottles with the same gas to liquid ratio (i.e. 60:100) as the experimental vessels, and measuring 100-μL headspace samples after 24-h equilibration. The total moles of chlorinated ethenes (PCE, TCE and cDCE) added to each vessel to prepare standard curves were calculated by the formula: total moles of chlorinated ethene = (volume of chlorinated ethene $\times$ density of chlorinated ethene)/molecular weight of chlorinated ethene. The total moles of VC and ethene were calculated using the ideal gas law (PV = nRT) with P = 1 atm, R = 0.08206 L·atm/(mol·K) and T = 293.15 K.

DNA extraction and qPCR

Microbial biomass was collected from 2-mL culture suspensions by vacuum filtration through 0.22 μm membrane filters (Millipore GVWP025000, Millipore Co., Bedford, MA, USA). Filter-trapped microbial cells were transferred into 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 min. Genomic DNA extraction using the PowerSoil DNA Isolation Kit followed the manufacturer's recommendations. DNA concentrations were quantified with a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE). DNA samples extracted from replicate cultures were stored at –20°C. qPCR assays were performed following established protocols (Ritalahti *et al.* 2006).

Contaminated site data and analysis

Gene copy abundance data (measured by qPCR) for *Dhc* 16S rRNA genes and the RDase genes *bvcA*, *vcrA* and *tceA*, along with groundwater pH information from 221 groundwater samples distributed among 23 sites impacted with chlorinated ethenes, were graciously provided by Microbial Insights, Inc. (www.microbe.com). Statistical analyses of the field data were conducted using R Statistical Software (version 3.2.4., R Foundation for Statistical Computing, Vienna, Austria). The two-sample t-test was used to compare the average of gene copy numbers of the selected pH intervals (https://stat.ethz.ch/R-manual/R-devel/library/stats/html/t.test.html).

RESULTS

Dhc dechlorination activity and growth at pH 7.2 and pH 5.5

The *Dhc*-containing consortium BDI did not degrade PCE to TCE, cDCE or VC in pH 5.5 medium, and *Dhc* cell numbers did not in-

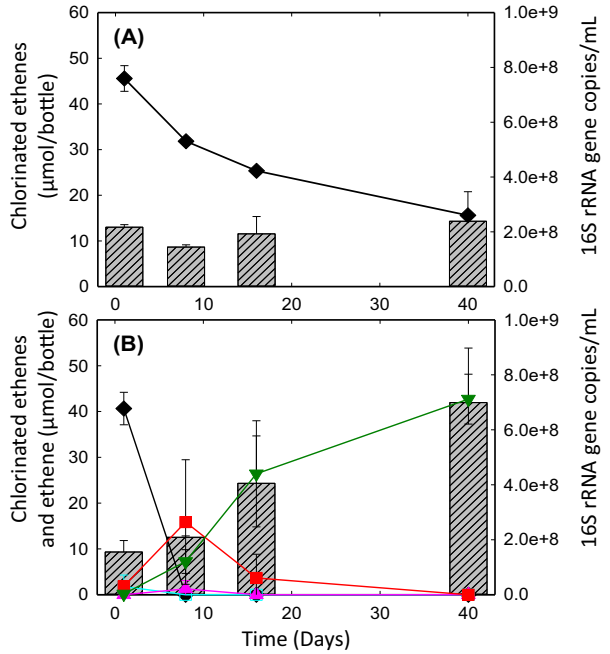

Figure 2. PCE dechlorination at pH 5.5 (panel **A**) and pH 7.2 (panel **B**) by the PCE-to-ethene-dechlorinating consortium BDI over a 40-day incubation period. No reductive dechlorination daughter product formation and no *Dhc* growth occurred at pH 5.5. The apparent PCE loss at pH 5.5 was due to abiotic loss (e.g. sorption to the rubber stoppers). At pH 7.2, PCE was degraded to ethene and *Dhc* growth was observed as determined by qPCR. Solid black diamonds—PCE; open cyan squares—TCE; solid pink triangles—cDCE; solid red squares—VC; solid dark green inverted triangles—ethene and shaded bar—*Dhc* 16S rRNA gene copy numbers.

crease during the 40-day incubation period (Fig. 2A). In contrast, consortium BDI dechlorinated PCE to ethene within 40 days at pH 7.2, and qPCR measurements demonstrated that the *Dhc* 16S rRNA gene copies increased from $1.55 \pm 0.42 \times 10^8$ mL^{-1} (cells introduced with the inoculum) to $6.99 \pm 1.99 \times 10^8$ mL^{-1} (Fig. 2B). The *vcrA* and *tceA* genes increased from $1.57 \pm 0.09 \times 10^8$ copies mL^{-1} and $1.29 \pm 0.11 \times 10^8$ copies mL^{-1} to $4.92 \pm 1.79 \times 10^8$ copies mL^{-1} and $2.31 \pm 0.47 \times 10^8$ copies mL^{-1}, respectively, following the 40-day incubation period at pH 7.2. *Dhc* strain BAV1 carrying the *bvcA* gene is also a part of consortium BDI, but this strain was not competitive in PCE-fed cultures (i.e. strain BAV1 lacks PCE and TCE RDase genes), and was consequently not detected (i.e. <20 gene copies mL^{-1}) after repeated transfers with PCE as electron acceptor.

pH tolerance and resilience

To explore the effects of low pH on *Dhc* growth and reductive dechlorination performance, consortium BDI biomass grown with PCE as the electron acceptor was suspended in pH 5.5 medium for 8, 16 and 40 days. Consortium BDI biomass exposed to pH 5.5 for 8 days recovered dechlorination activity following transfer to pH 7.2, and PCE was dechlorinated to VC and some ethene (10% of initial PCE amount) within the 40-day pH 7.2 recovery period (Fig. 3A). Based on qPCR analysis, the 16S rRNA, *tceA* and *vcrA* genes increased 39.2 ± 9.6-, 50.9 ± 8.9- and 14.2 ± 7.4-fold, respectively, within this 40-day recovery period at pH 7.2 following the 8-day exposure to pH 5.5 (Fig. 4). PCE-to-VC dechlorination was also observed in the pH 7.2 recovery experiments with consortium BDI biomass that experienced a 16-day exposure to pH 5.5 (Fig. 3C), and the *Dhc* 16S rRNA, *tceA* and *vcrA* genes increased 11.5 ± 3.9-, 41.3 ± 15.6- and 6.3 ± 2.5-fold, respectively, within the 40-day pH 7.2 recovery period (Fig. 4). Following a 40-day exposure to pH 5.5, the consortium degraded PCE to cDCE and small amounts of VC (6.2% of initial PCE amount) within the 40-day pH 7.2 recovery period (Fig. 3E), and the *Dhc* 16S rRNA, *tceA* and *vcrA* genes increased 6.0 ± 3.8-, 11.6 ± 8.8- and 2.5 ± 1.1-fold, respectively (Fig. 4).

Statistical analysis performed on average fold increases of 16S rRNA, *tceA* and *vcrA* genes indicated no differences in terms of *Dhc* growth yield upon transfer to pH 7.2 medium between the pH 5.5 (Fig. 3A) and pH 7.2 (Fig. 3B, control group) incubation vessels after an 8-day exposure period (16S rRNA gene: *P*-value = 0.211; *tceA* gene: *P*-value = 0.567 and *vcrA* gene: *P*-value = 0.242; Table 1). Similarly, no significant difference was seen between cultures maintained at pH 5.5 (Fig. 3C) versus pH 7.2 (Fig. 3D) during a 16-day exposure period and transferred to pH 7.2 medium (Table 1). These findings suggest that exposure to pH 5.5 for up to 16 days had minimal impact on *Dhc* survival and recovery of dechlorination activity to VC. Conversely, *Dhc* biomarker gene fold increase differences between the pH 5.5 group (Fig. 3E) and the pH 7.2 control group (Fig. 3F) after 40 days of pH 5.5 exposure were statistically significant (16S rRNA gene: *P*-value = 0.014; *tceA* gene: *P*-value = 0.034 and *vcrA* gene: *P*-value = 0.000; Table 1). These data indicate that the longer 40-day exposure period significantly affected the recovery of *Dhc* from low pH stress. The VC-to-ethene dechlorination step was most strongly inhibited, and only the cultures initiated with biomass exposed to pH 5.5 for 8 days produced some ethene (10% of the initial PCE amount) within the 40-day pH 7.2 recovery period (Fig. 3A). In contrast, the pH 7.2 control cultures produced ethene (67% of the initial PCE amount), demonstrating that manipulation of the biomass (e.g. centrifugation and resuspension) was not the reason for the limited reductive dechlorination activity recovered from biomass following pH 5.5 exposure (Fig. 3B, D and F).

After an extended 200-day recovery period, consortium BDI biomass from 8 and 16 days of pH 5.5 exposure regained the capability of complete degradation of all chlorinated ethenes to ethene (Fig. 3A and C); however, no ethene was produced in the cultures derived from biomass that experienced 40 days of pH 5.5 exposure (Fig. 3E). These findings indicate that the duration of exposure to low pH influences the ability of *Dhc* to recover from low pH induced stress. Furthermore, these data reveal a *Dhc* strain-specific response, suggesting that *Dhc* strain GT carrying the VC RDase *vcrA* was more susceptible to pH stress than *Dhc* strain FL2 carrying the *tceA* gene.

Relationship between groundwater pH and *Dhc* abundance

To assess whether pH affects the distribution and abundance of *Dhc* at sites impacted with chlorinated ethenes (Table S1, Supporting Information), a total of 221 groundwater samples from 23 sites were investigated. These groundwater samples were chosen for the availability of both pH and microbiological data. In 50 groundwater samples, *Dhc* 16S rRNA genes were below 100 copies L^{-1}, and these data were omitted from subsequent analyses. The pH of the remaining 171 groundwater samples ranged from 4.5 to 8.3, with a median pH of 6.4. *Dhc* 16S rRNA gene copy numbers in these 171 groundwater samples ranged from 100 to 4.0×10^6 copies L^{-1}, with a median of 6.96×10^5 copies L^{-1} (Fig. 5). Since the optimal pH range for *Dhc*-containing bioaugmentation consortia is 6.0–8.3 (Löffler *et al.* 2013), pH values were categorized into two intervals: acidic (4.5–6.0) and circumneutral (6.0–8.3). A survey of *Dhc* biomarker genes in groundwater samples with available groundwater pH information demonstrated

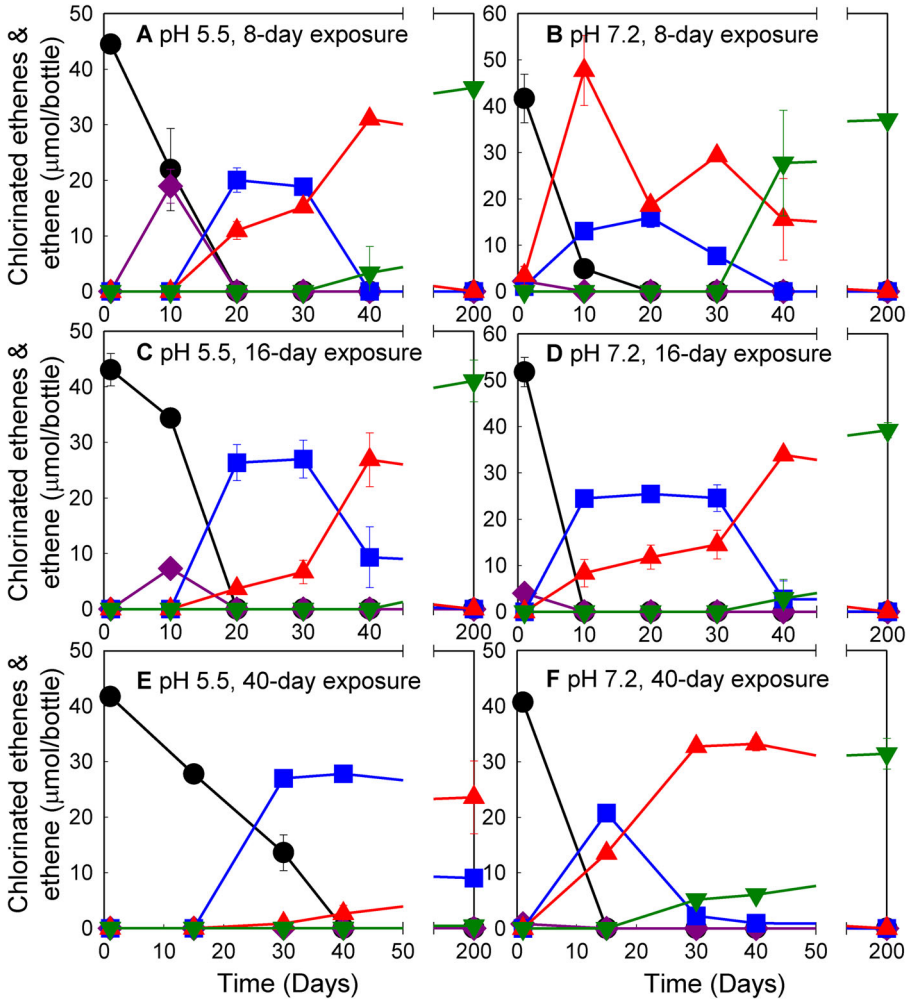

Figure 3. PCE degradation in consortium BDI pH 7.2 transfer cultures following pH 5.5 (left panels) or pH 7.2 (right panels) exposure for up to 40 days. Panels **A**, **C** and **E** show PCE degradation after 8, 16 and 40 days of pH 5.5 exposure, respectively. Panels **B**, **D** and **F** depict the control groups after 8, 16 and 40 days of pH 7.2 exposure, respectively. Final measurements were conducted on day 200. The error bars indicate one standard deviation. Solid black circles—PCE; solid dark purple diamonds—TCE; solid blue square—cDCE; solid red triangle—VC and solid dark green inverted triangles—ethene.

that the average *Dhc* abundance in the pH range 6.0–8.3 was approximately 1.52-fold higher than that in the pH range 4.5–6.0, suggesting pH affected the abundance of *Dhc* in the aquifer. Further, the comparison of the average *Dhc* abundances between the acidic and circumneutral pH ranges demonstrated a statistical difference (df [degree of freedom] = 67.4, *P*-value = 0.009). The mean *Dhc* abundance for the acidic pH range (i.e. pH 4.5–6.0) was 7.49×10^5 gene copies L^{-1}. By comparison, the average *Dhc* abundance for the circumneutral pH range was 1.14×10^6 gene copies L^{-1}. Although the abundances of the RDase genes *tceA* and *vcrA* between acidic and circumneutral pH ranges followed similar patterns (i.e. higher average *tceA* and *vcrA* gene copy numbers for the circumneutral pH range), the average *vcrA* gene copy numbers were 3.4- and 4.9-fold higher than the average *tceA* gene copy numbers in pH 4.5–6.0 and pH 6.0–8.3 groundwater samples, respectively (Fig. 5). Statistical analysis of the average *tceA* and *vcrA* gene abundances in the two pH ranges indicated significant differences (*vcrA*: *P*-value = 0.001 and *tceA*: *P*-value = 0.015, Table 2). The analysis of total bacterial 16S rRNA gene abundances indicated no significant differences between the 4.5–6.0 pH and the 6.0–8.3 pH categories (*P*-value = 0.314, Table 2).

DISCUSSION

Impacts of low pH on *Dhc* reductive dechlorination activity

Low pH conditions remain a challenge for *in situ* treatment of contaminated sites using microbial remedies. In a pilot-scale aquifer system, the rapid hydrolysis and subsequent fermentation of lactic acid esters used for biostimulation led to a pH decrease from circumneutral to close to pH 5.0, which impaired the establishment of robust dechlorination activity and ethene formation (Adamson, McDade and Hughes 2003). Biostimulation with whey powder to treat a TCE plume caused pH reduction below 5 in the treatment zone, which resulted in a cDCE stall and cessation of growth of native *Dhc* (Mora *et al.* 2008). To overcome the low pH challenge, adjusting and maintaining neutral pH by buffer additions is a feasible, but expensive strategy to sustain bioremediation (Philips *et al.* 2013). The addition of bicarbonate to raise the groundwater pH was implemented with some success (Schaefer, Lippincott and Steffan 2010), but the sustainability of this approach is unclear and repeated buffer additions add to the treatment cost. Silicate minerals were tested as an economical solution to adjust and maintain neutral pH, but sil-

Table 1. Average fold increase of *Dhc* biomarker genes in pH 5.5 and pH 7.2 batch culture incubations. Asterisks (*) denote statistically significant values.

		Average initial gene copy number (copies/mL)		Average fold increase		
Gene	Exposure time (days)	pH 5.5 (n = 6)	pH 7.2 (n = 4)	pH 5.5 (n = 6)	pH 7.2 (n = 4)	P-value of t-test
Dhc 16S rRNA	8	2.4E + 06	6.7E + 06	39.2	76.2	0.211
	16	6.24E + 06	9.38E + 06	11.5	29.9	0.304
	40	1.20E + 07	1.57E + 07	6.0	38.0	0.014*
tceA	8	4.73E + 06	1.17E + 06	50.9	70.2	0.567
	16	1.06E + 07	1.94E + 07	41.3	32.8	0.743
	40	1.89E + 07	1.99E + 07	11.6	58.7	0.034*
vcrA	8	1.29E + 06	2.01E + 07	14.2	41.7	0.242
	16	5.43E + 06	4.06E + 07	6.3	33.1	0.174
	40	8.90E + 06	4.45E + 07	2.6	28.7	0.000*

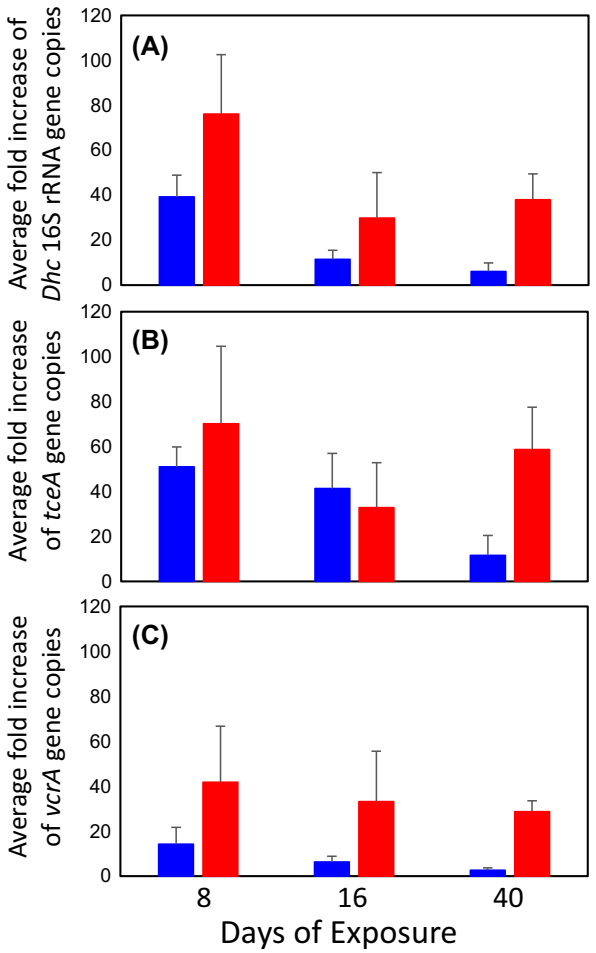

Figure 4. Average fold increases of 16S rRNA (**A**), *tceA* (**B**) and *vcrA* (**C**) genes measured after a 40-day incubation period in pH 7.2 transfer cultures that were prepared with biomass collected from cultures incubated for 8, 16 and 40 days at pH 5.5 (blue bars) or pH 7.2 (red bars). The error bars indicate standard error (for pH 5.5 n = 6; for pH 7.2 n = 4).

icate minerals affected *Dhc* dechlorinating activity (Lacroix *et al.* 2014a). Although pH control is a viable approach to achieve low pH site cleanup (Steffan and Schaefer 2016), additional research is warranted to determine the most productive pH adjustment approach at a given site, both in terms of sustained reductive dechlorination activity and cost.

At sites requiring pH adjustment, *Dhc* have inadvertently been exposed to pH stress since pH treatments are typically applied retroactively. Despite the ubiquity of low pH conditions in many aquifers undergoing bioremediation, the effects of low pH exposure on *Dhc* activity and resilience have been unclear. The outcomes of this study confirmed that *Dhc* does not grow at pH 5.5, and that the duration of low pH exposure affected the ability of *Dhc* strains in consortium BDI to recover dechlorination activity to ethene. Of particular importance for bioremediation is the finding that the duration of low pH exposure affected dechlorination end points following pH adjustment to pH 7.2. Under the experimental batch culture conditions used in this study, *Dhc* strains in consortium BDI did not recover the ethene-producing phenotype following a 40-day low pH exposure period. Batch cultures experiments do not resemble a dynamic aquifer system with biofilms forming on surfaces and dynamic flow. Still, the experiments indicate that the duration of exposure to low pH is a critical variable that determines the success of a pH adjustment strategy. At field sites where low pH conditions prevail for extended periods of time, bioaugmentation as a follow-up treatment to pH adjustment may be necessary to initiate reductive dechlorination activity.

Dhc strain-specific responses to pH stress

Dhc strain-specific responses towards environmental stressors such as oxygen and temperature have been reported (Amos *et al.* 2008; Fletcher *et al.* 2011). For example, of the three *Dhc* strains in consortium BDI, strain FL2 carrying the *tceA* gene exhibited higher robustness towards oxygen exposure and increased temperature compared to strain GT carrying the *vcrA* gene and strain BAV1 carrying the *bvcA* gene (Amos *et al.* 2008; Fletcher *et al.* 2011). Similarly, this study found that the *Dhc* strain carrying the *tceA* gene was less affected by low pH exposure than the *Dhc* strain carrying the *vcrA* gene. Independent of the stressor (e.g. oxygen, temperature, pH), these studies have revealed that the VC-to-ethene step is more susceptible to perturbations than the prior reductive dechlorination steps. This sensitivity of the final dechlorination step is problematic for contaminated site cleanup because VC is a human carcinogen and is more stringently regulated. The reason why the VC-to-ethene step is more sensitive to stressors is not immediately apparent because the same enzyme system (e.g. VcrA, BvcA) that dechlorinates cDCE to VC also dechlorinates

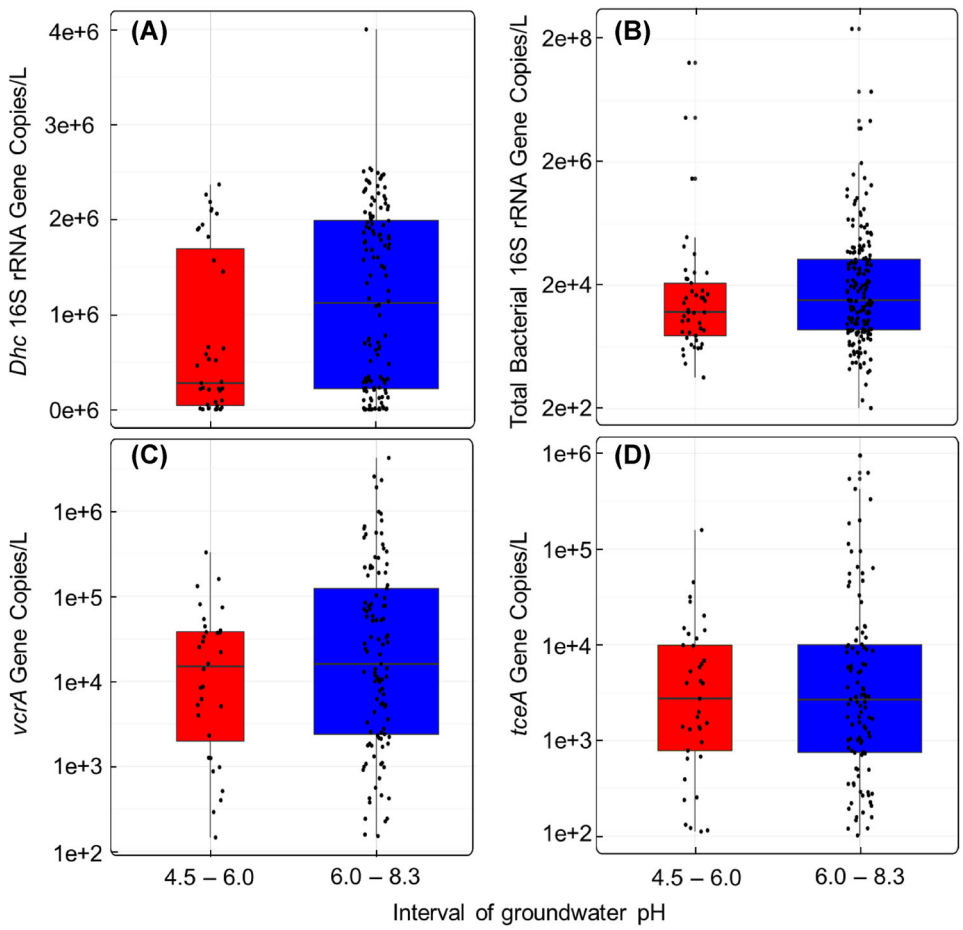

Figure 5. Distribution of *Dhc* 16S rRNA (**A**), total bacterial 16S rRNA (**B**), *vcrA* (**C**) and *tceA* (**D**) genes in 171 groundwater samples impacted with chlorinated ethenes. The pH 4.5–6.0 and pH 6.0–8.3 categories represent unfavorable versus favorable pH ranges, respectively, for *Dhc* growth and reductive dechlorination activity.

Table 2. One-way t-test of *Dhc* 16S rRNA, *tceA*, *vcrA* and total bacterial 16S rRNA gene abundances in 171 groundwater samples collected from sites with pH values ranging between 4.5 and 8.3. Samples were categorized into pH intervals of 4.5–6.0 and 6.0–8.3. The higher pH range reflects the optimal pH range for *Dhc*-containing consortia.

	Average copies/L				
Gene	pH 4.5–6.0	pH 6.0–8.3	t-value	df[a]	*P*-value
Dhc 16S rRNA	7.49×10^5	1.14×10^6	–2.44	67.4	0.009**
tceA	1.11×10^4	4.15×10^4	–2.19	121.8	0.015*
vcrA	3.77×10^4	2.05×10^5	–3.04	117.2	0.001**
Total bacterial 16S rRNA	1.06×10^7	1.20×10^7	–0.49	126.1	0.314

[a]df: degree of freedom.
Asterisks indicate significance levels: ** 0.01, * 0.05.

VC to ethene (Müller *et al.* 2004; Tang *et al.* 2013). Since incomplete reductive dechlorination is not a desirable outcome, a more complete understanding of the mechanisms underlying the susceptibility of final VC-to-ethene dechlorination step to inhibition is necessary.

The observation that *Dhc* could recover dechlorination activity to VC and ethene after a 16-day or shorter exposure period at pH 5.5 indicated that *Dhc* tolerates short-term exposure to low pH conditions. Microbial response mechanisms to low pH can be categorized into acid tolerance responses (ATR) for mildly acidic pH (>pH 3.0) and extreme acid resistance (XAR) for extremely acidic pH (<pH 2.0) (Lund, Tramonti and De Biase 2014). *Escherichia coli* (*E. coli*) has been a model microorganism to study the mechanisms of both ATR and XAR; however, *E. coli* may not offer comparable insights regarding the acid resistance mechanisms in *Dhc*. Mechanisms for low pH stress resistance include chloride channels or periplasmic carbonic anhydrase, the latter of which converts carbon dioxide to bicarbonate (Foster 2004). This enzyme was suggested to help the Gram-negative bacterium *Helicobacter pylori* to survive in the acidic environment of the stomach (Lund, Tramonti and De Biase 2014). All available genomes of *Dhc* strains possess putative carbonic anhydrase genes, indicating *Dhc* may be capable of using carbonic anhydrase to raise the periplasmic pH above 6.0 when the pH of the surrounding medium is lower.

Field measurements and implications for *in situ* bioremediation

It is well documented that pH is a relevant environmental factor that shapes microbial communities (Lauber *et al.* 2009). The survey of site data performed as part of the current study revealed that the overall *Dhc* abundance and the relative abundance of *Dhc* strains with different RDase genes (i.e. *tceA*, *vcrA*) were influenced by groundwater pH. *Dhc* 16S rRNA and RDase genes were less abundant in pH 4.5–6.0 compared with pH 6.0–8.3 groundwater. In contrast, the average total number of bacterial 16S rRNA genes did not differ in pH 4.5–6.0 versus pH 6.0–8.3 groundwater samples, indicating that pH gradients may not affect the overall bacterial abundances. A recent study, in which geochemical and microbial data sets collected from 35 wells at five contaminated sites were analyzed, did not reveal a strong correlation between *Dhc* abundance and groundwater pH (Lee *et al.* 2016). This prior study (Lee *et al.* 2016) concluded that comprehensive understanding of the physical and chemical parameters that affect *Dhc* dechlorination activity and ethene formation at contaminated sites has not been attained, suggesting efforts correlating specific environmental parameters with *Dhc* strain abundances must be carefully interpreted.

Dhc does not grow in low pH environments (Yang *et al.* 2017), implying that sustained *in situ Dhc* dechlorination activity under low pH conditions can not be expected. Based on the laboratory findings with the BDI consortium, *Dhc* strains carrying the *vcrA* gene are more susceptible than strains carrying the *tceA* gene towards pH stress; however, the average *vcrA* gene copies measured in in both 4.5–6.0 and 6.0–8.3 groundwater samples exceeded the average *tceA* gene copies 3.4- and 4.9-fold, respectively. A possible explanation is that these sampling locations were influenced by bioaugmentation with *Dhc*-containing consortia dominated by strains carrying the *vcrA* gene. Another possible explanation is that high VC concentration (Table S1, Supporting Information) may have selected for specialized *Dhc* strains carrying *vcrA*.

Taken together, this study demonstrated that *Dhc* strains in consortium BDI can survive low pH stress and recover at least partial dechlorination capability, but the duration of low pH exposure has a strong effect on recovery of complete dechlorination activity to ethene. These findings have implications for *in situ* bioremediation and suggest that extended periods of low pH exposure are detrimental to *Dhc*. As a consequence, bioaugmentation with *Dhc* capable of VC-to-ethene dechlorination after adjusting the groundwater to circumneutral pH may be required to reach cleanup goals. Furthermore, environmental stressors (e.g. temperature, oxygen, pH) disproportionally impact *Dhc* strains with VC RDase genes, which may partially explain the frequency of VC stalls observed at contaminated sites. Therefore, a priority goal to more successfully implement enhanced reductive dechlorination at sites impacted by chlorinated solvents is to develop mechanistic understanding that explains the observed sensitivity of the final VC-to-ethene reductive dechlorination step to environmental stressors, and possibly implement remedial strategies (standalone or treatment trains) that specifically overcome this bottleneck.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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