# Normalized Quantitative PCR Measurements as Predictors for Ethene Formation at Sites Impacted with Chlorinated Ethenes

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**S** Supporting Information

**ABSTRACT:** Quantitative PCR (qPCR) targeting *Dehalococcoides mccartyi* (*Dhc*) biomarker genes supports effective management at sites impacted with chlorinated ethenes. To establish correlations between *Dhc* biomarker gene abundances and ethene formation (i.e., detoxification), 859 groundwater samples representing 62 sites undergoing monitored natural attenuation or enhanced remediation were analyzed. *Dhc* 16S rRNA genes and the vinyl chloride (VC) reductive dehalogenase genes *bvcA* and *vcrA* were detected in 88% and 61% of samples, respectively, from wells with ethene. *Dhc* 16S rRNA, *bvcA*, *vcrA*, and *tceA* (implicated in cometabolic reductive VC dechlorination) gene abundances all positively correlated with ethene formation. Significantly greater ethene concentrations were observed when *Dhc* 16S rRNA gene and VC RDase gene abundances exceeded $10^7$ and $10^6$ copies $L^{-1}$, respectively, and when *Dhc* 16S rRNA- and *bvcA* + *vcrA*-to-total bacterial 16S rRNA gene ratios exceeded 0.1%. *Dhc* 16S rRNA gene-to-*vcrA*/*bvcA* ratios near unity also indicated elevated ethene; however, no increased ethene was observed in 19 wells where *vcrA* and/or *bvcA* gene copy numbers exceeded *Dhc* cell numbers 10- to 10 000-fold. Approximately one-third of samples with detectable ethene lacked *bvcA*, *vcrA*, and *tceA*, suggesting that comprehensive understanding of VC detoxification biomarkers has not been achieved. Although the current biomarker suite is incomplete, the data analysis corroborates the value of the available *Dhc* DNA biomarkers for prognostic and diagnostic groundwater monitoring at sites impacted with chlorinated ethenes.
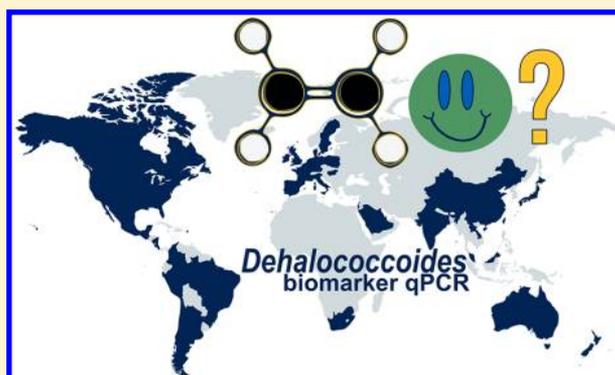
## INTRODUCTION

*Dehalococcoides mccartyi* (*Dhc*) strains are adept at removing chlorine substituents from a wide variety of chlorinated priority contaminants while conserving energy for growth.[1] In particular, the presence of *Dhc* has been linked to the reductive dechlorination of chlorinated ethenes to nontoxic ethene and inorganic chloride.[2−5] Quantitative real-time PCR (qPCR) targeting *Dhc* reductive dehalogenase (RDase) and *Dhc* 16S rRNA genes is the tool of choice for assessment of monitored natural attenuation (MNA) and enhanced bioremediation.[6−8] Detoxification of chlorinated ethenes at numerous contaminated sites occurs when *Dhc* abundances exceed $10^7$ *Dhc* $L^{-1}$.[4,7,9] Of the 444 distinct *Dhc* putative RDase proteins listed in the NCBI Identical Protein Groups database, PceA, TceA, PteA, MbrA, and PcbA1, PcbA4, and PcbA5 have been linked to reductive dechlorination of tetrachloroethene (PCE) and/or trichloroethene (TCE).

PceA and PteA dechlorinate PCE to TCE,[10,11] and MbrA, PcbA1, PcbA4, and PcbA5 were reported to dechlorinate PCE and TCE to a mixture of *cis*-1,2-dichloroethene (cDCE) and *trans*-1,2-dichloroethene (tDCE).[12,13] *Dhc* strains harboring the *tceA* gene can use TCE, cDCE, and tDCE as respiratory electron acceptors but cannot grow with VC; however, *Dhc* strains harboring *tceA* have been implicated in cometabolic transformation of VC to ethene.[14,15] The only *Dhc* RDases implicated in growth-linked reductive dechlorination of dichloroethenes to VC and to ethene are VcrA and BvcA.[16−18] Of note, strain BAV1 harboring BvcA comet-

abolizes TCE, and possibly PCE, in the presence of a growth-supporting electron acceptor (i.e., DCEs and VC).[18]

Molecular tools have become invaluable for monitoring processes of interest in environmental systems, and extracting DNA from groundwater has become routine laboratory practice.[4] qPCR has emerged as a mainstay technology to detect and quantify target genes in clinical and environmental samples.[19−21] On the basis of the available *Dhc* genome information, *Dhc* RDase and 16S rRNA genes occur as single copy genes,[22−24] and the application of both phylogenetic and functional gene-based qPCR measurements provides information about the abundance of *Dhc* strains carrying specific RDase genes capable of reductive dechlorination of different chlorinated ethenes.[5,6,8,25] The analysis of these biomarker genes can be used to predict a plume's trajectory without treatment, establish the need for enhanced bioremediation treatment, and track progress during bioremediation applications.[26,27] The value of *Dhc* biomarker gene analysis for in situ monitoring programs has been demonstrated and is commonly applied at sites slated for MNA or enhanced bioremediation treatment.[4,7,8] To further validate the *Dhc* quantitative biomarker approach and establish correlations between qPCR data and reductive dechlorination, we performed an integrated analysis of biomarker gene abundances, contaminant concentrations and ethene formation in 859 groundwater samples representing 62 sites impacted with chlorinated ethenes undergoing physical/chemical treatment, MNA, or enhanced bioremediation (i.e., biostimulation or biostimulation combined with bioaugmentation).

## ■ MATERIALS AND METHODS

**Materials.** The MO BIO PowerSoil kit (MO BIO Laboratories, Carlsbad, CA) was used to extract DNA from groundwater following previously described modifications and reagents for qPCR analysis were obtained from Life Technologies (Carlsbad, CA).[6] Millipore Sterivex cartridges (SVGPL10RC) were used to collect biomass from groundwater. Chemicals for medium preparation were purchased from Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich (St. Louis, MO).

**Data Collection.** Microbial Insights (MI, www.microbe.com) maintains an internal database of qPCR results of the abundances of 16S rRNA genes of microorganisms and functional genes (e.g., RDase genes) involved in biodegradation of common groundwater contaminants. At the time of manuscript preparation, the MI database contained site samples from across the U.S. and 33 countries around the world, including 27 707 groundwater samples that had been analyzed for *Dhc* 16S rRNA genes. Of these groundwater samples, a subset of 859 samples that had associated geochemical data, including ethene, methane, and/or sulfate concentrations as well as concentrations of chlorinated ethenes, was used in the current study (Figure S1 of the Supporting Information, SI).

Of these 859 groundwater samples, 573 were collected from monitoring wells at sites undergoing MNA, 157 were from wells influenced by electron donor addition (biostimulation), and 73 were from wells in areas where biostimulation was applied in combination with bioaugmentation (i.e., injection of a *Dhc*-containing consortium). The remainder of the groundwater samples were collected from monitoring wells at sites undergoing treatment with zerovalent iron (37 samples), chemical oxidation (8 samples), or unspecified treatment (11

samples). Dissolved ethene and methane concentrations were available for 625 and 554 groundwater samples, respectively. Sulfate concentrations were reported for 685 groundwater samples, including 148 samples from monitoring wells at sites undergoing biostimulation or bioaugmentation. Detection limits were in the ranges of 2 to 10 mg L$^{-1}$ for sulfate, 5 to 50 $\mu$g L$^{-1}$ for dissolved methane, and 1 to 5 $\mu$g L$^{-1}$ for dissolved ethene.

**Statistical Analysis and Data Processing.** An analysis of variance (ANOVA) was performed to reveal significant differences in concentrations of ethene, sulfate, and methane (dependent variables) with *Dhc* and RDase gene abundances as categorical predictors. Due to non-normal distributions and/or nonequal variances, the nonparametric method Kruskal−Wallis ANOVA was used to determine significant differences among groups based on ranks or medians.[28,29] The Kruskal−Wallis test is an alternative to parametric one-way ANOVA and used to test the null hypothesis that different samples in the comparison were drawn from distributions with the same median. As an example, in Figure 1A the Kruskal−Wallis test was used to determine whether the mean ranks of ethene concentrations were the same for different *Dhc* abundance groups. For some statistical analyses, such as the calculation of Spearman rank order coefficients ($\rho$) between the logarithm of *Dhc* and RDase gene abundances and chlorinated ethene concentrations, the data set was constrained to samples in which *Dhc* abundances exceeded the practical quantitation limit (typically 10$^2$ 16S rRNA gene copies L$^{-1}$). Kruskal−Wallis ANOVA with sulfate and methane as the dependent variables and log *Dhc* 16S rRNA gene copies as the categorical predictor was limited to groundwater samples from locations undergoing biostimulation alone or combined with bioaugmentation. This approach focused the analysis on samples where electron donor limitation was unlikely, thereby isolating geochemical conditions and providing the opportunity to assess the impact of methanogenesis as the primary variable impacting *Dhc* biomarker gene abundances. Sample sizes and selection criteria for each analysis are included in the respective figure legends. The strengths of Spearman rank order coefficients were qualified as follows: $\rho < 0.2$ was very weak; $\rho = 0.2−0.39$ was weak; $\rho = 0.4−0.59$ was moderate; $\rho = 0.6−0.79$ was strong; $\rho = 0.8−1.0$ was very strong. All statistical analyses were performed using Statistica v12.0 (StatSoft, Inc., Tulsa, OK) with $\alpha = 0.05$ (95% confidence level) unless otherwise noted.

**Groundwater Sampling, DNA Extraction and Quantitative Real-Time PCR (qPCR).** Groundwater collected by low-flow pumping techniques was shipped to MI in 1-L containers on ice as previously described,[30] or alternatively biomass was collected *on site* using Sterivex cartridges.[4] DNA from groundwater samples and Sterivex cartridges was extracted as described.[4] Protocols to prepare qPCR calibration curves and quantify total bacterial 16S rRNA genes, *Dhc* 16S rRNA genes, and the RDase genes *tceA*, *bvcA*, and *vcrA* followed established procedures.[4,6]

**Quality Assurance.** Field samples were immediately placed in a cooler with blue ice, shipped overnight to the analytical laboratory, and DNA extractions were performed upon receipt. DNA quantification using an UV/vis spectrophotometer was not possible for the majority of DNA samples due to low A260 nm readings. For qPCR, eight-point standard curves were generated with 10-fold dilutions of linearized plasmid DNA carrying single copies of the respective target
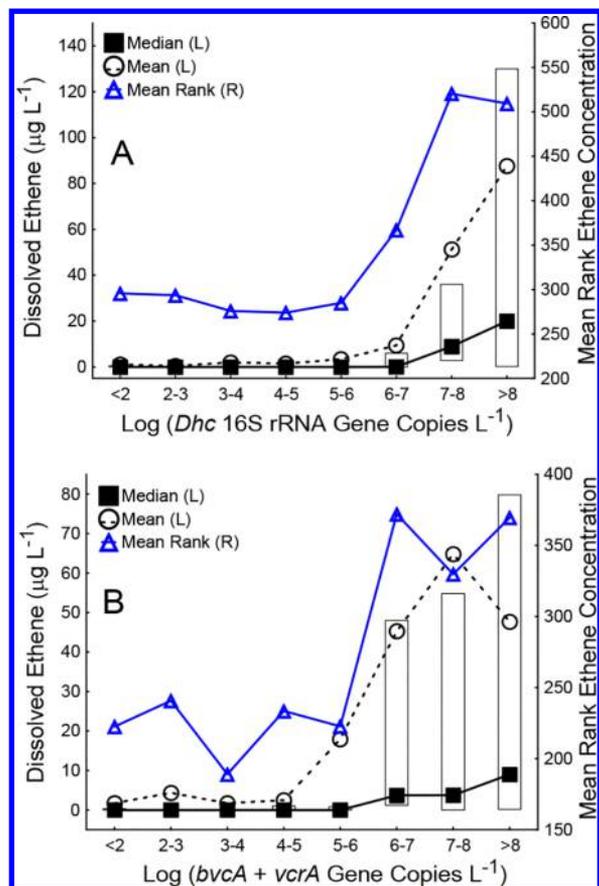
**Figure 1.** Correlations between *Dhc* biomarker gene abundances and ethene concentrations. Ethene concentrations were significantly greater in samples with (A) *Dhc* 16S rRNA gene and (B) VC RDase gene abundances greater than $10^7$ copies $L^{-1}$ and $10^6$ copies $L^{-1}$, respectively. The Kruskal−Wallis H test, also known as the one-way ANOVA on ranks, was performed with ethene concentration as the dependent variable (*y* axis) and order of magnitude ranges of log *Dhc* or log *bvcA* + *vcrA* abundance as the independent, categorical factor (*x* axis). For each category (e.g., log *Dhc* 16S rRNA gene copies $L^{-1}$ < 2), the median, mean, and mean ranks of ethene concentrations are shown. Boxes represent the interquartile range. The top and the bottom of each box represent the 25th and the 75th percentiles, respectively. Kruskal−Wallis tests [H(k−1,N)] for nonparametric one-way ANOVA indicated significant differences in mean ranks of ethene concentrations with log *Dhc* abundance [H(7,N = 625) = 141.3, *p* < 0.05] and log *bvc+vcrA* abundance [H(7,N = 479) = 83.1, *p* < 0.05] as the independent variables.

genes at 1 ng $\mu L^{-1}$ starting concentration,[6] and accompanied each qPCR plate.[20] Undiluted and 1:10 diluted DNA samples were analyzed to achieve two data sets for each sample and to recognize PCR inhibition.[4] Nonexponential fluorescence signal

increase or other than a 10-fold difference in target gene enumeration in the dilutions of template DNA indicated PCR inhibition,[31] and those reactions were not included in the analysis.

## RESULTS

***Dhc* Biomarker Gene and RDase Gene Detection Frequencies.** *Dhc* 16S rRNA genes were detected in 88% (755) of the 859 groundwater samples analyzed. Of those 755 groundwater samples with detectable (i.e., > $10^2$ gene copies $L^{-1}$) *Dhc* 16S rRNA genes, 625 had also been analyzed for *tceA*, *bvcA*, and *vcrA*, with detection frequencies of 47%, 32%, and 42%, respectively. In 301 groundwater samples with quantifiable (i.e., > $5 \times 10^2$ 16S rRNA gene copies $L^{-1}$) *Dhc* and with at least one VC RDase gene detected, both *bvcA* and *vcrA* were present in 52% (156) of samples. In this subset of 301 groundwater samples, *bvcA*, but not *vcrA*, was detected in 13% (40) and *vcrA*, but not *bvcA*, was detected in 35% (105) of samples. Overall, the high *Dhc* 16S rRNA gene and RDase gene detection frequencies demonstrate that *Dhc* are commonly present in chlorinated solvent-impacted aquifers although their abundance may be low (i.e., < $10^3$ 16S rRNA genes $L^{-1}$).

**Correlation of *Dhc* Biomarker Gene Abundances with Concentrations of Chlorinated Ethenes and Ethene.** Spearman rank order correlation coefficients were calculated between contaminant concentrations and the logarithm of quantifiable *Dhc* 16S rRNA gene and RDase gene abundances (Table 1). This analysis revealed that PCE concentrations were very weakly correlated with *tceA* and *vcrA* gene abundances and not significantly correlated with *Dhc* 16S rRNA gene or *bvcA* abundances. In contrast, TCE, cDCE, tDCE, VC, and ethene concentrations were all significantly positively correlated with *Dhc* 16S rRNA, *tceA*, *bvcA*, or *vcrA* gene abundances. Correlations between TCE concentrations and *Dhc* 16S rRNA gene and RDase gene abundances were weak. For cDCE, correlations with *Dhc* 16S rRNA, *tceA*, and *vcrA* genes were moderate while the correlation with *bvcA* was weak. A strong correlation was noted between VC and *tceA* gene abundance, and moderate correlations were observed with *Dhc* 16S rRNA and *vcrA* gene abundances. The correlation between VC and *bvcA* was relatively weak. Ethene concentration was moderately correlated with *tceA* gene abundance. While weak, ethene concentrations positively correlated with the abundances of *Dhc* and both VC RDase genes.

**Kruskal−Wallis ANOVA with *Dhc* 16S rRNA Gene and RDase Gene Abundances and Ethene Concentrations.** Dissolved ethene, an indicator for detoxification, was analyzed in 625 and detected in 237 groundwater samples. *Dhc* was present in 87% (207) of the 237 samples with detectable ethene. The *bvcA* and/or *vcrA* genes or the *tceA* gene were

**Table 1. Spearman Rank Order Correlation Coefficients (ρ) between the Logarithm of *Dhc* 16S rRNA Gene and RDase Gene Abundances and Chlorinated Ethene Concentrations in Groundwater Samples[a]**

| target gene | Log (biomarker gene copies $L^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | PCE | TCE | cDCE | tDCE | VC | ethene |
| *Dhc* 16S rRNA | 0.04 (682) | **0.28 (713)** | **0.46 (722)** | **0.29 (528)** | **0.57 (723)** | **0.34 (535)** |
| *tceA* | **0.14 (255)** | **0.30 (266)** | **0.46 (279)** | **0.23 (195)** | **0.62 (277)** | **0.49 (188)** |
| *bvcA* | 0.06 (185) | **0.20 (187)** | **0.32 (187)** | **0.38 (116)** | **0.37 (189)** | **0.26 (172)** |
| *vcrA* | **0.17 (236)** | **0.33 (246)** | **0.52 (257)** | **0.34 (168)** | **0.55 (260)** | **0.39 (236)** |

[a]Bold denotes significant correlations at α = 0.05. Number of observations for each pair (n) is provided in parentheses next to the coefficient.

present in 61% (107 out of 175) and 50% (87 out of 175), respectively, of samples where RDase genes were analyzed and ethene was detected. Kruskal−Wallis ANOVA was performed to determine the minimum *Dhc* and RDase gene abundances that correlated with elevated ethene concentrations. At *Dhc* abundances greater than $10^7$ 16S rRNA gene copies $L^{-1}$, the mean rank of ethene concentrations was significantly higher than observed at lower *Dhc* abundances (Figure 1A, *p* values <0.009). The mean rank of ethene concentrations was significantly higher when the sum of *bvcA* + *vcrA* gene abundances was greater than $10^6$ copies $L^{-1}$ (Figure 1B, *p* values <0.004).

Ethene concentrations noticeably increased when *Dhc* 16S rRNA gene copies were on the order of 0.1−1% of total bacterial 16S rRNA gene copies. Statistically, the mean rank of ethene concentrations was significantly greater in groundwater samples with *Dhc* greater than 1% of total bacterial 16S rRNA gene copies (Figure 2A, *p* values <0.002). The overall variability in bacterial 16S rRNA gene abundances was low based on the standard deviation (1.03) and semi-interquartile range (0.59) of the logarithm of bacterial 16S rRNA gene copies. Thus, the ratio of *Dhc*-to-bacterial 16S rRNA gene copies could serve as a means for evaluating the potential for complete reductive dechlorination. At $\alpha = 0.10$, the mean rank of ethene concentrations was significantly greater in groundwater samples when *bvcA* + *vcrA* gene copies were greater than 0.1% of total bacterial 16S rRNA gene copies (Figure 2B).

**Ethene Detection in Groundwater Samples with Low *Dhc* Abundance.** Out of 237 groundwater samples with measurable ethene, 30 samples (21 from MNA and nine from biostimulation sites) had fewer than $10^2$ 16S rRNA gene copies $L^{-1}$ and *tceA*, *vcrA*, and *bvcA* (with one exception) genes were not detected. The average total concentrations of chlorinated ethene exceeded 6700 $\mu$g $L^{-1}$ in 27 samples, and only three samples had average total concentrations of less than 50 $\mu$g $L^{-1}$. Of these 30 samples, only five had reported ethene concentrations of greater than 5 $\mu$g $L^{-1}$. One of these samples was collected from a site where zerovalent iron (ZVI) treatment had been implemented, suggesting that abiotic degradation may have contributed to ethene production. In addition, three of the five samples were collected from sites where adjacent sampling locations showed *Dhc* abundances greater than $10^6$ 16S rRNA gene copies $L^{-1}$. Thus, ethene detection in wells with low *Dhc* abundances at these three sites may have been the result of advective transport of dissolved ethene from upgradient areas with active reductive dechlorination to ethene.

**Lack of Ethene Detection in Groundwater Samples with High *Dhc* Abundance.** Significantly higher ethene concentrations were detected in 87% (62 samples) of the 71 samples with *Dhc* abundances exceeding $10^7$ copies $L^{-1}$ (Figure 1A, *p* values <0.009). Of the remaining nine samples with high *Dhc* but no observed ethene, four (three from biostimulation sites and one from an MNA site) had VC RDase genes greater than $10^5$ copies $L^{-1}$. In these cases, PCE, TCE, or DCE isomers were the predominant chlorinated ethenes ($0.75 < X_{PCE} + X_{TCE} + X_{DCE} < 0.96$); and for three of these four samples, VC concentrations were below 5 $\mu$g $L^{-1}$. With high *Dhc* abundances and detection of VC RDase genes at $10^5$ copies $L^{-1}$, ethene would have likely been detected in subsequent groundwater monitoring events as reductive dechlorination proceeded. In the remaining five samples (all MNA) with high *Dhc* but no ethene, the total VC RDase gene
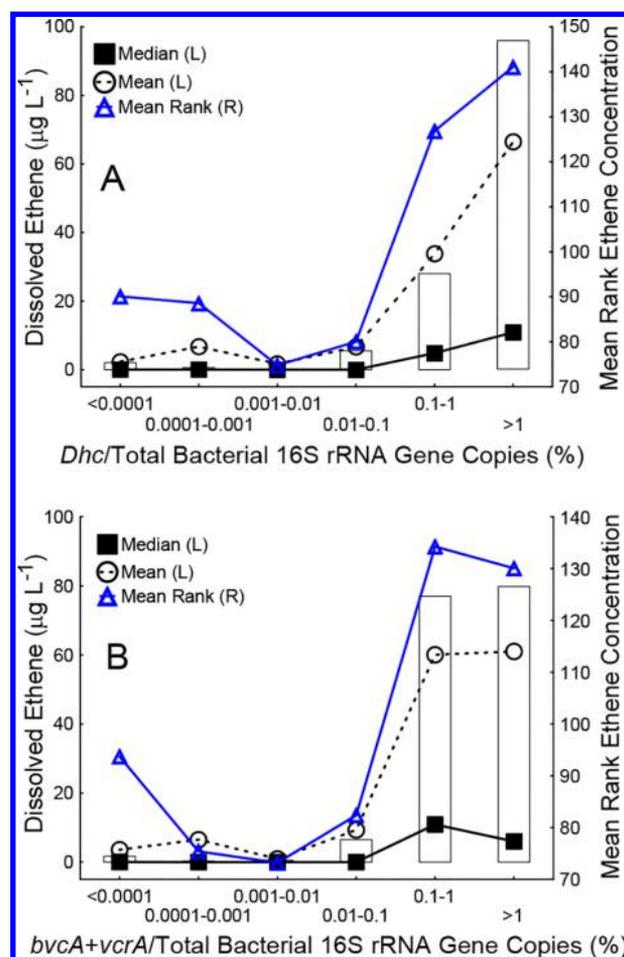


**Figure 2.** *Dhc* biomarker gene abundances normalized to total bacterial 16S rRNA gene abundances inform about ethene formation potential. Ethene concentrations were significantly greater in samples with (A) *Dhc* 16S rRNA gene and (B) VC RDase gene abundances greater than 1% and 0.1% of the bacterial 16S rRNA gene abundance, respectively. The Kruskal−Wallis H test was performed with ethene concentration as the dependent variable (*y* axis) and ranges of (A) percent *Dhc*/bacterial 16S rRNA gene abundance and (B) percent *bvc* +*vcrA* /bacterial 16S rRNA gene abundance as the independent, categorical factor (*x* axis). For each category (e.g., percent *Dhc*/bacterial 16S rRNA gene abundance >1%), the median, mean, and mean rank of ethene concentration are shown. Boxes represent the interquartile range. The top and the bottom of each box represent the 25th and the 75th percentiles, respectively. Kruskal−Wallis tests [H(*k*−1,*N*)] for nonparametric one-way ANOVA indicated significant differences in mean ranks of ethene concentrations with percent *Dhc*/bacterial 16S rRNA gene abundance [H(5,*N* = 197) = 39.0, *p* < 0.05] and percent *bvc*+*vcrA*/bacterial 16S rRNA gene abundance [H(5,*N* = 197) = 25.8, *p* < 0.05] as the independent variables.

abundances (i.e., *bvcA* + *vcrA*) were less than $10^4$ copies $L^{-1}$, but *tceA* abundances ranged from $10^4$ to $10^6$ copies $L^{-1}$. As shown in Figure 1B, ethene concentrations generally increased when VC RDase gene abundances exceeded $10^5$ gene copies $L^{-1}$ and were significantly higher at $10^6$ VC RDase gene copies $L^{-1}$. Thus, a lower abundance of *Dhc* strains harboring VC RDase genes may explain limited ethene formation in these samples.

**Influence of Treatment Approach on *Dhc* and RDase Gene Abundances.** Kruskal−Wallis ANOVA was performed to identify any significant differences in the abundances of *Dhc* and RDase genes between treatments (Table 2). When present

**Table 2. Differences in the Abundances of *Dhc* 16S rRNA and RDase Gene Copies in Groundwater from Sites Experiencing Different Treatments**[a]

| | gene abundance | | | |
|---|---|---|---|---|
| treatment | *Dhc* 16S rRNA | *tceA* | *bvcA* | *vcrA* |
| bioaugmentation | A ($<10^{-3}, <10^{-7}$) | A ($<10^{-3}, <10^{-7}$) | A (0.03, 0.006) | A ($<10^{-3}, <10^{-7}$) |
| biostimulation | B ($<10^{-6}$) | B (0.007) | B | B |
| MNA | C | C | B | B |

[a]The letters A, B, and C denote significant differences in the abundance of a given target gene between treatments. The same letter in a given target gene column denotes that no significant differences in target gene abundances were observed. Values in parentheses are *p* values for significant differences in target gene abundances between treatments. For bioaugmentation, *p* values are listed versus biostimulation and versus MNA. For biostimulation, the *p* value listed is versus MNA.

at quantifiable abundances, *Dhc* 16S rRNA genes and *tceA*, *bvcA*, and *vcrA* genes were each significantly more abundant in samples influenced by bioaugmentation than with biostimulation alone. At biostimulation sites that received fermentable substrate (i.e., electron donor) addition(s), the *Dhc* 16S rRNA ($p < 10^{-6}$) and *tceA* ($p < 0.007$) genes were significantly more abundant compared to MNA sites. In contrast, the *bvcA* and *vcrA* genes were not significantly more abundant in samples from biostimulation sites than MNA sites.

At bioaugmentation sites, the *Dhc* abundance met or exceeded the $10^7$ gene copies L$^{-1}$ threshold for strong ethene formation in 59% (43/73) of the samples, and the *vcrA* abundances exceeded the $10^6$ copies L$^{-1}$ threshold in over 70% (52/73) of the samples (Figure S2). Ethene was analyzed and detected in 27 of the samples meeting the *Dhc* threshold. At biostimulation sites, the *Dhc* abundances met the $10^7$ gene copies L$^{-1}$ threshold in 23% (36/157) of the samples analyzed. Unfortunately, ethene was analyzed in only 9 of these 36 samples and was detected in six of them. VC RDase gene abundances exceeded $10^6$ gene copies L$^{-1}$ in less than 10% of the samples from sites undergoing biostimulation or MNA treatment.

**Effect of Sulfate and Methane Concentrations on *Dhc* Abundances.** The relatively low number of samples with desirable *Dhc* 16S rRNA gene (i.e., > $10^7$ copies L$^{-1}$) and VC RDase gene (i.e., >$10^6$ copies L$^{-1}$) abundances from sites undergoing biostimulation and even bioaugmentation was surprising. A Kruskal–Wallis ANOVA on ranks was performed to examine the relationship between sulfate and methane concentrations and *Dhc* abundances for samples influenced by biostimulation and bioaugmentation. The mean ranks of dissolved sulfate concentrations were significantly lower in samples ($n = 142$) with *Dhc* abundances greater than $10^5$ 16S rRNA gene copies L$^{-1}$ (Figure 3A, *p* values <0.0001) corresponding to a mean sulfate concentration of 20–25 mg L$^{-1}$. Dissolved sulfate was largely absent in groundwater samples undergoing biostimulation or bioaugmentation treatments at higher *Dhc* abundances. With the limited number of samples from biostimulation and bioaugmentation sites with available methane data ($n = 43$), statistical analysis with samples from all treatment approaches revealed that the mean rank of methane concentrations was significantly greater in samples with *Dhc* abundances greater than $10^7$ 16S rRNA gene copies L$^{-1}$. The average methane concentration was ~1450 µg L$^{-1}$ in samples with high *Dhc* abundances, whereas in samples with lower methane concentrations of ~180 µg L$^{-1}$, $10^2$ or fewer *Dhc* 16S rRNA gene copies L$^{-1}$ were detected (Figure 3B, *p* value <0.0001).

**RDase Gene Copies Exceeding *Dhc* 16S rRNA Gene Copies.** In axenic *Dhc* cultures, RDase genes occur in a 1:1 ratio with the *Dhc* 16S rRNA gene (Figure 4A; solid line). Interestingly, the abundances of one or more RDase gene(s) exceeded *Dhc* 16S rRNA gene copies by at least an order of magnitude in 3% (19) of the 626 groundwater samples with quantifiable *Dhc* 16S rRNA and RDase genes (Figure 4A; dashed line). The frequency, with which the *tceA*, the *vcrA*, or the *bvcA* RDase gene abundances substantially exceeded the *Dhc* cell numbers was similar (approximately 2%), and often more than one RDase gene exceeded the *Dhc* cell number in a given sample. In the 13 samples where *bvcA* gene copies were at least 10-fold greater than the *Dhc* abundance, *tceA* and *vcrA* also exceeded the *Dhc* numbers in six and 11 samples, respectively. Regardless of how frequently RDase gene copies substantially exceeded the *Dhc* abundances, ethene production was not enhanced in samples exhibiting VC RDase genes abundances 10- to 10 000-fold higher than the *Dhc* 16S rRNA gene abundance (Figure 4B). Of note, in a few groundwater samples with *bvcA* + *vcrA*/*Dhc* 16S rRNA gene ratio <0.01, substantial ethene concentrations were observed (Figure 4B). This observation suggests that yet to be discovered RDases contribute to VC to ethene reductive dechlorination.

## ◼ DISCUSSION

A suite of remediation technologies is available to tackle cleanup at sites impacted with chlorinated solvents, particularly chlorinated ethenes. Bioremediation has emerged as a productive stand-alone remedial approach or is applied in combination with a physical or chemical treatment approach.[32−35] At sites impacted with chlorinated ethenes, *Dhc* strains play key roles in catalyzing the reductive dechlorination and detoxification to environmentally benign ethene under anoxic conditions.[1,5] A number of site-specific studies have demonstrated the value of *Dhc* biomarker-targeted site monitoring, (e.g.,[8,36,37]) and the quantitative analysis of *Dhc* biomarker genes is commonly applied during site assessment and bioremediation monitoring regimes at sites impacted with chlorinated ethenes.

***Dhc* Detection Frequency in Groundwater.** When first discovered, *Dhc* was considered to have limited distribution due to its restricted lifestyle that hinges on hydrogen as electron donor and specific chloroorganic compounds as electron acceptors.[2,5] The data analysis presented here demonstrated that *Dhc* are commonly present in contaminated aquifers (detected in 682 out of 786 [87%] samples examined) without bioaugmentation treatment. This observation is consistent with an increasing number of studies reporting 16S rRNA gene sequences representing organohalide-respiring Chloroflexi in environments not impacted by chlorinated solvent contamination.[38,39] Apparently, *Dhc* strains are members of microbial communities in pristine environments
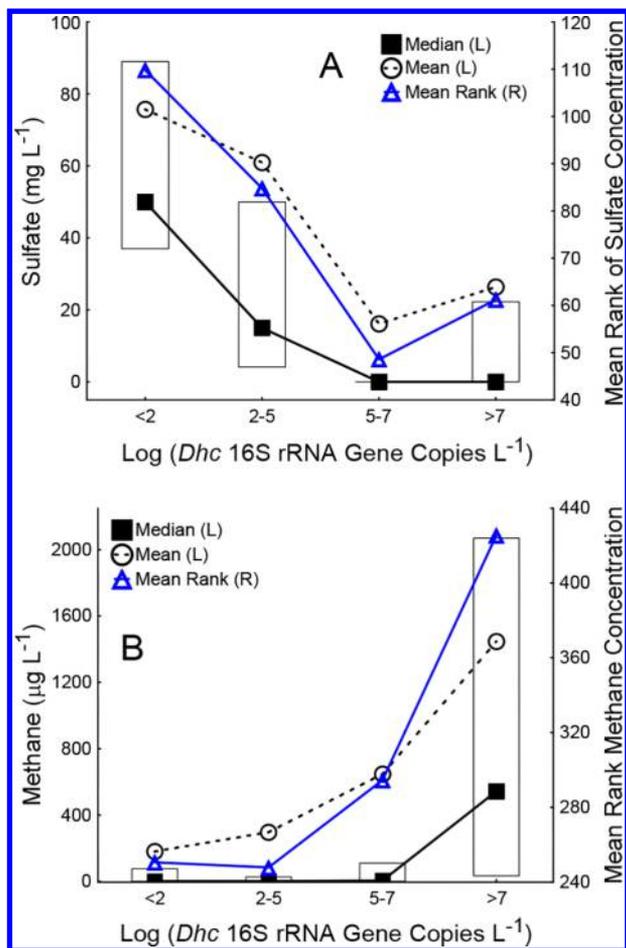
**Figure 3.** Effect of geochemical conditions on *Dhc* abundance. (A) Sulfate concentrations were significantly lower in samples with *Dhc* abundances greater than $10^5$ copies $L^{-1}$, and (B) *Dhc* abundances greater than $10^7$ copies $L^{-1}$ correlated with elevated methane concentrations. The Kruskal–Wallis H test was performed with (A) sulfate concentration and (B) methane concentration as the dependent variables (*y* axis) and order of magnitude ranges of log *Dhc* abundance as the independent, categorical factor (*x* axis). For each category (e.g., log *Dhc* 16S rRNA gene copies $L^{-1}$ < 2), the median, mean, and mean rank of ethene concentration are shown. Only samples from sites undergoing biostimulation or bioaugmentation were considered for analysis with sulfate as the dependent variable. Due to the limited number of samples from biostimulation and bioaugmentation sites where methane was reported, all available data were included during analysis with methane as the dependent variable. Kruskal–Wallis tests $[H(k-1,N)]$ for nonparametric one-way ANOVA indicated significant differences in mean ranks of sulfate $[H(3,N = 142) = 40.9]$ and methane $[H(3,N = 554) = 69.8]$ concentrations with log *Dhc* abundance as the independent variable.

where naturally occurring organohalogens support their energy metabolism. Since many *Dhc* strains use chlorinated ethenes as respiratory electron acceptors, the high detection frequency of *Dhc* in groundwater impacted with chlorinated ethenes is not surprising.

**Correlation between Ethene Formation and *Dhc* Biomarker Gene Abundances.** The complete dechlorination of chlorinated ethenes generally coincided with greater abundances of the *Dhc* 16S rRNA genes and VC RDase genes. The observed threshold *Dhc* abundance of $10^7$ 16S rRNA gene copies $L^{-1}$ for significantly greater ethene production (Figure 1A) is consistent with previous reports that suggested an
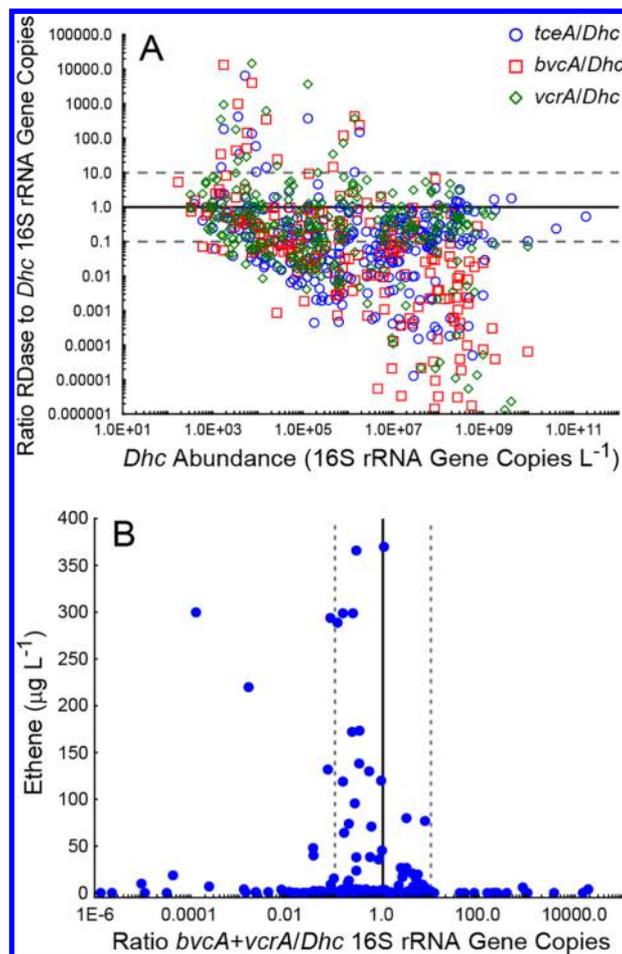


**Figure 4.** Correlations between RDase gene and *Dhc* 16S rRNA gene abundances with detoxification potential. (A) Ratio of RDase to *Dhc* 16S rRNA gene copies. The solid black line indicates a 1:1 ratio of RDase to *Dhc* 16S rRNA gene copies. Points above the dashed line are from the 19 groundwater samples where the abundance of RDase genes exceeded *Dhc* by 10-fold or greater. (B) Ethene concentration as a function of the ratio of the sum of VC RDase gene copies to *Dhc* 16S rRNA gene copies (*bvcA* + *vcrA*/*Dhc*). The highest ethene concentrations were observed when the ratio of RDase to *Dhc* 16S rRNA genes was near 1.0. The solid black line indicates that the sum of the RDase genes (i.e., *bvcA* + *vcrA*) equals the number of *Dhc* 16S rRNA genes. The dashed lines outline a 10-fold difference from a 1:1 ratio as an estimated maximum uncertainty associated with target gene quantification.

abundance of $10^7$ *Dhc* cells $L^{-1}$ could be used as a screening criterion for generally useful rates of reductive dechlorination.[4,9] To date, *vcrA* and *bvcA* are the only known *Dhc* genes that encode for RDases with confirmed VC reductive dechlorination activity. Remarkably, in 40% (68 out of 175) of samples with confirmed presence of ethene, the known *Dhc* VC RDase genes *bvcA* and *vcrA* were not detected. The *tceA* gene was not detected in 34% (60 out of 175) of the samples indicating that cometabolic transformation of VC to ethene by TceA cannot explain ethene formation. Possible explanations for the detection of ethene in samples where the known VC RDase genes were absent include advective transport from zones where complete reductive dechlorination to ethene occurred, abiotic transformation reactions,[14] or the presence of yet-to-be-identified VC RDases.

**Evidence for Additional VC RDases and other Bacteria Contributing to VC Reductive Dechlorination.** In 3% (6 out of 175) of groundwater samples with ethene formation, the *Dhc* abundances exceeded $10^7$ 16S rRNA gene copies L$^{-1}$ but the VC RDase gene copies were orders of magnitude lower ($\leq 10^4$ copies L$^{-1}$). The *tceA* gene implicated in cometabolic VC transformation to ethene[2,40] was detected in half of the samples, indicating that cometabolic VC transformation to ethene could not explain ethene formation. Because the known *Dhc* are obligate organohalide-respiring bacteria, this observation suggests that *Dhc* not possessing the VC RDases *bvcA* and *vcrA* thrive in aquifers impacted with chlorinated ethenes and contribute to ethene formation. Since the first report linking *Dhc* to the complete reductive dechlorination of chlorinated ethenes,[2] the reductive dechlorination to environmentally benign ethene has been solely attributed to certain *Dhc* strains. This dogma is being challenged by a recent report demonstrating that *Candidatus* Dehalogenimonas etheniformans harboring a novel VC RDase, *cerA*, dechlorinates TCE to ethene.[41] Apparently, the diversity of organisms and genes involved in VC reductive dechlorination exceeds the known *Dhc* strains carrying *vcrA* or *bvcA*, suggesting that the current diagnostic and prognostic PCR monitoring tools do not provide comprehensive information. Therefore, efforts to enrich and isolate organisms that utilize toxic chlorinated alkanes and alkenes as growth substrates should not be abandoned; however, innovative cultivation approaches are needed because the contemporary enrichment and isolation approaches have not expanded the taxonomic diversity of anaerobic VC dechlorinators.

These observations further suggest that the currently available bioaugmentation consortia do not take advantage of the existing genetic diversity of organisms that contribute to anaerobic VC detoxification. The commercially available bioaugmentation consortia are produced with PCE or TCE provided as an electron acceptor and contain predominantly *Dhc* strains harboring *vcrA*. Not surprisingly, *vcrA* was generally much more abundant than *bvcA* at bioaugmentation sites. *Dhc* strains that possess *bvcA* rely on DCEs and VC as electron acceptors, as does *Dhc* strain BAV1, an isolate that can cometabolize but not conserve energy from PCE or TCE reductive dechlorination to cDCE.[3,5,17,23] *Dhc* strains that harbor both the *bvcA* and *vcrA* genes have not been found, and *Dhc* strains with the *vcrA* genotype outcompete *Dhc* harboring *bvcA* in bioaugmentation consortia fed with TCE as a growth substrate and possibly at sites with PCE and TCE as the primary contaminants.[8,42,43]

In addition to a weak positive correlation with ethene formation, elevated numbers of *bvcA* were found at MNA sites where tDCE and VC were the predominant contaminants. No correlation was observed between tDCE and the *vcrA* gene, although enzyme assays have suggested that *vcrA* of *Dhc* strain VS is able to dechlorinate tDCE; however, this activity has not been demonstrated in vivo.[16] *Dhc* strain GT, which carries the *vcrA* gene, failed to grow with tDCE as an energy source[1] lending credence to the notion that tDCE is a substrate for *Dhc* strains with *bvcA* but not for those carrying *vcrA*.

**Value of Relative Abundance Ratios Based on Quantitative qPCR Data.** All samples in this study were processed using the same protocols for sample handling, DNA extraction, and qPCR; however, no EPA-approved standard protocols exist. A systematic investigation of errors that can affect qPCR data recommended standard operating proce-

dures,[44] and methodologies to minimize operator-induced biases are available.[45,46] Still, qPCR data for the same sample can differ between laboratories due to laboratory-specific procedures. Using relative abundance ratios like the ratio of *Dhc* to total bacterial 16S rRNA gene copies could circumvent the potential impacts of procedural differences. In the current study, ethene concentrations were noticeably higher when ratios of *Dhc* 16S rRNA genes to total bacterial 16S rRNA genes were greater than 0.1% and were significantly greater when ratios of *Dhc* to total bacterial 16S rRNA genes were at least 1% (Figure 2A). Likewise, ethene concentrations were higher in samples where the ratio of the sum of VC RDase genes *bvcA* and *vcrA* to total bacterial 16S rRNA genes was at least 0.1% (Figure 2B). The frequencies of ethene detection at threshold levels or higher based on absolute abundance of *Dhc* 16S rRNA genes ($\geq 10^7$ copies L$^{-1}$), absolute VC RDase gene abundance ($\geq 10^6$ copies L$^{-1}$), and ratios of *Dhc* to total bacterial 16S rRNA gene copies ($\geq 0.1\%$) were similar (87%, 78%, and 82%, respectively) suggesting the abundance ratios based on qPCR data provide meaningful information.

Relative abundance information can also be obtained using 16S rRNA gene amplicon sequencing, a common approach to survey bacterial community structure,[47] or shotgun metagenomics, which avoids amplification altogether. In contrast to qPCR, amplicon sequencing does not provide absolute quantitative information,[48] and absolute quantification in metagenomics is challenging and has yet to be established.[49] Further, by nature of the polymerase chain reaction, qPCR is at least 1 order of magnitude more sensitive than current shotgun metagenomics approaches. While deeper sequencing can improve sensitivity, the cost for sequencing and analysis increase substantially. In summary, a qPCR strategy offers the required sensitivity, sufficient throughput, and generates absolute quantitative data that are directly comparable between samples collected over time and from different sites.

**VC RDase Genes Outnumber *Dehalococcoides* 16S rRNA Genes.** An interesting observation was that the gene copy numbers of a single, two or all three RDase genes (i.e., *tceA*, *vcrA*, and *bvcA*) exceeded the total *Dhc* cell abundance 10- to 10 000-fold in 19 samples (Figure 4B). Consistent with this observation, a 100-fold excess of *vcrA* genes relative to the total *Dhc* abundance was seen at 11 sites impacted with chlorinated ethenes in The Netherlands and one aquifer in the U.K.[50] Further, a 6.5 fold higher *vcrA* gene ($9.96 \times 10^{11}$ L$^{-1}$) than *Dhc* 16S rRNA gene ($1.54 \times 10^{11}$ L$^{-1}$) abundance was reported in a TCE-dechlorinating bioreactor.[51] While two- or three-fold differences in RDase to *Dhc* cell abundances could be the result of qPCR variation or due to gene duplication within a dominant *Dhc* strain, neither explains the up to 10 000-fold difference in gene enumeration (Figure 4B). In the 859 sample survey reported here, the greatest fold-differences between the *Dhc* 16S rRNA gene and RDase gene copy numbers occurred when *Dhc* cells were present in low abundances (i.e., in the range of $10^3$ to $10^4$ cells L$^{-1}$) and mostly at MNA sites. *Dehalogenimonas*, another genus within the family *Dehalococcoidia*, has been implicated in reductive dechlorination of chlorinated alkanes,[52−54] TCE,[41] and tDCE.[55] Horizontal gene transfer between *Dhc* and *Dehalogenimonas* can occur;[56] however, there is no evidence to date for the presence of *bvcA* and *vcrA* in *Dehalogenimonas* genomes. Since the overabundance of RDase genes did not correlate with heightened dechlorination activity (i.e., ethene formation), an external RDase gene reservoir must be considered. An

intriguing possibility is the occurrence of these RDase genes on phage genomes[57] or as components of gene transfer agents,[58] which is an exciting aspect of *Dhc* biology that warrants exploration.

**Effect of Treatment Strategies and Sulfate Concentrations on *Dhc* Abundances.** Enhanced anaerobic bioremediation is an established treatment at sites impacted by chlorinated ethenes. Ethene production was observed in nearly 70% of the groundwater samples obtained from sites undergoing biostimulation alone or combined with bioaugmentation. At a number of sites undergoing enhanced anaerobic bioremediation, in situ ethene formation occurred below the $10^7$ *Dhc* 16S rRNA gene copies $L^{-1}$ threshold for sustaining generally useful rates of reductive dechlorination.[4,9] The percentage of sites experiencing biostimulation treatment and reaching the $10^7$ *Dhc* 16S rRNA gene copies $L^{-1}$ threshold was 23% (36 out of 157), which is substantially lower than what is generally observed at field sites undergoing biostimulation treatment.[59]

On the basis of the data set available for this study, *Dhc* biomarkers were detected in the majority of groundwater samples from MNA sites (86%, 495/573) suggesting that lower than desired *Dhc* abundances after biostimulation were not due to a lack of indigenous *Dhc*. A recent case study reported significantly greater *Dhc* abundances when sulfate concentrations were below 50 mg $L^{-1}$[60] and sulfate concentrations below 20 mg $L^{-1}$ have been considered favorable to reductive dechlorination.[61] The analysis of 685 samples with reported sulfate concentrations revealed that high *Dhc* abundances correlated with sulfate depletion (Figure 3A) and also with substantial methane generation (Figure 3B). For samples collected at biostimulation sites, sulfate concentrations were less than 20 mg $L^{-1}$ in 64% (91 of 142) of samples, and methane concentrations exceeded 50 μg $L^{-1}$ in 12% (5 of 43) of samples from biostimulation sites. Overall, the findings demonstrate the value of biostimulation combined with bioaugmentation or biostimulation alone to establish generally useful rates of reductive dechlorination, but also underscore the role of proper implementation to achieve favorable geochemical conditions for successful bioremediation.

**Database Limitations and Opportunities.** The integrated analysis of microbiological, geochemical, physical, hydrological, etc. data collected from chlorinated solvent-impacted aquifers can unravel unrecognized correlations and reveal new insights into the reductive dechlorination process. Although the current MI database contains qPCR data from more than 30 000 groundwater samples, associated site information is often incomplete, and only a subset of the data could be included in the analysis. A recent study demonstrated the value of machine learning-based data mining approaches to predict in situ detoxification potential of chlorinated ethenes, but highlighted the issue of data quality, completeness and accessibility.[62] The authors of this prior study emphasized the value of a curated, open-access, up-to-date and comprehensive collection of biogeochemical groundwater monitoring data.[62] The analyses performed in the current study were also limited to a subset of data included in the MI database, and three main issues were recognized: First, site-specific data are generated by different (commercial) laboratories and information is generally not recorded in a single depository. Second, public data access should not infringe on privacy rights, that is, the identity of a site under litigation cannot be released until a settlement has been reached. Third, inconsistent monitoring schedules and parameter sets, and a lack of standardized sampling protocols hamper integrated data analysis. Thus, building a systematic, comprehensive and open-access database that combines microbiological, geochemical, physical and hydrogeological aquifer/plume data, as well as information about treatment, should be a high priority. A number of U.S. states have implemented online data management systems for impacted sites (e.g., Geotracker; https://geotracker.waterboards.ca.gov), and a mechanism for data reporting in an open-access repository is available. What is currently lacking is a guidance document that provides information about the parameters analyzed and the frequency (i.e., time windows) of sample collection, so that the efforts generate the information necessary for productive, site-specific decision making. As a next step, remediation and regulatory communities should partner to include microbial information in established (public) repositories and develop mechanisms for data curation, retrieval and analysis. Such a resource will enable fundamental discoveries about the reductive dechlorination process and can be a pillar for efforts aimed at developing predictive understanding about the trajectory of a chlorinated solvent plume under different treatment scenarios.

**Implications for bioremediation practice.** The integrated data analysis reported here highlights the value of *Dhc* biomarker gene enumeration, and further demonstrates that ratios of *Dhc* to total bacterial 16S rRNA genes and of *bvcA* + *vcrA* to total bacterial 16S rRNA genes exceeding 0.1%, and a ratio of *vcrA* + *bvcA* to *Dhc* 16S tRNA genes near unity are useful normalized, measurable parameters for predicting detoxification (i.e., ethene formation) at sites impacted with chlorinated ethenes where *Dhc* are key dechlorinators. Thus, qPCR monitoring at sites impacted with chlorinated ethenes should include 16S rRNA genes of relevant dechlorinators (i.e., *Dhc*), key RDase genes such as *tceA*, *vcrA*, and *bvcA*, and general primers quantifying total bacterial 16S rRNA genes, ideally in concert with the collection of relevant geochemical information, including contaminant and ethene concentration data. The analysis revealed ethene production in wells that lacked the known *vcrA* or *bvcA* VC RDase genes, demonstrating that comprehensive understanding of VC detoxification biomarkers has not yet been obtained. Consequently, complete reductive dechlorination to environmentally benign ethene can occur in the absence of the known VC RDase genes *vcrA* and *bvcA*. The lack of open-access data management systems for groundwater monitoring data sets is a major hindrance, and remediation and regulatory communities should partner to include microbial information in established (public) repositories and develop mechanisms for data curation, retrieval and analysis.

## ■ ASSOCIATED CONTENT

**ⓢ Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b04373.

> Breakdown of microbial and geochemical data for the 859 groundwater samples by treatment, Figure S1; and illustration of *Dhc* 16S rRNA gene copy abundances by treatment, Figure S2 (PDF)

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**Notes**

The authors declare no competing financial interest.

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