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Correlation of *Dehalococcoides* 16S rRNA and Chloroethene-Reductive Dehalogenase Genes with Geochemical Conditions in Chloroethene-Contaminated Groundwater▽†

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Quantitative analysis of genes that code for *Dehalococcoides* 16S rRNA and chloroethene-reductive dehalogenases TceA, VcrA, and BvcA was done on groundwater sampled from 150 monitoring wells spread over 11 chlorinated ethene polluted European locations. Redundancy analysis was used to relate molecular data to geochemical conditions. *Dehalococcoides* 16S rRNA- and vinyl chloride (VC)-reductase genes were present at all tested locations in concentrations up to 10^6 gene copies per ml of groundwater. However, differences between and also within locations were observed. Variation in *Dehalococcoides* 16S rRNA gene copy numbers were most strongly correlated to dissolved organic carbon concentration in groundwater and to conditions appropriate for biodegradation of chlorinated ethenes (U.S. Environmental Protection Agency score). In contrast, *vcrA* gene copy numbers correlated most significantly to VC and chlorinated ethene concentrations. Interestingly, *bvcA* and especially *tce*A were more correlated with oxidizing conditions. In groundwater microcosms, dechlorination of 1 mM VC was correlated to an increase of *vcrA* and/or *bvcA* gene copies by 2 to 4 orders of magnitude. Interestingly, in 34% of the monitoring wells and in 40% of the active microcosms, the amount of individual VC-reductase gene copies exceeded that of *Dehalococcoides* 16S rRNA gene copies. It is concluded that the geographical distribution of the genes was not homogeneous, depending on the geochemical conditions, whereby *tceA* and *bvcA* correlated to more oxidized conditions than *Dehalococcoides* 16S rRNA and *vcrA*. Because the variation in VC-reductase gene numbers was not directly correlated to variation in *Dehalococcoides* spp., VC-reductase genes are better monitoring parameters for VC dechlorination capacity than *Dehalococcoides* spp.

Chlorinated ethenes, such as tetrachloroethene (PCE) and trichloroethene (TCE), are persistent groundwater pollutants (15, 22). Because these compounds are toxic and mobile in groundwater systems, they form a serious risk for human health and the environment. PCE and TCE can be dechlorinated by microorganisms under anaerobic conditions by reductive dehalogenation to dichloroethene (DCE), vinyl chloride (VC), and ethene (20). Bioremediation strategies for chloroethene-contaminated sites are often based on (stimulation of) reductive dechlorination of the chlorinated ethenes to ethene (7, 12, 14). In practice, reductive dechlorination of PCE and TCE can be incomplete, resulting in accumulation of DCE or VC. Since VC is much more mobile, toxic, and carcinogenic than PCE and TCE (9), monitoring and stimulation of VC dechlorination are essential steps in bioremediation strategies.

Only members of *Dehalococcoides* spp. are known to be able to reductively dechlorinate VC. Therefore, 16S rRNA genes of these species are often used as molecular target to indicate and monitor DCE and VC dechlorination capacity at contaminated sites. However, previous studies showed different dechlorination capacities for individual *Dehalococcoides* species, and only a few strains are known to metabolically dechlorinate VC (6, 8, 10, 17, 21). As a consequence, 16S rRNA gene-based detection can lead to overestimation of VC dechlorination capacity. In contrast, although metabolic reductive dechlorination of VC has mostly been linked to *Dehalococcoides* spp., it cannot be excluded that other microbial species that perform this dechlorination exist. Genes coding for DCE and VC reductases may be exchangeable between different microbial species via horizontal gene transfer. This is plausible since it has been shown that the metabolic genes for VC dechlorination, *vcrA* and *bvcA*, have a different evolutionary history than most other *Dehalococcoides* genes (16). Consequently, *Dehalococcoides* 16S rRNA gene-based detection can also lead to underestimation of VC dechlorination capacity.

To more precisely determine VC dechlorination capacity, genes directly involved in reductive dechlorination of VC should be used as a molecular target, in addition to *Dehalococcoides* 16S rRNA genes. A quantitative method was described to detect genes coding for VC-reductases VcrA and BvcA identified in *Dehalococcoides* sp. strains VS and GT and in *Dehalococcoides* sp. strain BAV1, respectively (10, 17, 21). Different studies showed direct correlation of *vcrA* and *bvcA* gene copy numbers with reductive dechlorination of VC in batch cultures, soil columns, and contaminated sites (2, 11, 19).

Quantification of genes that encode VC-reductases can be a useful method to monitor reductive dechlorination of VC in

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TABLE 1. Overview of amount of analyzed monitoring wells, the redox conditions, the vinyl chloride concentration, the presence of other pollutants, and the performed remediation activities for 11 sample locations[a]

Location	No. of wells	pH range	Eh (mV)	DOC (mg/liter)	VC (μg/liter)	Other pollutants present	Remediation actions
A	27	6.5–6.9	–166 to −70	8–110	<1–434	1,2-Dichloroethane	Natural attenuation
B	12	6.6–7.2	–130 to −59	9–47	2–14,000		Natural attenuation
C	9	5.8–6.3	–43 to +31	NA	<1–150	Mineral oil, cresol, heavy metals	Natural attenuation
D	11	5.8–6.4	–22 to +95	10–41	<1–870		Natural attenuation
E	12	6.7–7.3	–182 to −86	4–41	<1–4,978		Natural attenuation
F	12	5.1–6.6	–139 to +208	3–15	<1–19	Mineral oil, aromatics, heavy metals, pesticides	Natural attenuation
G	10	NA	NA	<1–48	<1–5		Natural attenuation
H	16	NA	NA	NA	74–160,417	1,2-Dichloroethane	Substrate addition
I	15	NA	NA	NA	<1–27		Substrate addition
J	16	5.5–6.6	–110 to +170	NA	<1–2,742		Substrate addition
K	10	6.7–7.2	–114 to +295	NA	<1–1,671		Substrate addition

[a] The values represent the lower and upper extremes of the individual monitoring wells at the locations. NA, information not available.

chloroethene-contaminated groundwater during enhanced natural attenuation activities (4, 19). However, little is known about the presence, dispersion, and importance of specific dehalogenase genes in chlorinated ethene polluted groundwater and their correlation to biogeochemical conditions and reductive dechlorination.

The objective of the present study was therefore to identify the relative importance of TCE-reductase gene *tceA* and VC-reductase genes *vcrA* and *bvcA* in chloroethene-polluted groundwater and to identify geochemical parameters that contribute to variation in copy numbers of these genes. To this end, groundwater of 150 monitoring wells from 11 European polluted sites was analyzed. Furthermore, microcosms with groundwater from 6 locations were started to test whether VC dechlorination is directly correlated to an increase of *vcrA* or *bvcA* genes.

MATERIALS AND METHODS

Sample locations. Eleven sample locations (Table 1) were selected based on the availability of groundwater for the present study, the presence of chlorinated organic pollutants, the presence of different monitoring wells at the same location, and the availability of geochemical data from the monitoring wells. All locations are situated in the Netherlands except location H, which is in the United Kingdom. Pollution with chlorinated ethenes originated from (former) industrial activities and was present for several decades. At locations A, C, F, and H, other pollutants such as 1,2-dichloroethane, pesticides, and mineral oil were also present. The natural bioremediation process of chlorinated ethenes was enhanced at locations H, I, J, and K by the addition of electron donor into the groundwater. For all locations, monitoring wells within and outside the polluted zone were included in the study. An overview of the geochemical parameters in the monitoring wells is presented in Table S1 in the supplemental material.

Groundwater sampling. Groundwater was collected between 2007 and 2008 from monitoring wells by using a peristaltic pump and Marprene tubing. Before duplicate samples were taken, three water volumes of the monitoring well were discarded to refresh the well water. Sterilized 1-liter glass bottles were filled completely and stored at 4°C. The samples were used within 1 week for DNA extraction and within 1 month for chemical analyses and preparation of microcosms.

Microcosm incubation. Anaerobic microcosms B_{mw421}, C_{mwdb2}, E_{mw403}, E_{mw100}, F_{mw401}, F_{mw54}, G_{mwRH05}, G_{mwRH48}, H_{mw68}, and H_{mw267} were prepared with groundwater from monitoring wells pb-421 (location B); pb-db2-23,5 (location C); pb-403 and pb-100-61 (location E); pb-401 and pb-54 (location F); pb-RH05-22 and pb-RH48 (location G); and pb-mwsa68 and pb-BH267 (location H), respectively. Monitoring wells with both a high or low VC concentration, a high or low concentration of vinyl chloride reductase genes, and a high, low, or unknown biodegradation potential (U.S. Environmental Protection Agency [EPA] score) were selected to minimize a potential bias of site selection criteria. Samples of 200 ml of groundwater were incubated at 20°C and 150 rpm for 4 months in 220-ml serum bottles, closed with a Viton stopper. Protamylasses, which is a product from the potato industry (1 ml; Avebe, Veendam, the Netherlands), was added as an electron donor. The protamylasses solution contained 180 mg total organic carbon per liter. This corresponded to 75 mM organic carbon added to the bottles. Assuming an average composition as sugar $[CH_2O]_n$, this accounts for 300 mM added "electron equivalents." For each monitoring well, two microcosms were started, one of which received 1.5 mM VC. Headspace analyses (500 μl) for VC concentration and liquid samples (5 ml) for DNA extraction were taken monthly under sterile and anaerobic conditions with a syringe.

DNA isolation. Biomass from 50- to 250-ml groundwater samples was concentrated by vacuum filtration in triplicate on 0.2-μm-pore-size filters (ME 24 membrane filters, 25 mm in diameter; Schleicher & Schuell, Den Bosch, the Netherlands). Biomass was concentrated from 5-ml microcosm samples in duplicate by centrifugation (20 min at 4,000 rpm), and the supernatant was filtrated through 0.2-μm-pore-size filters (Schleicher & Schuell). All filters were crushed with a sterilized wooden toothpick. Total DNA was extracted from the concentrated biomass with Bio 101 Fast DNA kit for soil (Qbiogene, Inc.). Other than the addition of one extra one-quarter-inch ceramic sphere (Qbiogene) to Lysing Matrix E of the DNA isolation kit, no modifications to the manufacturer's protocol were applied. The quality and quantity of the extracted DNA was determined by using a Nanodrop ND-1000 spectrophotometer (Isogen, de Meern, the Netherlands) and by electrophoresis on a 1% agarose gel. DNA was stored at −20°C.

qPCR analysis. Copy numbers of the VC-reductase genes *vcrA* and *bvcA*, *tceA* and the *Dehalococcoides* 16S rRNA genes were quantified in 25-μl quantitative PCR (qPCR) assays by using IQ-Supermix (Bio-Rad, Veenendaal, the Netherlands) and 3 μl of template DNA isolated from the filters. For all filters, 10- and 100-fold-diluted DNA extracts were analyzed in duplicate. Sterilized MilliQ water and an environmental sample without target genes were used as negative controls. As positive controls, genes of interest cloned into a pGEM-T vector (Promega) were used. The coefficient of variance was below 5% for all qPCR assays. The specific primers and probes used for detection of the *vcrA*, *bvcA*, *tceA*, and *Dehalococcoides* 16S rRNA genes were described by Ritalahti et al. (18). The temperature program for all PCR assays on an IQ5 real-time PCR system (Bio-Rad) was performed as follows: 3 min at 95°C; followed by 35 cycles of 30 s at 95°C, 30 s at 58°C, and 1 min at 72°C; with a final elongation step of 5 min at 72°C. The number of gene copies was calculated by comparing the threshold cycle (C_T) values of the individual samples to the C_T values of a calibration curve. *Dehalococcoides* 16S rRNA, *vcrA*, *bvcA*, and *tceA* gene calibration was done by using 10-fold serial dilutions of 10^1 to 10^7 genes/ml of pGEM-T Easy vectors (Promega, Madison, WI) containing a single copy of the gene of interest. The amount of gene copies in the DNA samples was extrapolated to the amount of genes copies per ml of groundwater or microcosm culture. The lower detection limit in groundwater of *Dehalococcoide*s 16S rRNA and reductase genes differed because of different PCR efficiencies and were 10 and 100 gene copies/ml of groundwater, respectively. The detection limits were determined based on the gene concentration present in the most strongly diluted standard of the calibration curve, which gave a positive signal in the qPCR analysis. This concentration was divided by the amount of groundwater used for DNA extraction. Every single qPCR run had its own calibration curve. The detection limits were 100-fold higher for microcosm samples due to a lower

sample volume used for DNA extraction. Regularly, the size of the obtained amplicons were checked on a 2% agarose gel. The standard error for quantification the individual genes differed from a 0.52 to a 0.75 order of magnitude for the *Dehalococcoides* spp. 16S rRNA and VC or TCE reductase genes.

Chemical analyses. Chlorinated hydrocarbons (PCE, TCE, DCE, and 1,2-dichloroethane) were analyzed in 8-ml diluted groundwater samples on a Varian 3800 gas chromatographic (GC) system equipped with a mass spectrometry (MS) detector and a Porabond-Q column (0.32 mm by 25 m) (Varian, Middelburg, the Netherlands). The GC settings were as follows: injector temperature, 200°C; detector temperature, 300°C; oven temperature, 3 min at 40°C, followed by an increase of 10°C/min to 70°C, followed by an increase of 15°C/min to 250°C for 7 min; and carrier gas helium with a flow rate of 2 ml/min. External standards at six different concentrations from 0 to 30 μM were used for calibration.

VC, ethene, ethane, and methane in groundwater samples were quantified in 1-ml samples on a Varian 3800 GC equipped with a flame ionizing detector (FID) and a Porabond-Q column (0.32 mm by 25 m) (Varian). The GC settings were as follows: injector temperature, 200°C; detector temperature, 300°C; oven temperature, 3 min at 40°C, followed by an increase of 10°C/min to 70°C, followed by an increase of 15°C/min to 250°C for 7 min; and a flow rate of 2 ml/min. For microcosm analyses, 500-μl headspace samples, obtained by using a 1-ml Pressure-Lock gas syringe (Alltech, Breda, the Netherlands) with a sterile needle, were analyzed on the GC-FID described above. External standards at six different concentrations from 0 to 3 mM were used for calibration. The lower detection limit was 1 μM for all analyzed compounds.

Chloride, nitrate, and sulfate ion analyses were done on 1 ml of diluted groundwater and microcosm samples on a Dionex ICS-1500 equipped with an Ionpac AS14 anion-exchange column and an A SRS-Ultra 14-mm suppressor (Dionex Corp., Sunnyvale, CA). The eluent (2.0 mM Na_2CO_3 and 0.75 mM $NaHCO_3$) flow rate was 1.3 ml/min. The injection needle was preflushed with 100 μl of MilliQ water, and 50-μl samples were injected. External standards at six different concentrations from 0 to 250 μM were used for calibration. Groundwater and microcosm samples were diluted 10-, 100-, and 500-fold before analysis.

Dissolved organic carbon (DOC) content was determined in 0.5 ml of groundwater by flash combustion, followed by CO_2 analysis by an IR detector (SC-632; Leco, Mönchengladbach, Germany). The iron content of the samples was determined by using ICP-OES (inductively coupled plasma-optical emission spectrometer) analysis (Spectro Ciros^{CCD} (Spectro, Kleve, Germany), after destruction with a mixture of HF, $HClO_4$, and HNO_3.

Hydrogen concentration in the groundwater was analyzed onsite using a gas chromatograph with a reduction gas detector (Trace Analytical, Bester, Amstelveen, the Netherlands), which was equipped with a Carbosieve II column. The carrier gas was N_2 with a flow rate of 20 ml/min. The column and detector temperatures were 104°C and 265°C, respectively.

Temperature, pH, redox potential, and salinity were measured in groundwater that was pumped through a flow cell (Eijkelkamp B.V., Groesbeek, the Netherlands) onsite, with a pH/mV/EC/Sal/TDS/T/O_2 multiparameter (Eijkelkamp) and corresponding probes (Eijkelkamp).

Biodegradation potential. Biodegradation potential of the individual monitoring wells was quantified by the EPA score, based on 9 to 10 critical geochemical parameters, as described by the U.S. EPA in the *Protocol for Evaluating Natural Attenuation* (23). For the EPA score, positive or negative values are provided for geochemical parameters that are presumed critical for reductive dechlorination of chlorinated ethenes (e.g., an oxygen concentration greater than 0.5 mg/liter is provided with a score of −3 and a sulfate concentration less than 20 mg/liter is provided with a score of 2). The sum of the scores for the individual parameters results in the EPA score. In groundwater with an EPA score greater than 15, reductive dehalogenation of chloroethenes is likely.

RDA. Redundancy analysis (RDA) is a multivariate regression analysis, whereby variation in one set of variables is explained by another set of variables. In the present study, gene copy numbers were selected as one set of variables (species data), and geochemical data were selected as the explaining set of variables (environmental data). The RDA was performed with Canoco 4.5 software (Microcomputer Power, Ithaca, NY) on data of all monitoring wells from locations A, B, E, F, and K and monitoring wells pb103, pb104, pb105, and pb1022 from location C, since for these monitoring wells an almost complete data set was available. For the RDA, a linear distribution of the "species" (in this case, gene copy numbers) was assumed to explain their variation by environmental variables. The data were log transformed [$Y = \log(AY+B)$, where $A = 1$ and $B = 1$] for parameters with more than 10-fold variation within the data. In the analysis, data below the detection limit were substituted by values of 70% of the detection limit. To test the significance of the relationship of gene copy number variance with geochemical parameters, unrestricted Monte Carlo permutation tests were performed with 499 random permutations and a significance level (P) of 0.05.

RESULTS

Molecular detection of chloroethene-reductive dehalogenase and *Dehalococcoides* 16S rRNA genes. VC-reductase genes (*vcrA* and *bvcA*), TCE reductase gene (*tceA*), and *Dehalococcoides* 16S rRNA genes were detected in 150 groundwater samples obtained from 11 contaminated locations. At all tested locations, *Dehalococcoides* 16S rRNA genes and VC-reductase genes were present in the groundwater, albeit not in all monitoring wells. Moreover, differences between and within locations were observed with respect to the ratio of *vcrA* and *bvcA* with *Dehalococcoides* 16S rRNA genes in the individual monitoring wells (Fig. 1). The concentration of *Dehalococcoides* 16S rRNA genes ranged for most monitoring wells from below the detection limit (10^1 gene copies/ml) up to 10^4 gene copies/ml but exceeded 10^5 gene copies/ml at locations H, I, and K, where enhanced bioremediation techniques were applied.

Of the two VC-reductase genes, *vcrA* was usually more abundant than *bvcA*. At five different locations, the *vcrA* concentration was greater than 10^5 gene copies/ml, although for most locations concentrations up to 10^4 gene copies/ml were found. Except for monitoring wells pb-307 (location D) and pb-605 (location J), in all samples *vcrA* genes were detected when the concentration of *Dehalococcoides* 16S rRNA gene copies was greater than 10^3/ml. For samples with more than 10^3 *Dehalococcoides* 16S rRNA gene copies, the average ratio of *vcrA* to *Dehalococcoides* 16S rRNA gene copies was found to be 1.3 ± 0.5, with a correlation coefficient of 0.62. For monitoring wells with *Dehalococcoides* 16S rRNA concentrations less than 10^3 gene copies/ml, *vcrA* gene copy numbers were 11.5 ± 6.9 times higher than *Dehalococcoides* 16S rRNA genes. However, the difference between both genes is smaller than the standard error for quantification of the individual genes, which differed from 0.52 to 0.75 order of magnitude for the *Dehalococcoides* 16S rRNA and VC- or TCE-reductase genes. Remarkably, at location B, the amount of *vcrA* gene copies was approximately 10 to 100 times greater than the detected amount of *Dehalococcoides* 16S rRNA genes ($P < 0.05$).

bvcA genes could not be detected in 71% of the analyzed monitoring wells. In most other monitoring wells the *bvcA* concentration was lower than that of the *Dehalococcoides* 16S rRNA genes ($P < 0.05$). At locations G and J, *bvcA* was below detection limit in all monitoring wells. Only in 15 monitoring wells were *bvcA* copy numbers greater than 10^3 copies/ml. Of these 15 wells, 11 were from locations where bioremediation was stimulated by addition of electron donor. At locations C, F, and J, *bvcA* but no *vcrA* was detected. At locations A, B, E, H, I, and K, *vcrA* and *bvcA* were simultaneously present. Here, the *vcrA* concentration was always 1 to 2 orders of magnitude higher than the *bvcA* concentration ($P < 0.01$).

tceA genes were most abundant in monitoring wells of locations F and G at concentrations of greater than 10^3 copies/ml. For the other locations, *tceA* genes were present in one or more monitoring wells at concentrations of up to 10^4 copies/ml; however, the genes were not detected at locations D and H.

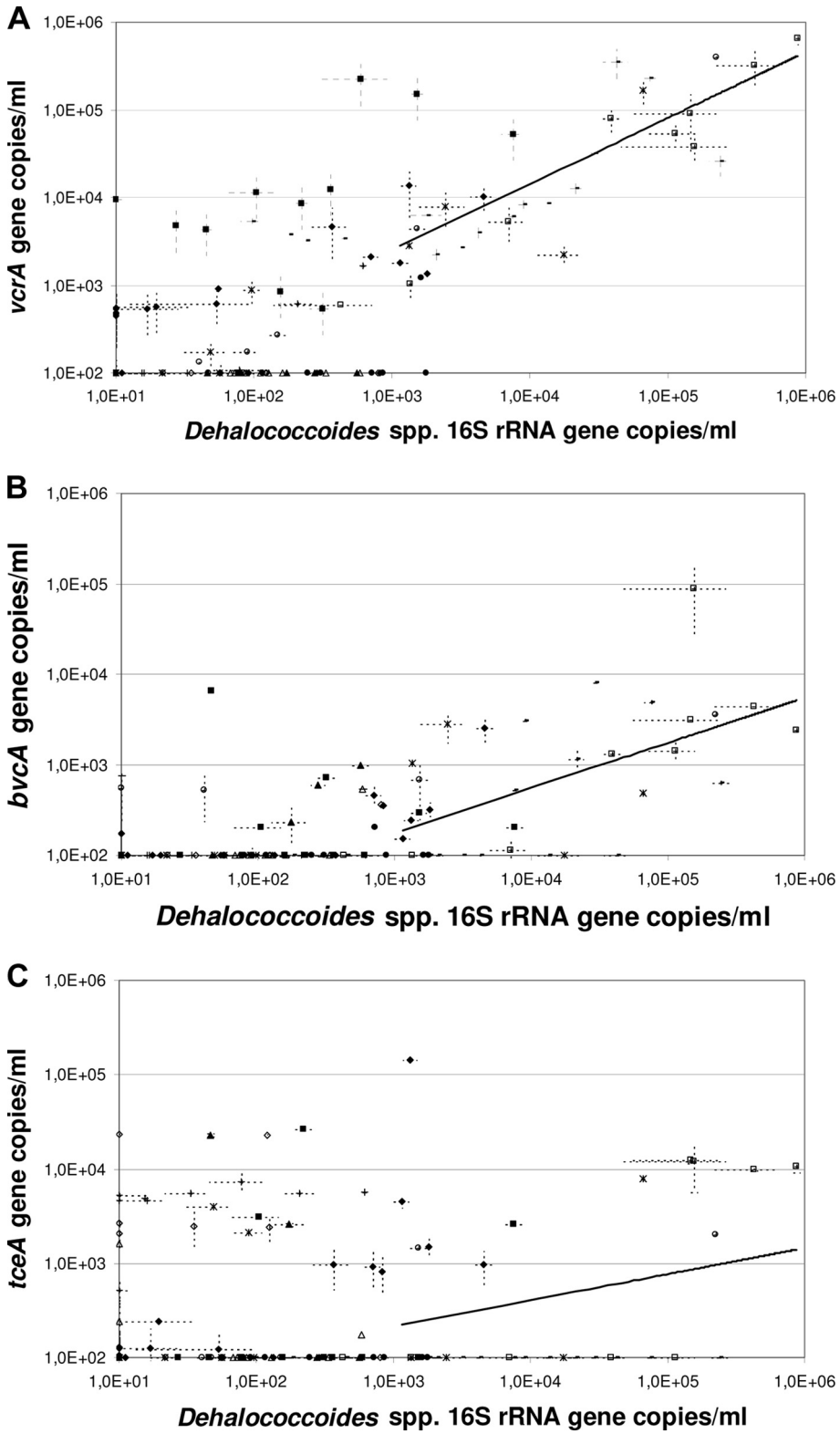

FIG. 1. Total amount of *Dehalococcoides* 16S rRNA gene and specific reductase *vcrA* (A), *bvcA* (B), or *tceA* (C) gene copies, respectively, in groundwater of the individual monitoring wells. Symbols represent the data of monitoring wells from the different locations (◆, location A; ■, location B; ▲, location C; ●, location D; ∗, location E; ◇, location F; +, location G; –, location H; □, location I; △, location J; ○, location K). *x*- and *y*-axis error bars indicate the standard deviations of the *Dehalococcoides* 16S rRNA or reductase genes, respectively, and were only visualized when larger than the symbols. Regressions lines (A, $y = 13.19x^{0.7526}$ [$R^2 = 0.62$]; B, $y = 5.64x^{0.4977}$ [$R^2 = 0.33$]; C, $y = 31.29x^{0.2777}$ [$R^2 = 0.06$]) were given for monitoring wells with $>10^3$ 16S rRNA gene copies/ml of groundwater.

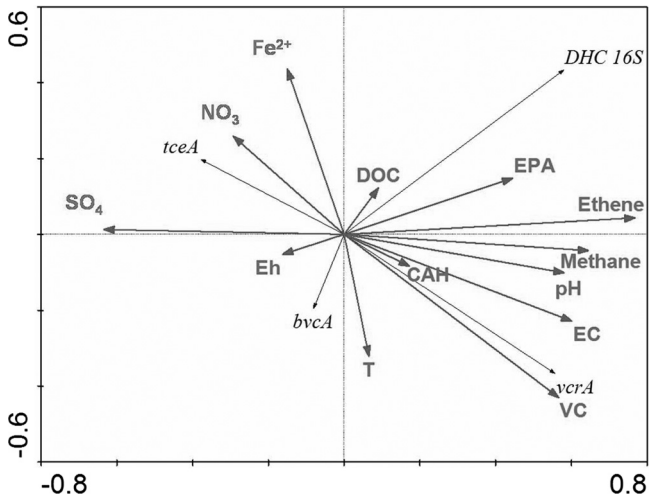

FIG. 2. RDA of geochemical parameters and gene copy numbers. Multivariate analysis was used to explain correlation between geochemical parameters on the concentration of *Dehalococcoides* 16S rRNA (DHC 16S), *vcrA*, *bvcA*, and *tceA* gene copy numbers. The data are presented as vectors, whereby the bold arrows represent the geochemical parameters (EPA, EPA score; methane, methane concentration; T, temperature; pH, pH; EC, conductivity; VC, VC concentration; ethene, ethene concentration; CAH, total concentration of PCE, TCE, DCE, and VC in units of potential available ethene; SO_4, sulfate; NO_3, nitrate; DOC, dissolved organic carbon; E_h, redox potential; Fe^{2+}, concentration of Fe^{2+}). The angle between vectors represents correlation of those vectors, whereby vectors pointing in the same direction (angle $< 90°$) are positively correlated while vectors in opposite direction are negatively correlated. The eigen values of the first (x) and second (y) canonical axes are 0.238 and 0.114, respectively.

No correlation was observed between *tceA* and the *Dehalococcoides* 16S rRNA or VC-reductase genes.

Geochemical parameters. RDA showed that variation in *Dehalococcoides* 16S rRNA, VC-reductase, and TCE-reductase gene copy numbers was explained by different geochemical parameters (Fig. 2), since the vectors representing the different genes show different angles with the vectors that represent specific geochemical conditions. In general, the smaller the angle between vectors in the RDA-plot, the better the correlation between the parameters these vectors represent. Vectors that point in opposite direction indicate that corresponding variables are negatively correlated. Along the first canonical axis (i.e., the *x* axis in Fig. 2), the *Dehalococcoides* 16S rRNA and *vcrA* genes were both positively correlated with pH, EPA score, and the methane, ethene, and VC concentrations. Along the second canonical axis (y-axis) both VC-reductase genes correlated negatively with *Dehalococcoides* spp. 16S rRNA. *Dehalococcoides* 16S rRNA correlated best with Fe^{2+}, NO_3, DOC and biodegradation potential. Variation in VC and CAH concentrations and conductivity of the groundwater had the highest correlation with *vcrA* genes. B*vcA* genes were on the *y* axis correlated to the same environmental parameters as *vcrA* genes, but on the *x* axis the *bvcA* genes were more positively correlated to Fe^{2+}, NO_3, and E_h (a high redox potential). Sulfate concentration was strongly negatively correlated to *Dehalococcoides* 16S rRNA and *vcrA* genes. *tceA* genes correlated to different, more oxidized, geochemical parameters than the other analyzed genes and even opposite to *vcrA* genes.

Microcosms. In microcosms with groundwater from locations B, C, and E, incubated with protamylasses and VC, dechlorination of VC to ethene was observed (Table 2). No VC dechlorination was observed in groundwater from locations F and G during the incubation period of 4 months. In microcosm H_{MW68}, VC was dechlorinated to ethene, whereas in microcosm H_{MW267} no dechlorination occurred. In the absence of VC dechlorination, no increase of *Dehalococcoides* 16S rRNA, *vcrA*, or *bvcA* gene copy numbers was found. In contrast, an increase of 2 to 4 orders of magnitude in the gene concentrations was observed in VC-dechlorinating microcosms during the incubation period. After incubation, total gene concentrations for both *Dehalococcoides* 16S rRNA and VC-reductase genes greater than 10^7 copies/ml were observed. In the VC-dechlorinating microcosms, *vcrA* gene copy numbers were 1 to 2 orders of magnitude greater than the *bvcA* gene copy numbers, as in the groundwater. However, in microcosm C_{mwdb2} a significant increase of *bvcA* occurred, whereas no *vcrA* was detected. This corresponded to the absence of *vcrA* and presence of *bvcA* in groundwater of location C. After incubation, the *vcrA*/*Dehalococcoides* 16S rRNA gene ratio in dechlorinating microcosms was 0.49 ± 0.06 and thus lower than that observed in the groundwater.

DISCUSSION

The aim of the present study was to identify the relative importance of the *Dehalococcoides* TCE-reductase TceA and VC-reductases VcrA and BvcA in chlorinated ethene polluted groundwater and to identify the geochemical parameters that most significantly contribute to variation in corresponding 16S rRNA, *tceA*, *vcrA*, and *bvcA* genes. Although a few environmental studies reported the presence of *tceA*, *vcrA*, or *bvcA* genes in combination with *Dehalococcoides* spp. for one or two locations (2, 3, 11), this is the first study that described quantities of all those genes at multiple locations and in combination with geochemical data, allowing for robust multivariate analysis. At all tested locations, VC-reductase-encoding genes were detected, and the highest gene copy numbers were observed at locations where enhanced bioremediation techniques were applied. In general, *vcrA* genes were detected in more monitoring wells and at higher concentrations than *bvcA* genes. This is in accordance with the results of Carreon-Diazconti et al. (3). At only one of the locations investigated in the present study (location C) was *bvcA* present whereas *vcrA* apparently was not. This contrasts to other environmental studies (2, 11), where the amount of *bvcA* gene copies numerically exceeded the amount of *vcrA* genes. Thus, since our results showed that the geographical distribution of *vcrA* and *bvcA* is heterogeneous, it can be concluded in combination with other studies that at different locations, the dominant VC-reductase need not be the same. Therefore, both *vcrA* and *bvcA* genes should be monitored when VC-reductase-encoding genes are used to identify VC dechlorination capacity.

In monitoring wells with more than 10^3 *Dehalococcoides* 16S rRNA gene copies/ml, the correlation coefficient with *vcrA* gene copy numbers was 0.62 (Fig. 1A). This indicates that both genes are correlated to each other in the tested monitoring

TABLE 2. VC dechlorination activity and gene copy numbers of *Dehalococcoides* 16S rRNA, *vcrA*, and *bvcA* genes detected in the microcosms before and after incubation with protamylasses and 1.5 mM VC

Microcosm	Monitoring well	VC dechlorination	No. of gene copies/ml					
			Before incubation			After incubation		
			16S-DHC	*vcrA*	*bvcA*	16S-DHC	*vcrA*	*bvcA*
B1	pb-421	+	$(1.7 \pm 0.2) \times 10^5$	$(6.5 \pm 2.5) \times 10^3$	$(5.3 \pm 1.6) \times 10^2$	$(1.4 \pm 1.8) \times 10^7$	$(6.5 \pm 1.3) \times 10^6$	$(1.7 \pm 0.7) \times 10^4$
C1	pb-DB2-23,5	+	$(2.9 \pm 0.3) \times 10^2$	<100	$(3.1 \pm 0.0) \times 10^2$	$(7.3 \pm 4.6) \times 10^6$	<10,000	$(3.3 \pm 0.4) \times 10^6$
E1	pb-403	+	$(1.8 \pm 3.5) \times 10^4$	$(2.2 \pm 1.2) \times 10^3$	<100	$(1.7 \pm 0.3) \times 10^7$	$(7.8 \pm 5.5) \times 10^7$	$(9.7 \pm 3.9) \times 10^5$
E2	pb-100-61	+	$(2.4 \pm 1.8) \times 10^3$	$(7.9 \pm 6.8) \times 10^3$	$(2.8 \pm 2.1) \times 10^3$	$(1.7 \pm 0.3) \times 10^6$	$(4.8 \pm 0.6) \times 10^6$	$(2.4 \pm 0.1) \times 10^5$
F1	pb-401	−	<10	<100	<100	<1,000	<10,000	<10,000
F2	pb-54	−	<10	<100	<100	<1,000	<10,000	<10,000
G1	pb-RH05-22	−	<10	<100	<100	<1,000	<10,000	<10,000
G2	pb-RH48	−	$(6.1 \pm 1.1) \times 10^2$	$(1.7 \pm 0.2) \times 10^3$	<100	<1,000	<10,000	<10,000
H1	pb-mwsa68	+	$(3.5 \pm 0.6) \times 10^2$	<100	<100	$(3.8 \pm 0.5) \times 10^6$	$(2.1 \pm 0.4) \times 10^6$	$(5.2 \pm 1.0) \times 10^4$
H2	pb-BH267	−	$(6.3 \pm 1.5) \times 10^2$	$(1.2 \pm 1.8) \times 10^3$	$(4.8 \pm 0.1) \times 10^2$	<1,000	<10,000	<10,000

wells. Different factors can be the reason for this relatively low correlation coefficient, e.g., variation in numbers of gene copies per *Dehalococcoides* cell. This should be addressed in future studies, e.g., by assessing 16S rRNA/VC-reductive dehalogenase gene ratios at the single-cell level. The relative amount of VC-reductase genes to *Dehalococcoides* 16S rRNA genes is relatively high (>1) compared to the studies of Lee et al. (11) and Scheutz et al. (19), which reported ratios of less than 1.

The amount of *Dehalococcoides* 16S rRNA genes correlated well with the EPA score for the biodegradation potential of chlorinated ethenes (Fig. 2). The EPA score was developed to identify for a polluted location whether it is likely that chlorinated ethenes are transformed via reductive dechlorination, for example, by *Dehalococcoides* spp. Our study confirms that for predicting the presence or absence of *Dehalococcoides* spp. at a location, the EPA score is a robust and better parameter than individual geochemical parameters, such as the total concentration of CAHs and the sulfate or nitrate concentrations. In contrast, TCE- and VC-reductase genes appeared to have a weaker correlation with the EPA score than the *Dehalococcoides* 16S rRNA genes. This indicates that under favorable conditions for reductive dechlorinating bacteria, *Dehalococcoides* strains with genes encoding for *vcrA* or *bvcA*, such as strains VS, BAV1, and GT, are not necessarily enriched. Thus, the capacity for TCE and VC dechlorination is not necessarily high when the EPA score is high or when high numbers of *Dehalococcoides* spp. are present. *Dehalococcoides* 16S rRNA genes are an effective predictor of overall reductive dechlorination capacity; however, these phylogenetic marker genes cannot predict the biodegradation potential for a specific chlorinated ethene, such as VC. Rather, the specific reductase genes are more direct predictors of, respectively, TCE and VC dechlorination capacity. However, it should always be kept in mind that groundwater samples might not fully represent the *in situ* conditions, since bacteria can have different affinities for the groundwater or soil matrix.

Variation in the presence and abundance of VC-reductase *vcrA* appeared to be more strongly related with concentrations of VC, CAH, and methane than *Dehalococcoides* 16S rRNA genes. However, both genes had a positive correlation with ethene. Carreon-Diazconti et al. (3) also found a positive relation between high concentrations of ethene and *vcrA* but not with *bvcA*. However, since ethene can be produced from other contaminants than VC in the groundwater, it cannot be concluded that ethene alone is a good indicator for VC dechlorination. *Dehalococcoides* spp. and *vcrA* correlated with a low redox potential. In contrast, *bvcA* and *tceA* correlated with a high redox potential, sulfate, nitrate, and iron, and least with methane and the EPA score. In particular, *tceA* had a more positive correlation with more oxidized conditions than *Dehalococcoides* 16S rRNA genes and *vcrA*. Possibly, *Dehalococcoides* strains containing *tceA*, which dechlorinate higher chlorinated ethenes (PCE and TCE), tolerate more oxidized conditions than VC dechlorinating *Dehalococcoides* strains. This is in agreement with the findings of Amos et al. (1), who showed that *Dehalococcoides* strains respond differently to oxygen exposure: strains with VC-reductase genes are more susceptible to oxygen inhibition than others (1). Lu et al. (13) showed that for dechlorination

of VC, the hydrogen threshold values were in the range of methanogenesis (2 to 24 nM), whereas these threshold values for PCE and TCE were in the range of denitrification and ferric iron reduction (0.1 to 0.4 nM) (13). Unfortunately, hydrogen data were only available for locations A, I, and J and could therefore not be included in the RDA. However, these data also showed that in monitoring wells with a hydrogen concentration above 1 nM, VC-reductase gene concentrations greater than 10^3/ml were observed.

In the monitoring wells, a relatively low correlation between concentrations of *vcrA* and *bvcA* genes was found. RDA suggested that microorganisms that contain *vcrA* or *bvcA* genes have different niches, where *Dehalococcoides* spp. with *bvcA* genes prefer higher redox potential. Niche differentiation of these genes was also observed in soil column experiments, where specific enrichment of *vcrA* or *bvcA* genes was observed after the addition of different carbon sources (unpublished data).

In line with previous studies (11, 19), VC dechlorination in microcosms was found to be correlated to an increase of *vcrA* and *bvcA* gene copies. The mere presence of VC-reductase or *Dehalococcoides* 16S rRNA genes in low concentrations (10^3 gene copies/ml) was not in all cases related to the occurrence of dechlorination of VC. Rather, an increase of the VC reductase gene copy numbers with an order of magnitude or more during microcosm incubation correlated with biodegradation. In the present study, *in situ* monitoring of VC dechlorination over time was only possible at one location. In this case, VC dechlorination also correlated with increase of *vcrA* and *bvcA* genes. As a robust indicator for VC dechlorination, an increase of VC reductase gene concentrations with an order of magnitude should be observed. In combination with other *in situ* parameters, such as ethene formation, redox potential, or a decrease in the VC concentration, the confidence in *in situ* VC dechlorination can be reinforced.

In groundwater and VC-dechlorinating microcosms, gene copy numbers for *vcrA* were structurally 10 to 100 times higher than for *bvcA*. This difference is significantly higher than the standard error within the replicate analyses. Thus, incubation in the presence of VC under anaerobic conditions did not influence the average ratio between *vcrA* and *bvcA* gene copies. In groundwater from location C, where *vcrA* genes appeared to be absent, *bvcA* genes were enriched to the same levels as *vcrA* in other microcosms. These results suggest that bacteria containing VC-reductase *vcrA* are in competition with bacteria containing VC-reductase *bvcA*, whereby species with *vcrA* are more efficient under tested groundwater conditions and therefore more readily enriched. In the literature, similar doubling times for *Dehalococcoides* strains VC and BAV1 have been reported (5, 8). It should be noted, however, that these values were determined under artificial laboratory conditions (e.g., culture medium), whereas in our study the populations were enriched in the original groundwater. This supports the idea of niche differentiation between *vcrA* and *bvcA* gene containing *Dehalococcoides*, as proposed above.

In the present study, we demonstrated that *Dehalococcoides* 16S rRNA and the specific reductase genes *tceA*, *vcrA*, and *bvcA* are widespread within different groundwater systems. The geographical distribution of the genes was not homogeneous, depending on the geochemical conditions, whereby *tceA* and *bvcA* correlated more to oxidized conditions than *Dehalococcoides* 16S rRNA and *vcrA* genes. Because variation in the VC-reductase gene numbers was not directly correlated to variation in *Dehalococcoides* spp., VC-reductase genes are better monitoring parameters for VC dechlorination capacity than *Dehalococcoides* 16S rRNA genes. To indicate VC dechlorination at contaminated locations, high concentrations ($>10^3$ copies/ml) of *vcrA* or *bvcA*, and an increase of more than an order of magnitude should be detected over time.

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REFERENCES

1. **Amos, B. K., K. M. Ritalahti, C. Cruz-Garcia, E. Padilla-Crespo, and F. E. Löffler.** 2008. Oxygen effect on *Dehalococcoides* viability and biomarker quantification. Environ. Sci. Technol. **42:**5718–5726.
2. **Behrens, S., M. F. Azizian, P. J. McMurdie, A. Sabalowsky, M. E. Dolan, L. Semprini, and A. M. Spormann.** 2008. Monitoring abundance and expression of "*Dehalococcoides*" species chloroethene-reductive dehalogenases in a tetrachloroethene-dechlorinating flow column. Appl. Environ. Microbiol. **74:**5695–5703.
3. **Carreon-Diazconti, C., J. Santamaria, J. Berkompas, J. A. Field, and M. L. Brusseau.** 2009. Assessment of in situ reductive dechlorination using compound-specific stable isotopes, functional gene PCR, and geochemical data. Environ. Sci. Technol. **43:**4301–4307.
4. **Cupples, A. M.** 2008. Real-time PCR quantification of *Dehalococcoides* populations: methods and applications. J. Microbiol. Methods **72:**1–11.
5. **Cupples, A. M., A. M. Spormann, and P. L. McCarty.** 2003. Growth of a *Dehalococcoides*-like microorganism on vinyl chloride and *cis*-dichloroethene as electron acceptors as determined by competitive PCR. Appl. Environ. Microbiol. **69:**953–959.
6. **Duhamel, M., K. Mo, and E. A. Edwards.** 2004. Characterization of a highly enriched *Dehalococcoides*-containing culture that grows on vinyl chloride and trichloroethene. Appl. Environ. Microbiol. **70:**5538–5545.
7. **Gerritse, J., O. Drzyzga, G. Kloetstra, M. Keijmel, L. P. Wiersum, R. Hutson, M. D. Collins, and J. C. Gottschal.** 1999. Influence of different electron donors and acceptors on dehalorespiration of tetrachloroethene by *Desulfitobacterium frappieri* TCE1. Appl. Environ. Microbiol. **65:**5212–5221.
8. **He, J., K. M. Ritalahti, M. R. Aiello, and F. E. Loffler.** 2003. Complete detoxification of vinyl chloride (VC) by an anaerobic enrichment culture and identification of the reductively dechlorinating population as a *Dehalococcoides* population. Appl. Environ. Microbiol. **69:**996–1003.
9. **Henschler, D.** 1994. Toxicity of chlorinated organic compounds: effects of the introduction of chlorine in organic molecules. Angew Chem. Int. Edit. **33:**1920–1935.
10. **Krajmalnik-Brown, R., T. Holscher, I. N. Thomson, F. M. Saunders, K. M. Ritalahti, and F. E. Löffler.** 2004. Genetic identification of a putative vinyl chloride reductase in *Dehalococcoides* sp. strain BAV1. Appl. Environ. Microbiol. **70:**6347–6351.
11. **Lee, P. K. H., T. W. Macbeth, K. S. Sorenson, Jr., R. A. Deeb, and L. Alvarez-Cohen.** 2008. Quantifying genes and transcripts to assess the *in situ* physiology of "*Dehalococcoides*" spp. in a trichloroethene-contaminated groundwater site. Appl. Environ. Microbiol. **74:**2728–2739.
12. **Lendvay, J. M.** 2003. Bioreactive barriers: bioaugmentation and biostimulation for chlorinated solvent remediation. Environ. Sci. Technol. **37:**1422–1431.
13. **Lu, X.-X., S. Tao, T. Bosma, and J. Gerritse.** 2001. Characteristic hydrogen concentrations for various redox processes in batch study. J. Environ. Sci. Health A **36:**1725–1734.
14. **Major, D. W., M. L. McMaster, E. E. Cox, E. A. Edwards, S. M. Dworatzek, E. R. Hendrickson, M. G. Starr, J. A. Payne, and L. W. Buonamici.** 2002. Field demonstration of successful bioaugmentation to achieve dechlorination of tetrachloroethene to ethene. Environ. Sci. Technol. **36:**5106–5116.
15. **McCarty, P. L.** 1997. Microbiology: breathing with chlorinated solvents. Science **276:**1521–1522.
16. **McMurdie, P. J., S. F. Behrens, S. Holmes, and A. M. Spormann.** 2007. Unusual codon bias in vinyl chloride reductase genes of *Dehalococcoides* species. Appl. Environ. Microbiol. **73:**2744–2747.
17. **Muller, J. A., B. M. Rosner, G. von-Abendroth, G. Meshulam-Simon, P. L.**

McCarty, and A. M. Spormann. 2004. Molecular identification of the catabolic vinyl chloride reductase from *Dehalococcoides* sp. strain VS and its environmental distribution. Appl. Environ. Microbiol. **70:**4880–4888.
18. **Ritalahti, K. M., B. K. Amos, Y. Sung, Q. Wu, S. S. Koenigsberg, and F. E. Loffler.** 2006. Quantitative PCR targeting 16S rRNA and reductive dehalogenase genes simultaneously monitors multiple *Dehalococcoides* strains. Appl. Environ. Microbiol. **72:**2765–2774.
19. **Scheutz, C., N. D. Durant, P. Dennis, M. H. Hansen, T. Jorgensen, R. Jakobsen, E. E. Cox, and P. L. Bjerg.** 2008. Concurrent ethene generation and growth of *Dehalococcoides* containing vinyl chloride reductive dehalogenase genes during an enhanced reductive dechlorination field demonstration. Environ. Sci. Technol. **42:**9302–9309.
20. **Smidt, H., and W. M. de Vos.** 2004. Anaerobic microbial dehalogenation. Annu. Rev. Microbiol. **58:**43–73.
21. **Sung, Y., K. M. Ritalahti, R. P. Apkarian, and F. E. Loffler.** 2006. Quantitative PCR confirms purity of strain GT, a novel trichloroethene-to-ethene-respiring *Dehalococcoides* isolate. Appl. Environ. Microbiol. **72:**1980–1987.
22. **Westrick, J. J., J. W. Mello, and R. F. Thomas.** 1984. The groundwater supply survey. J. Am. Water Resources **A:**52–59.
23. **Wiedemeier, T. H., M. A. Swanson, D. E. Moutoux, E. K. Gordon, J. T. Wilson, B. H. Wilson, D. H. Kampbell, P. E. Haas, R. N. Miller, J. E. Hansen, and F. H. Chapelle.** 1998. Technical protocol for evaluating natural attenuation of chlorinated solvents in ground water. U.S. Environmental Protection Agency, Washington, DC.