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Quantifying Genes and Transcripts To Assess the In Situ Physiology of "*Dehalococcoides*" spp. in a Trichloroethene-Contaminated Groundwater Site▽†

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Quantitative PCR (qPCR) was coupled with reverse transcription (RT) to analyze both gene copy numbers and transcripts of the 16S rRNA gene and three reductive dehalogenase (RDase) genes (*tceA*, *vcrA*, and *bvcA*) as biomarkers of "*Dehalococcoides*" spp. in the groundwater of a trichloroethene-dense nonaqueous-phase liquid site at Fort Lewis, WA, that was sequentially subjected to biostimulation and bioaugmentation. *Dehalococcoides* cells carrying the *tceA*, *vcrA*, and *bvcA* genes were indigenous to the site. The sum of the three identified RDase gene copy numbers closely correlated to 16S rRNA gene copy numbers throughout the biostimulation and bioaugmentation activity, suggesting that these RDase genes represented the major *Dehalococcoides* metabolic functions at this site. Biomarker quantification revealed an overall increase of more than 3 orders of magnitude in the total *Dehalococcoides* population through the 1-year monitoring period (spanning biostimulation and bioaugmentation), and measurement of the respective RDase gene concentrations indicated different growth dynamics among *Dehalococcoides* cells. The *Dehalococcoides* cells containing the *tceA* gene consistently lagged behind other *Dehalococcoides* cells in population numbers and made up less than 5% of the total *Dehalococcoides* population, whereas the *vcrA*- and *bvcA*-containing cells represented the dominant fractions. Quantification of transcripts in groundwater samples verified that the 16S rRNA gene and the *bvcA* and *vcrA* genes were consistently highly expressed in all samples examined, while the *tceA* transcripts were detected inconsistently, suggesting a less active physiological state of the cells with this gene. The production of vinyl chloride and ethene toward the end of treatment supported the physiological activity of the *bvcA*- and *vcrA*-carrying cells. A clone library of the expressed RDase genes in field samples produced with degenerate primers revealed the expression of two putative RDase genes that were not previously monitored with RT-qPCR. The level of abundance of one of the putative RDase genes (*FtL-RDase-1638*) identified in the cDNA clone library tracked closely in field samples with abundance of the *bvcA* gene, suggesting that the *FtL-RDase-1638* gene was likely colocated in genomes containing the *bvcA* gene. Overall, results from this study demonstrate that quantification of biomarker dynamics at field sites can provide useful information about the in situ physiology of *Dehalococcoides* strains and their associated activity.

Chlorinated ethenes, such as tetrachloroethene (PCE), trichloroethene (TCE), isomers of dichloroethene (DCE), and vinyl chloride (VC), are toxic and carcinogenic compounds (35) that pose a serious threat to public health when released into water sources. PCE and TCE are among the most frequently detected compounds in both non-point-source- and point-source-contaminated groundwater systems in the United States (26, 36). A promising solution to remediate chlorinated-ethene-contaminated groundwater is in situ bioremediation (28).

Complete reductive dechlorination of chlorinated ethenes to the innocuous end product ethene has been demonstrated only by organisms in the genus "*Dehalococcoides*" (33). To date, there are six described strains of *Dehalococcoides*, each of which has different dechlorination capabilities (1, 8, 9, 25, 27, 34). Of the six, the genomes of strain 195 (32), strain CBDB1 (16), and strain BAV1 (http://www.jgi.doe.gov/) have already been sequenced and annotated. From the annotations and other studies, insights have been gained into the physiology and biochemical pathways of *Dehalococcoides* spp., including the metabolically diverse reductive dehalogenase (RDase) genes that code for the proteins that are responsible for the respiration of chlorinated ethenes. For example, the *tceA* gene present in strains 195 and FL2 codes for a protein that catalyzes the sequential metabolic transformation of TCE to *cis*-DCE (cDCE) and VC and the cometabolic generation of ethene from VC (9, 21, 22, 32), while the gene product of *vcrA*, found in strains VS and GT, reduces DCE isomers and VC (27, 34) and the product of *bvcA* found in strain BAV1 is implicated in metabolic transformation of VC to ethene; however, the protein has not yet been fully characterized (8, 15).

In addition to the three aforementioned genes, whose func-

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tions are known, multiple nonidentical putative RDase genes have also been found in the different isolates (12, 15, 16, 32) and in a *Dehalococcoides*-containing enrichment culture (37). From the genome annotations, 17 intact putative RDase genes are present in strain 195 (32), while strain CBDB1, an organism that metabolizes chlorobenzene and polychlorinated dibenzo-*p*-dioxins, possesses 32 putative RDase genes (16). For strains BAV1 and FL2, 11 and 14 putative RDase genes, respectively, have been identified (12, 15) (http://www.jgi.doe.gov/). However, the substrate specificity, function, and regulation of these putative RDase genes are mostly unknown at this time.

Monitoring of the presence of *Dehalococcoides* strains at solvent-contaminated field sites has typically focused on the 16S rRNA gene, using such methods as quantitative PCR (qPCR), end-point PCR, and terminal restriction fragment length polymorphism (6, 10, 18, 23, 29), while the RDase genes are less frequently monitored (3, 31). However, analysis of the 16S rRNA gene sequences of different *Dehalococcoides* isolates, environmental samples, and enrichment cultures has revealed that despite differences in chlorinated-ethene metabolic capabilities, the *Dehalococcoides* 16S rRNA gene sequences are highly conserved (5, 9, 10, 16, 34). Consequently, metabolic function cannot be inferred from *Dehalococcoides* phylogeny, and the detection of 16S rRNA genes alone at field sites could lead to potential misinterpretations if the *Dehalococcoides* spp. present do not actually have the specific metabolic capabilities of interest. Therefore, genes that are specific to functions of interest can serve as useful biomarkers whose quantification can enable effective monitoring of different *Dehalococcoides* activities.

Monitoring the presence or absence of target genes may also be inadequate in some cases to predict microbial activity, since the presence of a gene does not guarantee its expression or its activity (19). Further, it has been demonstrated previously for strain 195 that growth and dechlorination activity can decouple (25), resulting in cell and gene concentrations that remain static while dechlorination continues. Consequently, quantifying expression of functionally important genes may give a more robust surrogate indicator of microbial activity. Previous findings have indicated that RDase genes are expressed in response to the presence of different chlorinated organics (7, 13, 14, 17, 30, 37), and RDase gene expression has been correlated to the physiological ability of *Dehalococcoides* spp. to dechlorinate beyond cDCE (17). Previous experiments have also shown that the half-life of RDase transcripts in the absence of chlorinated ethenes is between 4 and 6 h (17). The short-lived nature of the RDase mRNA suggests that it could serve as a sensitive biomarker to indicate the activity of *Dehalococcoides* spp.

In this study, qPCR and reverse transcription (RT-qPCR) were applied to quantify the DNA and RNA of targeted RDase genes (*tceA*, *bvcA*, and *vcrA*) and 16S rRNA genes of *Dehalococcoides* spp. at a TCE-dense nonaqueous-phase liquid (DNAPL) site during different phases of a bioremediation application. Biostimulation with whey to promote the activity of indigenous microorganisms, bioaugmentation with a *Dehalococcoides*-containing enrichment culture, and varying of the concentration of whey were strategies implemented at the site to enhance dechlorination. Nucleic acid data collected from the groundwater were used to diagnose the in situ physiology of *Dehalococcoides* spp. and to evaluate the progress of sequential treatments.

TABLE 1. Dates and concentration of whey injected at treatment plots 1 and 2

Date	Treatment plot 1		Treatment plot 2	
	Vol of water (gallons)	Concn of whey[b] (g/liter)	Vol of water (gallons)	Concn of whey (g/liter)
19 June 2005	3,200	30	3,900	30
26 June 2005	3,200	30	3,200	30
July 2005	1,700	100	4,000	10
Aug. 2005	0[a]	0[a]	1,800	10
Sept. 2005	1,700	100	4,000	10
Oct. 2005	1,900	100	1,800	10
Nov. 2005	1,800	10	1,800	100
Dec. 2005	1,800	10	1,800	100
Jan. 2006	1,800	10	1,800	100
Feb. 2006	1,300	10	1,800	100

[a] No recirculation or injection of whey due to equipment difficulties.
[b] Whey was a complex substrate with ~10 to 13% protein and ~70 to 75% lactose.

MATERIALS AND METHODS

Site description and treatment strategies. The field study was conducted at the Fort Lewis military base in Tacoma, WA, within the East Gate Disposal Yard (EGDY), a landfill where TCE-containing spent solvents were disposed. The EGDY was the source of a nearly 5-mile-long TCE groundwater plume. The EGDY treatment site was located within the area designated NAPL Area 3, characterized by high concentrations of TCE-DNAPL in soils. Two test plots were constructed within NAPL Area 3 to evaluate enhanced bioremediation using two whey injection strategies (see Fig. S1 in the supplemental material). The injection strategy was based on laboratory and field studies that demonstrated enhanced mass transfer of chlorinated solvents from the residual DNAPL to the aqueous phase using high-concentration whey powder to enhance the effective solubility of the DNAPL (20). The objective of the field study was to compare the effectiveness of low-concentration (10 g/liter) and high-concentration (100 g/liter) whey injections for treating DNAPL source areas. Each approximately 40-foot by 20-foot plot was constructed with an injection, extraction, and four multilevel continuous-multichannel-tubing monitoring wells. Samples were collected at three depths, approximately 12 to 15 feet, 17 to 20 feet, and 27 to 30 feet below the ground surface along the treatment plot axis at approximately 20 feet and 30 feet from the injection well. In addition, samples were collected from two wells located 20 feet down- and 10 feet cross-gradient of the treatment plot axis from the injection well at the 27- to 30-foot-depth interval for a total of eight sampling locations per treatment plot.

Periodic whey powder injections as described in Table 1 were performed as part of the treatment strategy within the two treatment plots. During injection, groundwater was extracted from the extraction wells and recirculated through a whey injection system, where whey powder was fed in-line at the desired feed rate, mixed using an in-line actuator, and reinjected into the injection wells to the final concentrations described in Table 1. This procedure typically took 3 h, and the recirculation system was only used during injections as a source of water for the injections. Ambient groundwater flow was the primary mechanism of whey distribution following injections. A groundwater sample was collected from each monitoring well on each treatment plot for molecular analysis prior to biostimulation with whey amendment as a baseline (April 2005), 1 month following whey amendment (July 2005), 1 month following bioaugmentation (August 2005), 4 months following bioaugmentation and modification of whey amendment (November 2005), 7 months following bioaugmentation (February 2006), and finally 9 months following bioaugmentation and 2 months after cessation of whey amendments (April 2006).

Bioaugmentation was achieved with a culture that was enriched from the groundwater of the TCE-contaminated Bachman Road aquifer (Oscoda, MI) and was fed TCE and lactate prior to use in this study. All culture enrichment was performed at Utah State University. The bioaugmentation culture was transferred from 18-liter stainless steel containers into each of the injection wells

under an argon gas atmosphere after anaerobic conditions were achieved in the groundwater of the treatment area as indicated by depleted dissolved oxygen and sulfate and elevated ferrous iron (data not shown). Anaerobic conditions were initially established by two 30-g/liter whey injections. Differential whey injections were applied subsequent to bioaugmentation to distribute the culture (Table 1). Since the injected bioaugmentation volume represented less than 0.01% of the effective aquifer pore volume, the bioaugmentation strategy was based on assumptions of growth following injection, distribution during whey injections, and transport due to ambient groundwater flow.

Groundwater samples were also collected to measure geochemical composition, organic carbon, redox parameters (data not shown), and chlorinated ethenes. Groundwater samples were collected using dedicated Teflon tubing in each port of the continuous-multichannel-tubing sampling systems and pumping using a peristaltic pump. Purging was performed by pumping at approximately 100 ml/min and until water quality indicator parameters (temperature, pH, conductivity, dissolved oxygen, and oxidation-reduction potential) stabilized based on measurements with a water quality instrument (Quanta G by Hydrolab) and a flow-through cell. For the volatile organic carbon results presented here, samples were sent to a fixed lab (Severn Trent Laboratories) for analysis according to standard EPA method SW846 8260B.

DNA and RNA isolation. Groundwater samples intended for genomic DNA (gDNA) isolation were collected from monitoring wells (MW) into autoclaved 1-liter bottles and were shipped overnight on ice to the laboratory at the University of California at Berkeley. Samples were stored at 4°C upon arrival and processed within 48 h. Between 100 ml and 1,000 ml of groundwater was filtered through a 0.2-μm, surfactant-free cellulose acetate filter (Fisher, Houston, TX) that was subsequently vortexed vigorously for 5 min in 2 ml of the sample groundwater in order to dislodge filtered particles. The resultant liquid was centrifuged at 21,000 × *g* at 4°C for 10 min, the supernatant was discarded, and the cell pellet was stored at −80°C until extraction. gDNA was isolated from frozen cell pellets using the UltraClean microbial DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. A negative control of 0.2-μm-filtered and autoclaved Milli-Q water was extracted in parallel with each set of samples. The gDNA concentration was quantified using the PicoGreen double-stranded-DNA quantitation kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Groundwater samples intended for RNA isolation were filtered on site at the time of sampling. Between 275 ml and 8 liters of groundwater was pressured through a sterile 142-mm-diameter, 0.22-μm hydrophilic polyvinylidene fluoride filter (Millipore, Billerica, MA) via a tripod collection system. The filtration process typically took less than 5 min. The filters were then placed into sterile 50-ml conical tubes and immediately frozen on dry ice. Samples were shipped overnight in dry ice to the University of California at Berkeley and upon arrival stored at −80°C until extraction. Total RNA was isolated from the frozen filters using the RNA PowerSoil total RNA isolation kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. Prior to the first step of the extraction procedure, the filters in the 50-ml conical tubes were cut into smaller pieces with scissors while the tubes were submerged in liquid nitrogen. After RNA isolation, contaminating DNA was removed by applying two successive DNase treatments via the DNA-*free* kit (Ambion, Austin, TX) according to the manufacturer's recommendation. The purified total RNA concentration was quantified with the RiboGreen RNA quantification kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In order to deal with the limitations inherent in imperfect DNA and RNA extraction efficiencies in this study, a single method was applied throughout the study (by the same operator for consistency) and results were compared over time for relative interpretation rather than absolute quantification.

Quantification of genes and gene expression. To quantify the three identified RDase genes (*tceA*, *vcrA*, and *bvcA*) and the 16S rRNA gene of *Dehalococcoides* spp., qPCR was applied to the gDNA as described previously (11). Briefly, each 25-μl reaction mixture contained 3 μl of sample or 10-fold serially diluted standard, 1× TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA), 0.7 μM of the appropriate forward and reverse primers (Table 2), and 0.2 μM of TaqMan probe (Applied Biosystems, Foster City, CA) (Table 2). Standard curves for qPCR were constructed using a single linearized plasmid containing all three RDase genes and the 16S rRNA gene fragments as described by Holmes et al. (11). All samples and standards were analyzed in triplicate, and gene concentrations were reported as copies of gene per liter of groundwater. Selected samples were routinely diluted 10- to 100-fold to test for the presence of PCR inhibitors in the groundwater, and a linear response was always obtained from the dilution series (see Fig. S2 in the supplemental material).

To quantify gene expression, transcripts from the total RNA were reverse transcribed using the RT core reagent kit (Applied Biosystems, Foster City, CA) as described previously (14, 17). Each 10-μl reaction mixture contained 2 μl of sample or serially diluted RNA standard and 0.5 μM of the appropriate reverse primer listed in Table 2. The reaction mixture was incubated at 48°C for 30 min followed by 5 min at 95°C. Contaminating gDNA in each sample was tested for by an additional RT reaction that did not contain reverse transcriptase. The reverse-transcribed samples and standards (3 μl) were then quantified via the qPCR method described above. Triplicate RT-qPCR was performed for each sample and duplicate RT-qPCR for the serially diluted RNA standard. Similar to gene quantification, selected RNA samples were routinely diluted 10- to 100-fold to test for inhibitors, and a linear response was always obtained (see Fig. S2 in the supplemental material). Expression data were reported as copies of transcripts per copy of genes. This value was calculated by dividing the quantity of transcripts per liter of groundwater by the corresponding quantity of gene per liter of groundwater.

RNA standards for the *tceA* and *vcrA* transcripts were in vitro transcribed as described previously (13, 17). Standards for the *bvcA* and 16S rRNA transcripts were synthesized in vitro in this study using the MEGAscript T7 kit (Ambion, Austin, TX) according to the manufacturer's instructions. Input to the RNA synthesis kit was a PCR product that contained the qPCR primers and probe target region and the T7 RNA polymerase promoter sequence. The PCR product for in vitro transcription was synthesized as follows. The T7 sequence was incorporated upstream of the coding sequence via a forward primer that was identical in sequence to the qPCR forward primer except with the T7 sequence added to the 5′ end (Table 2). The reverse primer (Table 2) was downstream of the qPCR target region to generate an amplicon of at least 1,000 bp to enhance the efficiency of in vitro transcription. The T7 sequences incorporating the forward primer and the reverse primer were used to amplify gDNA from strain 195 or strain BAV1 in a PCR according to the following protocol: an initial denaturation step at 94°C (12 min), followed by 35 cycles of 94°C (60 s), 50°C (45 s), and 72°C (120 s), with a final extension at 72°C for 12 min. The 30-μl PCR contained 1× PCR buffer II, 2.5 mM $MgCl_2$, 0.13 mg/ml bovine serum albumin, 1 mM of deoxynucleoside triphosphate (dNTP) mix, 0.05 μM of each primer, and 1.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA).

Following the PCR and the subsequent in vitro transcription, the synthesized RNA standard was purified using the MEGAclear kit (Ambion, Austin, TX) according to the manufacturer's instructions. Contaminating DNA within the synthesized transcripts was removed via the DNA-*free* kit (Ambion, Austin, TX). Complete DNA removal was confirmed by triplicate RT-qPCR reactions where the reverse transcriptase had been omitted and no fluorescence signal was detected after 40 qPCR cycles. The RNA standard concentration was quantified using the RiboGreen RNA quantification kit (Invitrogen, Carlsbad, CA), and the theoretical copy number was calculated according to the size of the input PCR amplicon and an average molecular weight of 340 Da per RNA nucleotide.

RDase gene cDNA clone library. In order to identify additional highly expressed RDase genes in the field samples, degenerate primers (15) were used to detect expression of putative RDase genes by means of a clone library. Total RNA from sample MW 1D4 (February 2006) was reverse transcribed using the SuperScript III First-Strand synthesis system (Invitrogen, Carlsbad, CA) and PCR amplified. In a reverse-transcription reaction of 10 μl, 2 μl of sample RNA was first incubated with 50 ng of random hexamers and 1 mM of dNTP mix at 65°C for 5 min and later on ice for 1 min. A 10-μl cDNA synthesis mix containing 2× RT buffer, 10 mM $MgCl_2$, 0.02 M dithiothreitol, 40 U RNaseOUT, and 200 U of reverse transcriptase was then combined with the previous 10-μl sample and incubated at 25°C for 20 min, 50°C for 60 min, and 65°C for 5 min. Parallel reactions with no reverse transcriptase and with no template were prepared as negative controls. Following reverse transcription, 2 μl of the cDNA product was used in a 25-μl PCR that contained 1× *Ex Taq* buffer, 0.8 mM dNTP mix, 0.5 μM (each) of the degenerate primers (15), and 0.625 U *Ex Taq* hot-start DNA polymerase (Takara Mirus Bio, Madison, WI). The same thermal cycling conditions as above were applied except that 40 cycles were used and the annealing temperature was 48°C. For sample MW 1D4, the expected PCR product of ~1,700 bp was detected on an Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA) using DNA 7500 chips according to the manufacturer's protocol, and the negative controls showed no band.

The clone library was constructed with the TOPO TA cloning kit (with the pCR2.1-TOPO vector) (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A total of 75 plasmid inserts were later digested with the restriction enzymes HhaI or MspI (Promega, Madison, WI) at 34°C for 3 h, and the resulting fragments were analyzed on an Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA) using DNA 7500 chips. Distinct clones were selected for sequencing at Elim Biopharmaceuticals, Inc. (Hayward, CA). Primers for sequencing of

TABLE 2. Primer and probe sequences used in this study[a]

Function or primer/probe	Sequence	Target gene (accession no.)	Reference(s)
Quantification of target mRNAs and genes concn			
tceA_For	5′-ATCCAGATTATGACCCTGGTGAA	*tceA* gene (AF228507)	11, 13
tceA_Rev	5′-GCGGCATATATTAGGGCATCTT	*tceA* gene (AF228507)	11, 13
tceA_Probe	5′-FAM-TGGGCTATGGCGACCGCAGG-TAMRA	*tceA* gene (AF228507)	11, 13
vcrA_For	5′-CTCGGCTACCGAACGGATT	*vcrA* gene (AY322364)	11
vcrA_Rev	5′-GGGCAGGAGGATTGACACAT	*vcrA* gene (AY322364)	11
vcrA_Probe	5′-FAM-CGCACTGGTTATGGCAACCACTC-TAMRA	*vcrA* gene (AY322364)	11
bvcA_For	5′- GGTGCCGCGACTTCAGTT	*bvcA* gene (AY563562)	11
bvcA_Rev	5′-TCGGCACTAGCAGCAGAAATT	*bvcA* gene (AY563562)	11
bvcA_Probe	5′-FAM-TGCCGAATTTTCACGACTTGGATGAAG-TAMRA	*bvcA* gene (AY563562)	11
Dhc16S_For	5′-GGTAATACGTAGGGAAGCAAGCG	*Dehalococcoides* sp. 16S rRNA gene (CP000027)	11
Dhc16S_Rev	5′-CCGGTTAAGCCGGGAAATT	*Dehalococcoides* sp. 16S rRNA gene (CP000027)	11
Dhc16S_Probe	5′-VIC-ACATCCAACTTGAAAGACCACCTACGCT CACT-TAMRA	*Dehalococcoides* sp. 16S rRNA gene (CP000027)	11
FtL-RD1638I_For	5′-CTGGGCCGAAACCAAAGA	Field sample *FtL-RDase-1638* gene (bp 381 to 449)[b]	This study
FtL-RD1638I_Rev	5′-CCCGTCCACTTCGGTTCAC	Field sample *FtL-RDase-1638* gene (bp 381 to 449)[b]	This study
FtL-RD1638I_Probe	5′-FAM-CCCGCTCCGGTAAAAACCCCTGA-TAMRA	Field sample *FtL-RDase-1638* gene (bp 381 to 449)[b]	This study
FtL-RD1638II_For	5′-TTCCTGTCAGAAGTGTGCAGATTC	Field sample *FtL-RDase-1638* gene (bp 1056 to 1129)[b]	This study
FtL-RD1638II_Rev	5′-GCAAATCCCATGAAGGTTCTTT	Field sample *FtL-RDase-1638* gene (bp 1056 to 1129)[b]	This study
FtL-RD1638II_Probe	5′-FAM-TGTCCGCCCCAGTGTATATCTAAAG-TAMRA	Field sample *FtL-RDase-1638* gene (bp 1056 to 1129)[b]	This study
RNA standard synthesis			
Dhc16S_T7_For	5′-TAATACGACTCACTATAGGGGGTAATACGTAGG GAAGCAAGCG	*Dehalococcoides* 16S rRNA gene (CP000027)	This study
Unv_1492_Rev	5′-GG(C/T)TACCTTGTTACGACTT	*Dehalococcoides* 16S rRNA gene (CP000027)	4
bvcA_T7_For	5′-TAATACGACTCACTATAGGGGGTGCCGCGACTTC AGTT	*bvcA* gene (AY563562)	This study
bvcA_1270_Rev	5′-ATTGTGGAGGACCTACCT	*bvcA* gene (AY563562)	15

[a] For, forward primer; Rev, reverse primer; underlined region, RNA polymerase T7 promoter sequence.
[b] Nucleotide position according to the sequenced clone.

internal regions of a clone were designed using the Primer Express v2.0 software program (Applied Biosystems), and the sequenced reads were assembled using the Cap3 Assembly software program (http://pbil.univ-lyon1.fr/cap3.php). After alignment and visual checking, the near-full-length RDase sequences were compared to sequences in GenBank using BLASTn and BLASTx (http://www.ncbi.nlm.nih.gov/).

Quantification of the *FtL-RDase-1638* gene. Two primer and probe sets (Table 2) were used to target the front and rear regions of the *FtL-RDase-1638* gene retrieved from the cDNA clone library. Two distinct regions were targeted in order to obtain an accurate placement of this gene, since a nucleotide BLAST analysis against the genome of strain BAV1 using the IMG 2.3 software at JGI (http://www.jgi.doe.gov/) indicated a possible truncation of an *FtL-RDase-1638*-like gene by an integrase in that isolate. Standards for the qPCR analysis of the *FtL-RDase-1638I* and *FtL-RDase-1638II* gene segments were synthesized by IDT (Coralville, IA) as two separate 80-bp double-stranded DNAs that contained the primer and probe target sequence. The theoretical copy number was calculated using the length of the duplex and an average molecular weight of 660 Da per DNA nucleotide.

Nucleotide sequence accession numbers. Sequences from the RDase gene cDNA clone library have been submitted to the GenBank database under accession no. EU137841 to EU137844.

RESULTS

Anaerobic reductive dechlorination performance. Chlorinated ethene concentrations were measured in groundwater samples collected from eight sampling locations throughout each treatment plot during the enhanced bioremediation demonstration conducted at the Fort Lewis EGDY. Molar concentrations of TCE, cDCE, VC, and ethene measured in ground-

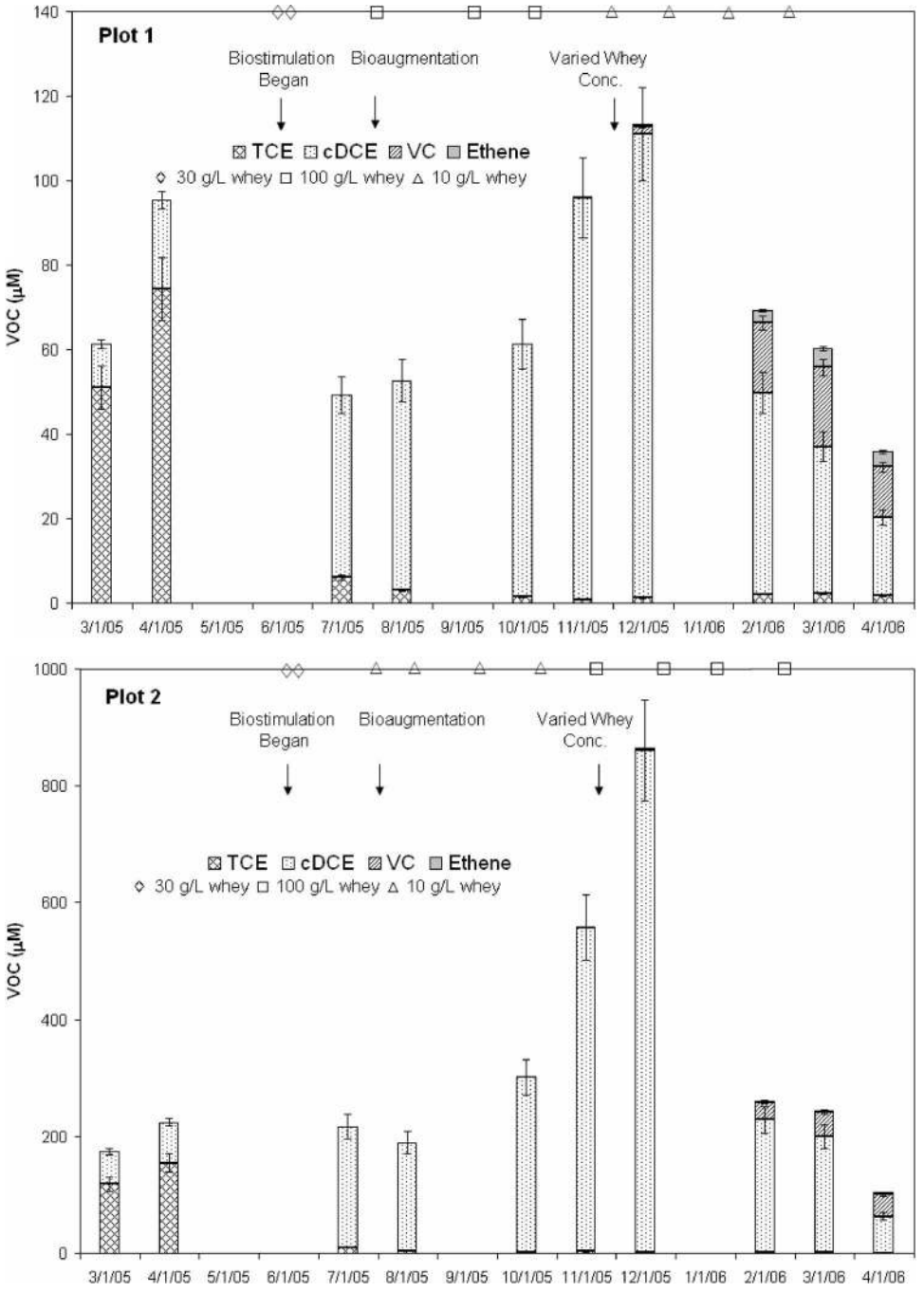

FIG. 1. Molar concentrations of chlorinated ethenes and ethene in groundwater samples over the 1-year monitoring period for treatment plots 1 and 2. Concentrations are reported as averages for eight sampling locations within each treatment plot, and error bars represent analytical error. The date and concentration of whey injected into the respective treatment plots are indicated on the top axis, and manipulations implemented at the site are indicated by arrows on the graphs. For clarity, only one of the two sampling events performed within 2 weeks of each other during March 2005 is plotted on the graph.

water samples are presented in Fig. 1. Three sampling events were conducted prior to whey amendment, two or three events were conducted in subsequent phases of whey amendment, and two sampling events were conducted 1 and 2 months following cessation of whey injections. Because of poor mass balances in the open system, the temporal changes in chlorinated ethenes were represented as a percentage of the total ethenes. The predominant chlorinated ethene observed during the three baseline sampling events (March to April 2005) conducted prior to whey injection was TCE, representing an average range of 78 to 82% of the molar concentration in plot 1 and 68 to 69% of the molar concentration in plot 2. No VC or ethene was detected in either treatment plot. In addition, the average chlorinated ethene concentration was a factor of 2.9 higher in

plot 2 (202 ± 26 μM) than in plot 1 (69 ± 23 μM). In order to stimulate more reducing conditions that would favor reductive dechlorination, whey was injected into the subsurface as an exogenous electron donor for biostimulation beginning in June 2005. One month following 30 g/liter whey injection (July 2005), the molar percentage of chlorinated ethenes in the form of cDCE increased significantly in plot 1 (88%) and plot 2 (96%). Immediately following the July 2005 sampling, each treatment plot was bioaugmented with 18 liters of a *Dehalococcoides*-containing enrichment culture along with injection of 100 g/liter whey into plot 1 and 10 g/liter whey into plot 2. One month following these injections (August 2005), the molar percentage of chlorinated ethenes in the form of cDCE again increased, to an average of 94% in plot 1 and 98% in plot 2, a trend that continued for 4 months after bioaugmentation (November 2005), with cDCE reaching 99% of the molar percentage in both plots 1 and 2. In plot 1, no significant VC or ethene was observed during this sampling, but in plot 2, quantifiable VC was observed, representing 0.3% of the molar concentration.

Approximately 5 months after the initial whey injection (November 2005), the whey injection strategy was altered so that plot 1 received 10-g/liter whey injections while plot 2 received 100-g/liter whey injections. Three months after this operational change and 7 months after bioaugmentation (February 2006), significant increases in VC and ethene were observed, with VC and ethene in both treatment plots representing an average of 24% and 4%, respectively, of the molar concentration in plot 1 and 12% and 1%, respectively, of the molar concentration in plot 2. The increase in VC and ethene fractions continued even after cessation of whey injections, with VC and ethene representing an average of 34% and 10%, respectively, of the molar concentration in plot 1 and 36% and 4%, respectively, of the molar concentration in plot 2 during the last sampling event (April 2006). Up to the April 2006 sampling event, residual carbon persisted within the treatment area as evidenced by chemical oxygen demand concentrations measuring between 67 to 538 mg/liter in plot 1 and 183 to 821 mg/liter in plot 2, indicating that the electron donor was available for reductive dechlorination.

Quantifying RDase and 16S rRNA genes. qPCR was used to monitor the concentrations of 16S rRNA, *tceA*, *bvcA*, and *vcrA* genes of *Dehalococcoides* spp. in treatment plots 1 and 2 over the 1-year monitoring period spanning biostimulation and bioaugmentation activities (Fig. 2). In order to focus on the temporal trends of the data, the respective gene concentrations were averaged spatially across the eight sampling wells on each treatment plot. At the baseline sampling event (April 2005), the *Dehalococcoides* 16S rRNA gene was detected at $1.2 \times 10^4 \pm 0.20 \times 10^4$ copies/liter and $1.1 \times 10^5 \pm 0.13 \times 10^5$ copies/liter in plots 1 and 2, respectively (Fig. 2). Redox conditions at the site during the baseline sampling event indicated predominantly aerobic conditions, with plot 2 exhibiting slightly more reducing conditions than plot 1 (data not shown). Genes associated with VC degradation, *bvcA* and *vcrA*, were present in both treatment plots 1 and 2, while the *tceA* gene was detectable only in plot 2 initially.

One month after the commencement of whey injection for biostimulation (July 2005), significant increases in gene copies for *Dehalococcoides* spp. were detected. Notably, the 16S rRNA genes increased 32-fold in plot 1 and 2.5-fold in plot 2. Furthermore, the *tceA* gene became detectable in plot 1, and the *bvcA* gene in both plots increased by over an order of magnitude (Fig. 2). The dominance of *bvcA* among the three monitored RDase genes was amplified following biostimulation, accounting for the majority of the total RDase genes measured.

Following the July 2005 sampling, bioaugmentation was implemented at the site with an enrichment culture that had a *Dehalococcoides* 16S rRNA gene concentration of $4.3 \times 10^7 \pm 0.72 \times 10^7$ copy/ml, and it contained the *tceA* ($5.1 \times 10^7 \pm 0.28 \times 10^7$ copy/ml) and *vcrA* ($3.6 \times 10^7 \pm 0.12 \times 10^7$ copy/ml) genes but no *bvcA* gene. For 3 months after bioaugmentation (August to November 2005), while all monitored genes increased to some extent, the most significant change was the order-of-magnitude increase in the *vcrA* gene in both plots (Fig. 2).

By February 2006, 3 months after the whey injection strategy was altered, the 16S rRNA gene concentrations in plot 1 increased by 2 orders of magnitude while those in plot 2 increased by 19-fold (Fig. 2). In parallel with the significant increase in the 16S rRNA gene was the increase in the three RDase genes, with the *bvcA* gene concentration continuing to dominate in numbers. During the 1-year biostimulation-and-bioaugmentation period, the 16S rRNA gene concentrations increased by more than 3 orders of magnitude in plots 1 and 2 (Fig. 2).

Accounting for the dominant *Dehalococcoides* cells. The 16S rRNA gene sequences of distinct *Dehalococcoides* strains are highly conserved, making it impossible to differentiate metabolic functions using this gene. Primers and probes that target the 16S rRNA gene in this study were designed to target 16S rRNA sequences of all known *Dehalococcoides* strains. The correlation between concentrations of 16S rRNA gene copies and the sum total of the *tceA*, *vcrA*, and *bvcA* genes is plotted in Fig. 3 in an attempt to identify the dominant *Dehalococcoides* cells within the populations. As illustrated in the regression analysis in Fig. 3, there was good linear correlation ($r^2 = 0.99$) between the 16S rRNA gene and the sum total of the three RDase gene concentrations, and the two variables were within a factor of 1.4 ± 0.064 (slope of regression line ± 95% confidence interval) of each other over concentrations that span several orders of magnitude. With the exception of a few samples analyzed during the July 2005 sampling in plot 2, the data were distributed randomly around the 1:1 ratio and the differences were within the analytical reproducibility and uncertainty of the qPCR technique. The close tracking of copy numbers between functional and ribosomal genes was observed both during the early phases of treatment, when *bvcA* was the only dominant RDase gene, and during the later phases of treatment, when both the *vcrA* and *bvcA* genes were dominant.

Expression of RDase and 16S rRNA genes. In order to verify that the detected genes were being expressed, RT-qPCR was applied to the more labile RNA molecules for the final two sampling events (February 2006 and April 2006). The 16S rRNA genes of *Dehalococcoides* were highly expressed in the selected monitoring wells for both sampling events, with concentrations between $9.7 \times 10^1 \pm 2.1 \times 10^1$ and $8.7 \times 10^2 \pm 1.4 \times 10^2$ transcripts/gene (Fig. 4A). The *tceA*, *vcrA*, and *bvcA*

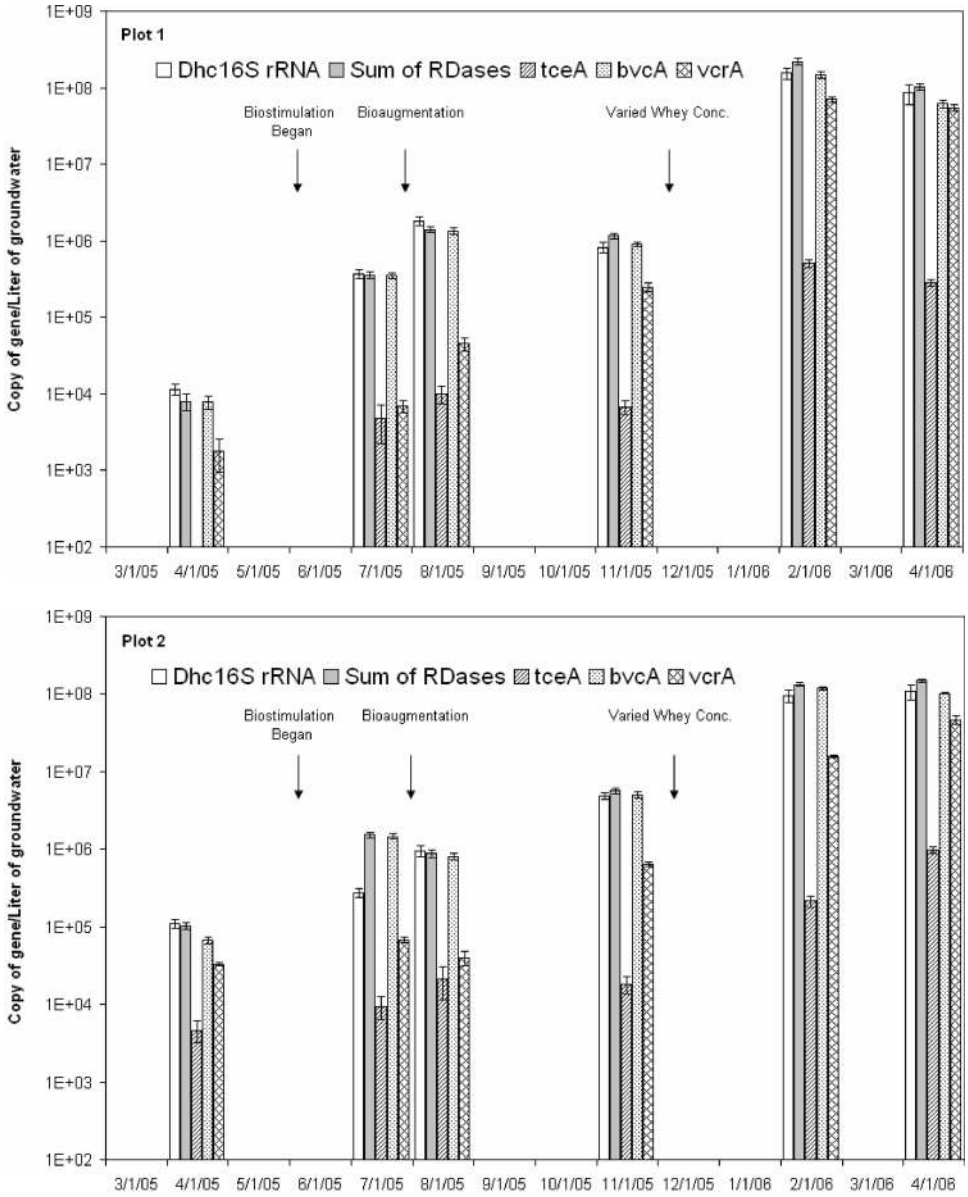

FIG. 2. Dynamics of the *Dehalococcoides* (Dhc) 16S rRNA gene and RDase gene concentrations over the 1-year monitoring period for treatment plots 1 and 2. Manipulations implemented at the site are indicated by arrows on the graphs. Data at each time point are averages for samples from the eight monitoring wells in the respective plot, and each error bar represents one standard deviation for the qPCR method.

transcripts were also detected, but the copies per gene were about 1 to 3 orders of magnitude lower than those for the 16S rRNA transcripts. Samples without detectable *tceA* gene copies (e.g., MW 1A4, February 2006) (Fig. 4B) unsurprisingly had no detectable transcripts, but there were two samples (MW 2D4, February 2006, and MW 1A4, April 2006) with no detectable transcripts despite the presence of a detectable gene. As negative controls, samples collected from two up-gradient aerobic monitoring wells that were not impacted by whey injection had no detectable *Dehalococcoides* 16S rRNA or RDase transcripts.

RDase gene cDNA clone library. In addition to monitoring the three characterized RDase genes, degenerate primers (15) were used to detect the expression of other putative RDase genes in the groundwater samples. A clone library was constructed using total RNA from MW 1D4 from the February 2006 sampling. Out of the 75 clones examined, 4 different RDase transcripts were identified (Table 3). As expected, the *bvcA* and *vcrA* transcripts were recovered, although the *vcrA* clones were present at relatively low abundance. In addition to the *bvcA* and *vcrA* transcripts, two sequences that share a high percent identity (99%) to two putative RDase genes in strain CBDB1 were recovered in the cDNA clone library (Table 3). Of the two, *FtL-RDase-1638* made up 56% of the clones while *FtL-RDase-1618* made up 2.7% (Table 3).

Quantification of the *FtL-RDase-1638* gene. In order to determine whether the *FtL-RDase-1638* gene was located on genomes with one of the RDase genes targeted for quantification

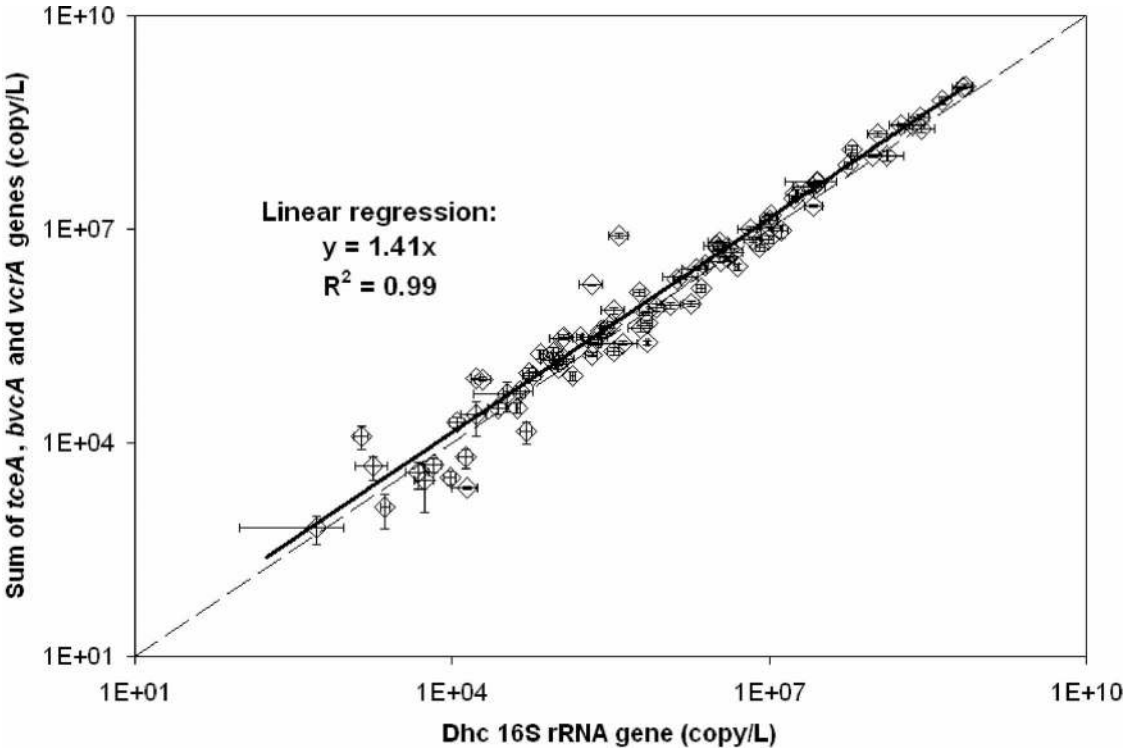

FIG. 3. The sums of RDase gene concentrations are compared against the *Dehalococcoides* (Dhc) 16S rRNA gene concentrations on a log-log scale. Each data point represents a sample collected at a monitoring well from treatment plots 1 and 2 during each sampling event. Each error bar represents one standard deviation for the qPCR method. The dashed line represents the hypothetical 1:1 correlation between the two variables.

in this study, the concentration of the *FtL-RDase-1638* gene was measured in the August 2005 and February 2006 samples. These samples were chosen because the targeted RDase gene numbers differed by orders of magnitude between the samples, facilitating the potential identification of cooccurring genes. As shown in Fig. 5, the gene concentration of *FtL-RDase-1638*, quantified using two distinct regions on the gene, was within twofold of the gene concentration of *bvcA* and about 2 orders of magnitude higher than both the *vcrA* and *tceA* gene concentrations in the August 2005 sample. Furthermore, the *FtL-RDase-1638* gene was detected in the February 2006 sample, while the *tceA* gene was absent, making it unlikely that *FtL-RDase-1638* was present in the *tceA*-carrying genomes. Comparison of the MW 2B4 sample at two time points showed that the increase over time of the *FtL-RDase-1638* gene was similar to the increase of the *bvcA* gene (Fig. 5). These results suggest that the *FtL-RDase-1638* gene is likely present in the genomes of *bvcA*-carrying cells at this site and may also be present in the genomes of other *Dehalococcoides* cells, as suggested by its detection at quantities greater than those of the *bvcA* gene.

DISCUSSION

Multiple strains of *Dehalococcoides* with different chlorinated-ethene-degrading abilities are commonly present in microbial communities (3, 11, 31, 37). At the Fort Lewis site, the *bvcA*, *vcrA*, and *tceA* genes of *Dehalococcoides* were detected prior to any site manipulation, indicating that *Dehalococcoides* cells containing those genes were indigenous to the site. The slightly aerobic conditions initially present likely inhibited growth of the indigenous *Dehalococcoides* populations. Using characterized *Dehalococcoides* isolates as models, TCE would be expected to serve as a metabolic electron acceptor for *tceA*-expressing strains (9, 21, 22, 32) while being cometabolized by *bvcA*-expressing strains (8). Similarly, cDCE would serve as an electron acceptor for *Dehalococcoides* strains expressing *bvcA*, *vcrA*, or *tceA* (8, 9, 21, 22, 27, 34), while VC would be respired by *vcrA*- and *bvcA*-expressing strains and cometabolized by *tceA*-expressing strains (15, 24, 27). Following the initiation of biostimulation and bioaugmentation, moderate increases in *Dehalococcoides* populations were observed during the first 5 months of operation, with the corresponding production and persistence of cDCE. Production of VC and ethene were eventually observed during the February and April 2006 sampling rounds (Fig. 1) and were concomitant with 2-orders-of-magnitude increases in *Dehalococcoides* populations, in particular the *bvcA*- and *vcrA*-carrying cells (Fig. 2).

The total number of *Dehalococcoides* 16S rRNA gene copies present in the field samples was compared to the sum of identified RDase gene copies (*tceA*, *bvcA*, and *vcrA*) in order to determine whether the dominant *Dehalococcoides* cells present at the site could be identified by quantification of the three identified RDase genes. The accuracy of the qPCR comparison was facilitated by the application of a four-gene plasmid standard that has been shown to minimize analytical variability among individual genes within single samples (11). Because of the highly conserved nature of the 16S rRNA gene among *Dehalococcoides* strains and its single copy in the genome, it

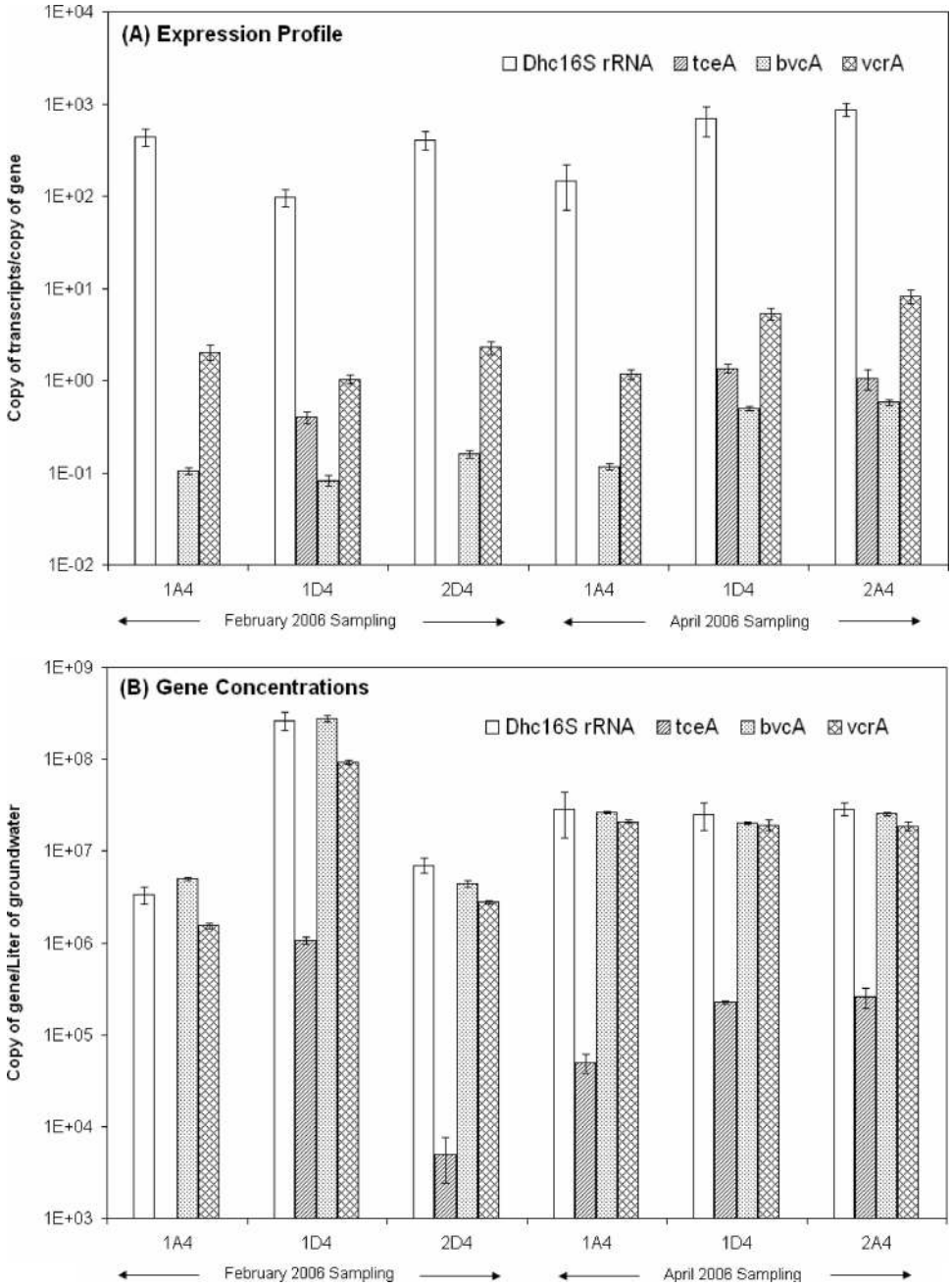

FIG. 4. (A) Expression profile of the *Dehalococcoides* (Dhc) 16S rRNA gene and the RDase genes during the February 2006 and April 2006 sampling events. Data were calculated from triplicate RT-qPCRs, and each error bar represents one standard deviation. Labels on the *x* axis represent monitoring well designations. (B) Gene concentrations at the corresponding monitoring wells. Data were calculated from triplicate qPCRs, and each error bar represents one standard deviation.

TABLE 3. RDase transcripts recovered from a cDNA-clone library

Fort Lewis RDase gene	Most similar RDase gene in *Dehalococcoides* isolates (% identity[a])	% of total clones
FtL-RDase-bvcA	Strain BAV1 *bvcA* gene (98)	40
FtL-RDase-vcrA	Strain VS *vcrA* gene (99)	1.3
FtL-RDase-1638	Strain CBDB1 cbdbA1638[b] (99)	56
FtL-RDase-1618	Strain CBDB1 cbdbA1618[b] (99)	2.7

[a] Based on GenBank BLASTn analysis with a query sequence coverage of at least 1,500 bp that includes the putative RDase gene and the partial anchoring gene.
[b] Locus tag for strain CBDB.

can be assumed that the measured 16S rRNA gene concentration represents the total *Dehalococcoides* concentration within a sample. Furthermore, it was assumed that each of the three identified RDase genes was present as a single copy in the genome, since this has been consistently observed in previously characterized *Dehalococcoides* isolates (9, 15, 27, 32; http://www.jgi.doe.gov/). Hence, significant discrepancies between the sum total of the three RDase genes and the 16S rRNA gene concentrations would then suggest the presence of *Deha-*

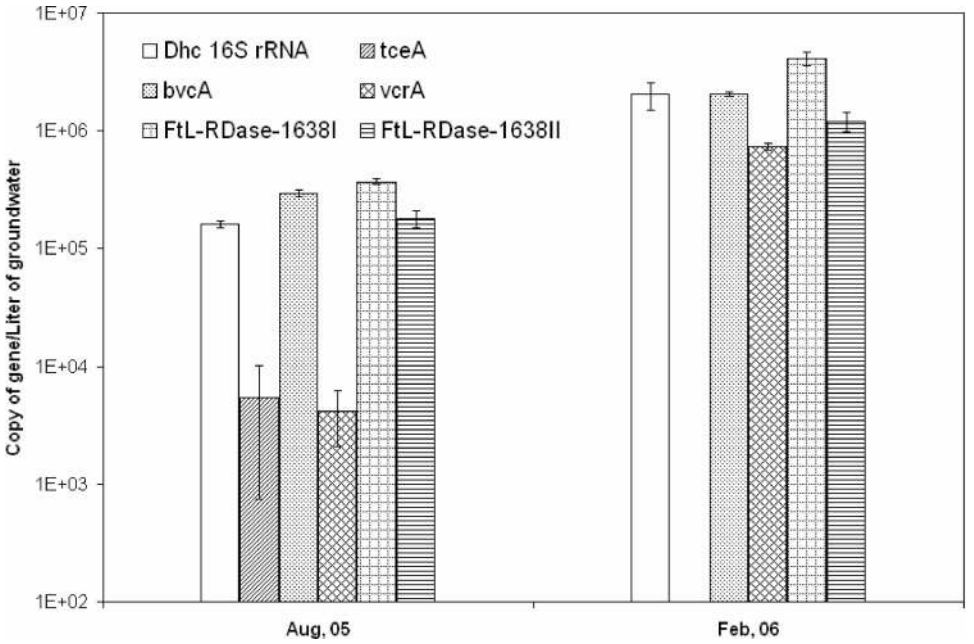

FIG. 5. Concentrations of the *FtL-RDase-1638* gene are compared against *Dehalococcoides* (Dhc) 16S rRNA gene and RDase gene concentrations. The data represent two sampling events for monitoring well 2B4. *FtL-RDase-1638I* and *FtL-RDase-1638II* represent regions from bp 381 to 449 and from bp 1056 to 1129, respectively, on the sequenced clone. Data were calculated from triplicate qPCRs, and each error bar represents one standard deviation.

lococcoides cells not detectable by *bvcA*-, *vcrA*-, and *tceA*-targeted primers/probes. In this study, the close agreement observed between the 16S rRNA gene concentration and the RDase gene sum across several orders of magnitude of detection (Fig. 3) suggests that the majority of *Dehalococcoides* cells at this field site carry a *tceA*, *vcrA*, or *bvcA* gene and that the number of *Dehalococcoides* cells with none of these RDase genes is relatively insignificant. Furthermore, the three RDase genes were determined to be present in separate *Dehalococcoides* cells, since independent variation in the ratio of gene concentration over time indicates that colocation of any of the genes is unlikely (Fig. 2). While a complete accounting of *Dehalococcoides* cells by quantifying known RDase genes was possible at this site and for other enrichment cultures (11, 31), this might not always be the case, as has been demonstrated with other environmental samples (31).

The simultaneous tracking of three *Dehalococcoides* RDases at this site also provided insights into their potential interactions under field conditions. In general, *Dehalococcoides* populations also compete against other TCE-respiring organisms outside of the *Dehalococcoides* genus that are only able to carry out incomplete TCE respiration to cDCE (33). In fact, a previous modeling study demonstrated that growth of *Dehalococcoides* populations could be restricted by other PCE- or TCE-to-cDCE dehalorespirers under certain growth conditions (2), with the competitive advantage of *Dehalococcoides* populations lying in their ability to metabolize cDCE and VC.

Dynamically, while the entire *Dehalococcoides* population showed a significant increase over time, the extent of the increase was unique for each targeted functional gene (Fig. 2). For both treatment plots, the *tceA*-carrying cells showed about a 2-orders-of-magnitude increase throughout the study but never represented more than 0.2 to 4.5% of the total *Dehalococcoides* population even though significant reduction of TCE was measured (Fig. 2), suggesting that these cells were outcompeted by other TCE-respiring organisms and other *Dehalococcoides* strains. In contrast, the *vcrA*-carrying cells in plot 1 increased by more than 4 orders of magnitude over the 1-year monitoring period, while the *bvcA*-carrying cells exhibited a more than 3-orders-of-magnitude increase (Fig. 2). The ratio of *bvcA*-to-*vcrA* cells changed from the initial value of 4:1 to 1:1 toward the end of the monitoring period in plot 1. For plot 2, both cells increased by more than 3 orders of magnitude and the 2:1 ratio that was initially observed between the *bvcA*- and *vcrA*-carrying cells was maintained toward the end. The *tceA*-, *bvcA*-, and *vcrA*-carrying cells occupied a unique niche in this subsurface environment as the only currently known organisms that can reduce chlorinated ethenes beyond DCE (33), and their significant overall increases were reflected in the production of VC and ethene during the latter phases of treatment.

In addition to biostimulation, bioaugmentation with a *Dehalococcoides*-containing culture was also implemented at this site. The rationale for implementing bioaugmentation was to ensure that the field site contained the appropriate metabolic capability for complete reductive dechlorination to ethene. In hindsight, this strategy might not have been necessary, since the three characterized genes that are vital for converting chlorinated ethenes to ethene were indigenous to the site. The addition of the *tceA*- and *vcrA*-containing cells from the bioaugmentation culture created a redundancy in metabolic functions, and it was not possible to differentiate the contribution from the two sources to the overall gene concentration in order to specifically evaluate the performance of the bioaugmentation culture in the field environment. In contrast, because the

bvcA gene was absent in the bioaugmentation culture, the measured increase in that gene concentration could be solely attributed to biostimulation of the indigenous cells.

Although net increases in 16S rRNA gene concentrations are indicative of increased *Dehalococcoides* cell numbers, the presence of a gene does not always guarantee expression (19). Further, changes in the gene concentration from net cell growth require considerable amounts of time for *Dehalococcoides* cells, as evidenced by doubling times reported under optimized laboratory conditions of 19 h for strain 195 (25) and 2.2 days for strain BAV1 (8). The doubling time in the less-controlled field conditions might be considerably longer. Laboratory experiments have also indicated that the half-life of *Dehalococcoides* RDase transcripts is on the order of 4 to 6 h in the absence of chlorinated ethenes (17) and the up-regulation response time after chlorinated ethenes exposure is approximately 3 h (14). This relatively short response time is ideal for diagnosing cell physiology.

Although a significant increase in the overall gene concentration was observed at the field site during the 1-year monitoring period, there were two periods during which significant growth was not detected (between July and November 2005 and between February and April 2006). However, the transcript data measured for the RDase genes and 16S rRNA gene during one of those periods verified that *Dehalococcoides* cells were indeed physiologically active even though no significant net increase in gene concentration was observed (Fig. 4). The quantity of the 16S rRNA transcripts was comparable to that for actively dechlorinating *D. ethenogenes* strain 195 cells grown in the laboratory (P. K. H. Lee and L. Alvarez-Cohen, unpublished data). Furthermore, the expression data indicated variable levels of activity among the *Dehalococcoides* populations. While the concentration of the *bvcA* gene was the highest in groundwater samples, its expression level was unexpectedly lower than those of both the *vcrA* and *tceA* genes when the transcripts were normalized to the respective gene copy number. Transcript-per-gene data normalize for the effects of cell concentration on the expression profile. The *bvcA* expression data suggested that not all *bvcA* genes were actually in metabolically active cells. On the other hand, the higher *vcrA* transcript-per-gene levels indicated that the *vcrA*-carrying cells were relatively more active. The expression of the *bvcA* and *vcrA* transcripts was consistent with the chlorinated ethene data, which showed significant increases in VC and ethene during the February and April 2006 samplings. Interestingly, the *tceA* transcripts were not uniformly detected in all samples that contained the *tceA* gene. The lack of expression suggests that not all *tceA*-carrying cells were metabolically active and they might not have played a major role in the TCE-to-VC reduction. These data could further explain why the *tceA* strain lagged behind the other *Dehalococcoides* cells in overall cell numbers.

In our examination of expressed RDase genes at the site detected using the cDNA clone library, it was surprising to recover a highly expressed gene (*FtL-RDase-1638*) that shared a high percent identity (99%) with a putative RDase gene in strain CBDB1 (Table 3). The close accounting between *Dehalococcoides* 16S rRNA gene copies and the three targeted RDase genes suggested that it was unlikely that strain CBDB1, an isolate that does not carry the *tceA*, *vcrA*, or *bvcA* gene targeted here, represented a significant fraction of the *Dehalococcoides* population (Fig. 3). Quantitative analysis of the copy numbers of the *FtL-RDase-1638* gene using two distinct regions of the sequence suggested that this gene is likely present in the genomes of *bvcA*-carrying cells at this site (Fig. 5), with the possibility that it is also present in other low-abundance *Dehalococcoides* cells. The discrepancy of less than a factor of 3.5 between the absolute quantities of the *FtL-RDase-1638* gene fragments is probably due to the application of two separately quantified standards in qPCR analysis (11). Clone libraries of a *Dehalococcoides*-containing enrichment, KB-1, also indicated the presence and expression of a gene (*KB1RdhAB5*) similar to *FtL-RDase-1638* when KB-1 was exposed to TCE, cDCE, or VC (37). While the genomic location of this gene was not determined, *bvcA*-carrying cells are present in KB-1. Interestingly, although an *FtL-RDase-1638*-like gene was not experimentally identified as 1 of the 11 putative RDase genes in strain BAV1 (15), a nucleotide-nucleotide BLAST analysis against the genome of strain BAV1 identified a 645-bp fragment that has 95% similarity to the amino terminus-coding region of the *FtL-RDase-1638* gene (http://www.jgi.doe.gov/). According to the annotation of strain BAV1, this 645-bp fragment is followed downstream by an integrase and a transposase (see URL above), indicating that this RDase gene has been truncated in this strain. Based on the data in this study together with the KB-1 and strain BAV1 results, the *FtL-RDase-1638* gene is likely present in the *bvcA*-carrying genome.

In summary, this study demonstrates that quantifying genes and transcripts of the 16S rRNA gene and three characterized RDase genes of *Dehalococcoides* during different stages of bioremediation can provide important and complementary information about the dynamics and activity of these organisms. Since *Dehalococcoides* spp. are the only organisms known to convert TCE to innocuous ethene, having techniques that can monitor and optimize their activity can substantially promote the success of bioremediation projects. The type of data collected in this study can also be useful to regulatory agencies to evaluate the effectiveness of in situ biological processes.

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