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Quantitative PCR Targeting 16S rRNA and Reductive Dehalogenase Genes Simultaneously Monitors Multiple *Dehalococcoides* Strains

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The 16S rRNA gene provides insufficient information to infer the range of chloroorganic electron acceptors used by different *Dehalococcoides* organisms. To overcome this limitation and provide enhanced diagnostic tools for growth measurements, site assessment, and bioremediation monitoring, a quantitative real-time PCR (qPCR) approach targeting 16S rRNA genes and three *Dehalococcoides* reductive dehalogenase (RDase) genes with assigned function (i.e., *tceA*, *bvcA*, and *vcrA*) was designed and evaluated. qPCR standard curves generated for the RDase genes by use of genomic DNA from *Dehalococcoides* pure cultures correlated with standard curves obtained for both *Bacteria*- and *Dehalococcoides*-targeted 16S rRNA genes, suggesting that the RDase genes are useful targets for quantitative assessment of *Dehalococcoides* organisms. RDase gene probe/primer pairs were specific for the *Dehalococcoides* strains known to carry the diagnostic RDase gene sequences, and the qPCR method allowed the detection of as few as 1 to 20 and quantification of as few as 50 to 100 *tceA*, *bvcA*, or *vcrA* gene targets per PCR volume. The qPCR approach was applied to dechlorinating enrichment cultures, microcosms, and samples from a contaminated site. In characterized enrichment cultures where known *Dehalococcoides* strains were enumerated, the sum of the three RDase genes equaled the total *Dehalococcoides* cell numbers. In site samples and chloroethane-dechlorinating microcosms, the sum of the three RDase genes was much less than that predicted by *Dehalococcoides*-targeted qPCR, totaling 10 to 30% of the total *Dehalococcoides* cell numbers. Hence, a large number of *Dehalococcoides* spp. contain as-yet-unidentified RDase genes, indicating that our current understanding of the dechlorinating *Dehalococcoides* community is incomplete.

Chlorinated solvents are a well-recognized class of groundwater (GW) contaminants (1, 7, 41, 53). Substantial knowledge regarding diverse groups of bacteria that partially dechlorinate tetrachloroethene (PCE) or trichloroethene (TCE) to *cis*-1,2-dichloroethene (*cis*-DCE) (5, 12, 19, 30, 50) or *trans*-DCE (13) has been accrued over the past decade. Partial reductive dechlorination contributes to the formation of DCEs and, in some cases, vinyl chloride (VC) (1, 26, 45). Environmental accumulation of VC is particularly troublesome because this compound is a proven human carcinogen; therefore, incomplete dechlorination of PCE and TCE does not result in detoxification.

Recently, *Dehalococcoides* organisms were discovered as a deeply branching group within the green nonsulfur bacteria (*Chloroflexi*) (44). This physiologically and phylogenetically distinct bacterial group requires hydrogen as an electron donor and specific chloroorganic compounds as electron acceptors to support their energy metabolism. *Dehalococcoides ethenogenes* strain 195 was the first isolate described to dechlorinate PCE to VC and ethene (35); however, the last transformation step from VC to ethene occurred slowly in a cometabolic process (34). Similarly, metabolic dechlorination of TCE to VC and cometabolic transformation to ethene occurs in *Dehalococcoides* sp. strain FL2 (17). Although these organisms can produce nontoxic ethene, the last transformation step is often incomplete, potentially leading to VC accumulation at contaminated sites. Other *Dehalococcoides* strains dechlorinate VC metabolically and more efficiently produce ethene. Strain BAV1, isolated from a contaminated aquifer in Michigan, respires all DCE isomers and VC (15, 16), and strains GT, VS, and KB-1(VC/H_2) are also capable of metabolic dechlorination of VC (9, 36, 49). The link between the presence of *Dehalococcoides* organisms and chloroethene detoxification (i.e., ethene formation) has been established (18, 27), and field studies demonstrated a cause-and-effect relationship between bioaugmentation with *Dehalococcoides*-containing consortia and ethene formation (10, 26, 33).

16S rRNA gene-targeted molecular tools proved valuable for assessing reductive dechlorination potential at contaminated sites and for monitoring *Dehalococcoides* organisms following the implementation of enhanced bioremediation approaches. Qualitative information regarding the presence of *Dehalococcoides* organisms has been obtained using PCR primers that targeted signature regions of the 16S rRNA gene (18, 29). Sensitivity was further improved using an initial amplification of community 16S rRNA genes with *Bacteria*-targeted primers, followed by PCR with *Dehalococcoides*-specific, internal primers (i.e., nested PCR), effectively increasing the detection limit by 2 orders of magnitude (29, 39). After the significance of *Dehalococcoides* to chloroethene detoxification was realized, quantitative PCR methods to enumerate *Dehalococcoides* 16S rRNA gene copies were developed and applied

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(8, 15, 16, 26, 47). Quantitative information was critical for establishing cause-and-effect relationships between specific treatments, changes in *Dehalococcoides* abundance, and chloroethene detoxification (26).

To overcome the limitations resulting from the high degree of *Dehalococcoides* 16S rRNA gene similarity (>98% identity) and an absence of correlation between 16S rRNA gene sequence and dechlorination activity (2, 9, 16, 17, 20, 34, 39, 49), a quantitative real-time PCR (qPCR) approach targeting three *Dehalococcoides* strain-specific reductive dehalogenase (RDase) genes was developed to more comprehensively characterize the chloroethene-dechlorinating *Dehalococcoides* community. The approach selectively targeted the TCE-to-VC RDase gene (*tceA*) of *Dehalococcoides ethenogenes* strain 195 (3, 32) and strain FL2 (17, 21); the *bvcA* gene, implicated in VC-to-ethene reductive dechlorination in strain BAV1 (23); and the *vcrA* gene, first discovered in strain VS (36) and later identified in strain GT, a pure *Dehalococcoides* culture capable of metabolic TCE-to-ethene dechlorination (49). The *tceA*, *bvcA*, and *vcrA* genes provide promising targets to complement existing 16S rRNA gene-based approaches to more comprehensively describe the chloroethene-dechlorinating *Dehalococcoides* community.

MATERIALS AND METHODS

Sources of DNA. DNA was obtained from *Dehalococcoides* pure cultures, *Dehalococcoides*-containing consortia, microcosms, and groundwater, and aquifer materials collected from a contaminated site near Montague, MI. The three pure cultures were grown in mineral salts medium (MM) amended with acetate, hydrogen, and a chlorinated electron acceptor (28, 50). Isolate BAV1 (16) was grown with VC, and isolates FL2 (17) and GT (49) were grown with TCE. *Dehalococcoides*-containing consortia included cultures SZ(PCE) and SZ(VC), which were derived from SuZi Creek sediment (Seoul area, South Korea), with PCE and VC as electron acceptors, respectively. Cultures KS(1,2-D) and RC(1,2-D), which do not utilize chloroethenes as electron acceptors, were derived from Alaska and Michigan river sediments, respectively, and were grown with 1,2-dichloropropane (1,2-D) (39). Bio-Dechlor INOCULUM (BDI), a commercially available PCE-to-ethene-dechlorinating consortium that has been used successfully for bioaugmentation at chloroethene-contaminated sites (40), was maintained in MM amended with lactate and TCE. GW and aquifer material were collected from a chloroethene (mainly TCE and PCE)-, hexachlorocyclopentadiene (C-56)-, and octachlorocyclopentene (C-58)-contaminated site near Montague, MI (Occidental Chemical Corporation). The aquifer materials contained 0.1 to 0.4 mg kg^{-1} C-56, 1.5 to 2.0 mg kg^{-1} C-58, and less than 0.11 μg g^{-1} PCE and TCE. Groundwater had less than 0.001 mg $liter^{-1}$ of either C-56 or C-58 but did contain up to 73 mg $liter^{-1}$ PCE and 2.3 mg $liter^{-1}$ TCE. Microcosms were initiated by placing ~4 g (wet weight) of homogenized aquifer material in sterile, 20-ml glass vials. Anoxic site GW (pH 7.1) or reduced, bicarbonate-buffered MM (pH 7.1) was added to replicate microcosms to a total volume of 10 ml, and the vials were sealed with Teflon-lined septa. The medium was prepared as described previously except that resazurin was omitted (28, 50). The microcosms were amended with C-56 and C-58 to 0.86 and 4.9 μg ml^{-1}, respectively, in the GW-containing microcosms and to 3 and 18 μg ml^{-1}, respectively, in the MM-amended samples. The microcosms were initiated in an anoxic glove box with a 96% (vol/vol) nitrogen atmosphere balanced with hydrogen and were incubated in the dark at room temperature and without shaking. Following a 206-day incubation period, two replicate microcosms were sacrificed for chemical examination and two were used for molecular analyses.

DNA isolation. Chromosomal DNA from *Dehalococcoides* pure and mixed cultures was collected from the biomass of 10 ml of culture fluid, which was collected by centrifugation (10,000 × *g*, 15 min at room temperature). DNA was extracted using a QIAamp DNA Mini kit (QIAGEN, Valencia, CA) according to the bacterial protocol, with the following modification. After addition of 180 μl of buffer ATL (provided with the QIAGEN kit) to the bacterial pellet, 20 μl of lysozyme (100 mg/ml) (Sigma, St. Louis, MO) and 10 μl of achromopeptidase (25 mg/ml) (Sigma) were added, and the mixture was incubated at 37°C for 1 h. Then, 45 μl of proteinase K (20 mg/ml) (Invitrogen, Carlsbad, California) was added, and the mixture was incubated at 55°C for 1 h. DNA was eluted from a column in 50 μl of Tris buffer (10 mM, pH 8.0) and quantified by spectrophotometry at a wavelength of 260 nm. The concentration of DNA was calculated according to the formula ng DNA/μl = optical density at 260 nm × 50 × dilution factor (4). Chromosomal DNA from *Dehalococcoides ethenogenes* strain 195 (60 ng/μl) was kindly provided by S. Zinder, Cornell University.

DNA from groundwater was obtained from the biomass of two 1-liter samples collected on polyethersulfone membrane filters (0.2-μm pore size), and DNA extractions were performed using a MoBio water DNA kit (MoBio, Carlsbad, California). A MoBio soil DNA kit was used to obtain DNA from duplicate 1-g aquifer material samples. DNA preparations from both the solid and the groundwater extractions were obtained in total volumes of 50 μl of 10 mM Tris buffer (pH 8.5) and quantified by spectrophotometry. The supernatant from the statically incubated microcosms was decanted from the aquifer slurry, and the biomass from the liquid fraction was concentrated by centrifugation in 1.5-ml plastic tubes. The cell pellets were applied to the MoBio soil DNA kit and extracted per the manufacturer's recommendations. Also, the MoBio soil DNA kit was used to obtain DNA from 0.75 ml of the aquifer material slurry.

Target DNA was also obtained from *Escherichia coli* host cells with plasmid Topo-TA pCR2.1 (Invitrogen), carrying cloned fragments of the strain BAV1 16S rRNA gene (pBAV1/16S), the strain BAV1 *bvcA* gene (pBAV1/bvcA), the strain GT *vcrA* gene (pGT/vcrA), and the strain FL2 *tceA* gene (pFL2/tceA) (16, 21, 49). The vectors were obtained from the *E. coli* host by using a QIAprep plasmid extraction kit according to the spin prep protocol. Plasmid DNA was eluted in 50 μl of Tris buffer (10 mM, pH 8.0) and quantified by spectrophotometry. The DNA was used for preparation of standard curves for qPCR, as indicated in Table 1. Table 1 also introduces the abbreviated nomenclature for genomic DNA and plasmids carrying 16S rRNA and RDase genes cloned from specific *Dehalococcoides* organisms.

PCR amplification. The presence of *Dehalococcoides* 16S rRNA genes in sample materials was first determined using a nested-PCR approach (29). Initially, a community's 16S rRNA genes were amplified using universal bacterial primers 8F and 1541R and 20 to 30 ng of community DNA as template, as described previously (55). Following amplification, 2 μl of PCR product was analyzed by agarose gel electrophoresis to verify that 16S rRNA genes were amplified from the community DNA. Then, 1.25 μl of 1:2 and 1:50 dilutions of the 16S rRNA gene amplicons were used as templates in a second round of PCR with a *Dehalococcoides* 16S rRNA gene-specific primer pair (15).

qPCR primer and probe design and reaction conditions. Previously, qPCR approaches using a linear hybridization (TaqMan) probe were designed to target the 16S rRNA gene of members of the *Dehalococcoides* group (15, 16, 26) and the *tceA* gene of *Dehalococcoides ethenogenes* strain 195 and *Dehalococcoides* sp. strain FL2 (3, 22). We expanded this procedure to include primers and TaqMan probes for the *bvcA* and *vcrA* genes implicated in VC reductive dechlorination in isolate BAV1 and isolates GT and VS, respectively (23, 36, 49). The specificity of primers and probes targeting regions of the RDase genes met the criteria of the Primer Express software (Applied Biosystems, Foster City, CA), including an amplicon length of 50 to 150 bp and the desired primer and probe melting temperatures of 58 to 60°C and 68 to 70°C, respectively (37). The G+C content was between 30 to 80 mol%, with no more than three consecutive G or C bases in either the primer or the probe sequence. The specificity of the primers and probes was also manually verified using BLAST analysis and comparison with the genome sequence of *Dehalococcoides ethenogenes* strain 195 (44). The probe and primer sets utilized in this study are summarized in Table 2. Their specificity was also tested with genomic DNA of *Dehalococcoides* cultures, including isolates BAV1 (16, 23), FL2 (17), CBDB1 (2, 21), 195 (34, 35), and strain KB-1(VC/H_2) present in consortium KB-1 (9). The selected primer and probe sequences were specific to the target sequences of the *tceA*, *bvcA*, and *vcrA* genes (GenBank accession numbers AY563562, AY165309, and AY322364, respectively).

In addition to determining the number of *Dehalococcoides* sp. 16S rRNA genes, the total number of bacterial 16S rRNA genes was estimated with a TaqMan-based qPCR approach. Published primer sequences designed to detect total bacterial 16S rRNA genes (i.e., "universal primers") (6, 14, 31, 37, 51, 52) were compared with an alignment of bacterial 16S rRNA genes that included bacterial genera frequently encountered in anoxic subsurface habitats (39, 43). The *Bacteria*-targeted forward primer 1055f described by Harms et al. (14) was modified because an alignment of 16S rRNA gene sequences revealed a T rather than a C in the fifth position from the 5′ end of the forward primer in some strains. The sequences with a T in this position included *Dehalobacter restrictus* (GenBank accession number Y10164), several *Acetobacterium* spp. (ARC05 clone, AY185312; *Acetobacterium carbinolicum*, X96956; *A. malicum*, X96957; and *A. woodii*, X96954), and *Sedimentibacter* sp. strain BRS2 (AY221992). Hence, the residue C in primer Bac1055F was synthesized as a Y to account for both nucleotide variations and to include these organisms in the total bacteria

TABLE 1. Quantities of chromosomal and plasmid DNA used to construct qPCR standard curves

Target DNA	Abbreviation	Length[a]	Tested concn range (ng/μl)[b]	No. of target gene copies tested (range [per μl DNA])[c]
Dehalococcoides sp. strain BAV1				
Genomic DNA	BAV1 DNA	1.5 Mb	2×10^{-6}–20	1.2×10^{0}–1.2×10^{7}
Cloned 16S rRNA gene	pBAV1/16S	5.4 kb	1×10^{-8}–1	1.4×10^{0}–1.4×10^{8}
Cloned *bvcA* gene	pBAV1/bvcA	5.0 kb	1×10^{-8}–1	1.7×10^{-1}–1.7×10^{8}
Dehalococcoides sp. strain GT				
Genomic DNA	GT DNA	1.5 Mb	1.2×10^{-6}–12	7.3×10^{0}–7.3×10^{6}
Cloned *vcrA* gene	pGT/vcrA	5.4 kb	1×10^{-8}–1	1.8×10^{0}–1.8×10^{8}
Dehalococcoides sp. strain FL2				
Genomic DNA	FL2 DNA	1.5 Mb	5×10^{-6}–50	3.0×10^{0}–3.0×10^{7}
Cloned 16S rRNA gene	pFL2/16S	5.4 kb	1×10^{-8}–1	1.7×10^{0}–1.7×10^{8}
Cloned *tceA* gene	pFL2/tceA	7.3 kb	1×10^{-8}–1	1.25×10^{0}–1.25×10^{8}
Dehalococcoides ethenogenes strain 195 genomic DNA	195 DNA	1.5 Mb	6×10^{-6}–60	3.6×10^{0}–3.6×10^{8}
Anaeromyxobacter dehalogenans strain 2CP-C genomic DNA[d]	2CP-C DNA	5.0 Mb	2.4×10^{-6}–24.5	8.9×10^{0}–8.9×10^{6}

[a] Based on the genome sizes of *Dehalococcoides ethenogenes* strain 195 (1.5 Mb) and *Anaeromyxobacter dehalogenans* strain 2CP-C (5.0 Mb) or calculated by adding the size of plasmid pCR2.1 (3.9 kb) to the cloned insert size (1.5 kb for the BAV1 16S rRNA and *bvcA* genes and 3.4 kb for the *tceA* gene).
[b] Based on spectrophotometric quantification of the DNA at 260 nm and 10-fold serial dilutions.
[c] Calculated by equation 1 as described in the text.
[d] Two 16S rRNA genes per genome.

enumerated by the technique (modified primer designated Bac1055YF). The reverse primer Bac1392R and the probe Bac1115Probe were used as described previously (14).

PCRs contained a forward primer, a reverse primer, and a probe that utilized 6-carboxyfluorescein (FAM) as a reporter fluorophore on the 5′ end, with N,N,N′,N′-tetramethyl-6-carboxyrhodamine (TAMRA) as a quencher on the 3′ end (Table 2). qPCR twofold concentrated master mix (Applied Biosystems, Foster City, CA), 300 nM probe, and 300 nM of each primer were combined in sterile, nuclease-free water (Invitrogen) prior to addition of any DNA template. The optimal probe and primer conditions were determined experimentally by using the protocol provided with the master mix. The vial containing the master mix was vortexed to assure a homogeneous solution and briefly spun down in a microcentrifuge. Aliquots (27 μl) of the reaction mix were dispensed to each ABI optical tube held on ice. All manipulations were performed in a dimly lit room to prevent light-activated degradation of the fluorescently labeled oligonucleotide probes. Template DNA (3 μl) was added to each tube, and the tubes were sealed with an 8-tube optical cap strip. DNA concentrations used for standard curve preparation are listed in Table 1; unknown samples contained 20 to 100 ng μl^{-1} DNA. All tubes were maintained on ice and in the dark during transport to the spectrofluorimetric thermal cycler (ABI Prism 7700 sequence detection system), which was used to detect FAM fluorescence. The 16S rRNA gene-, *tceA*-, *bvcA*-, and *vcrA*-targeted primer pairs all optimally utilized an annealing temperature of 58°C. All assays were identical in probe and primer concentrations and used the same two-step TaqMan PCR protocol as was employed with the *Dehalococcoides* 16S rRNA gene-targeted probe/primer set. PCR cycle parameters were as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 58°C (except for Bac1115Probe, which used an annealing temperature of 52°C). These studies all used sterile water, DNA from *Anaeromyxobacter dehalogenans* strain 2CP-C (42), or plasmid pCR2.1 DNA without insert as a negative control to assure identification of false positives. DNA from *Dehalococcoides* strains that did not possess a specific target RDase

TABLE 2. TaqMan probes and primers used to quantify *Dehalococcoides* spp. RDase genes and total bacterial 16S rRNA genes

Primer/probe	Sequence	Target gene	Reference(s) or source
Dhc1200F	5′-CTGGAGCTAATCCCCAAAGCT	*Dehalococcoides* sp. 16S rDNA	15
Dhc1271R	5′-CAACTTCATGCAGGCGGG	*Dehalococcoides* sp. 16S rDNA	15
Dhc1240Probe	5′-FAM-TCCTCAGTTCGGATTGCAGGCTGAA-TAMRA	*Dehalococcoides* sp. 16S rDNA	15
Bvc925F	5′-AAAAGCACTTGGCTATCAAGGAC	*bvcA* gene	This study
Bvc1017R	5′-CCAAAAGCACCACCAGGTC	*bvcA* gene	This study
Bvc977Probe	5′-FAM-TGGTGGCGACGTGGCTATGTGG-TAMRA	*bvcA* gene	This study
Vcr1022F	5′-CGGGCGGATGCACTATTTT	*vcrA* gene	This study
Vcr1093R	5′-GAATAGTCCGTGCCCTTCCTC	*vcrA* gene	This study
Vcr1042Probe	5′-FAM-CGCAGTAACTCAACCATTTCCTGGTAGTGG-TAMRA	*vcrA* gene	This study
TceA1270F	5′-ATCCAGATTATGACCCTGGTGAA	*tceA* gene	3, 22
TceA1336R	5′-GCGGCATATATTAGGGCATCTT	*tceA* gene	3, 22
TceA1294Probe	5′-FAM-TGGGCTATGGCGACCGCAGG-TAMRA	*tceA* gene	3, 22
Bac1055YF[a]	5′-ATGGYTGTCGTCAGCT	*Bacteria*	This study; 11
Bac1392R	5′-ACGGGCGGTGTGTAC	*Bacteria*	25
Bac1115Probe	5′-FAM-CAACGAGCGCAACCC-TAMRA	*Bacteria*	14, 25

[a] The C in the original reference was modified to a Y (which equals C/T).

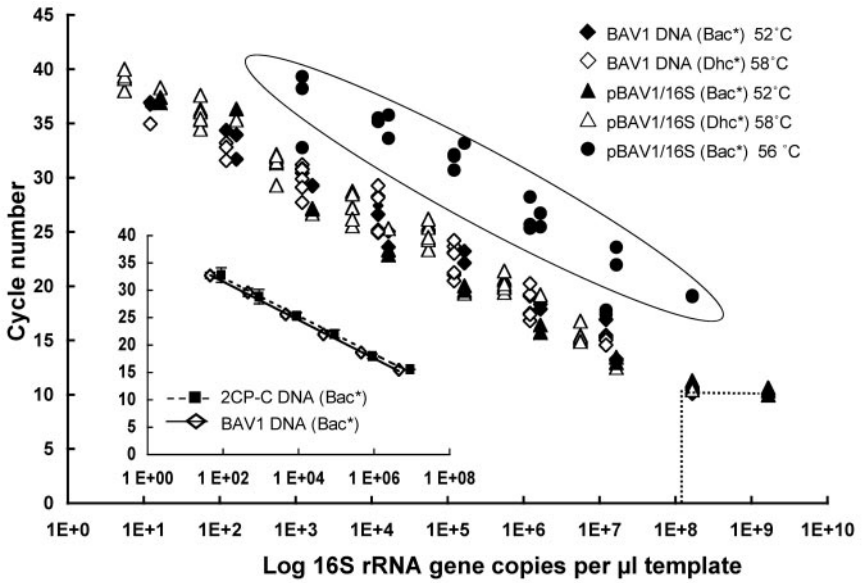

FIG. 1. qPCR with *Dehalococcoides* (Dhc*) and *Bacteria* (Bac*) 16S rRNA gene-targeted primer/probe sets, using *Dehalococcoides* genomic and plasmid DNA carrying a *Dehalococcoides* 16S rRNA gene fragment as the template. *Dehalococcoides* sp. strain BAV1 genomic DNA (diamond) and pBAV1/16S (triangle) interrogated with the *Dehalococcoides*-targeted (open) and universal bacterial (filled) probes were not statistically different under optimized conditions (95% confidence interval, $P > 0.05$). The dotted line at 2×10^8 gene copies per μl of template indicates the maximum copies per reaction mixture that could be quantified under the reaction conditions tested. The points enclosed in the ellipse (filled circles) indicate values generated with the universal probes and BAV1 plasmid DNA at 56°C, which differed from the standard curves generated at 52°C. The inset shows a comparison of standard curves generated with the bacterial 16S rRNA gene-targeted (Bac*) primer/probe set for *Dehalococcoides* (open diamond, solid line) and *Anaeromyxobacter* (filled square, dashed line). The data sets for both organisms were not statistically different ($n = 3$, $P > 0.05$).

gene served as an additional negative control for the RDase gene-targeted probes and primer sets.

qPCR calibration curves. Calibration curves (gene copy number versus the cycle number at which the fluorescence intensity reaches a set cycle threshold value) were obtained using serial dilutions of pure-culture genomic DNA and/or plasmids carrying a single, cloned *Dehalococcoides* target gene: pBAV1/16S, pFL2/16S, pBAV1/bvcA, pGT/vcrA, or pFL2/tceA. Equation 1 was used to calculate the number of gene copies in a known amount of DNA.

$$\text{gene copies} = (\text{DNA concentration [ng/µl]})\left(\frac{1\text{ g}}{1{,}000^3\text{ ng}}\right)\left(\frac{1\text{ mol bp DNA}}{660\text{ g DNA}}\right) \times \left(\frac{6.023 \times 10^{23}\text{ bp}}{\text{mol bp}}\right)\left(\frac{1\text{ copy}}{\text{genome or plasmid size [bp]}}\right) \times (\text{volume of template [µl]}) \quad (1)$$

Copy number estimates assume an average molecular weight of 660 for a base pair in double-stranded DNA and one gene copy per 1.5-Mbp-sized *Dehalococcoides* genome (16, 44). Standard curves based on genes cloned in the Topo-TA pCR2.1 vector used the respective length of the plasmid reported in Table 1 instead of 1.5 Mbp used for *Dehalococcoides* genomic DNA. The standard curve for *Anaeromyxobacter dehalogenans* strain 2CP-C assumes a genome size of 5.0 Mb and two copies of the 16S rRNA gene (GenBank accession no. CP000251).

Amplification efficiencies were calculated by the method of Pfaffl (38) and compared with previously published quantification methods for *Dehalococcoides* spp. (15, 47). The technique was optimized by using triplicate reactions and running independently diluted standard curves repeatedly under identical conditions. To enumerate gene copy numbers of unknown samples, one calibration curve was routinely run with each sample set and compared with previous standard curves to check for consistency between runs. The number of non-*Dehalococcoides* bacterial 16S rRNA gene copies was estimated by subtracting the *Dehalococcoides* copy number from the number of total 16S rRNA gene copies. To determine the number of uncharacterized RDases (i.e., the number of *Dehalococcoides* organisms that do not possess *tceA*, *bvcA*, or *vcrA*), the sum of the RDase gene copies contributed by *tceA*, *bvcA*, and *vcrA* was subtracted from the total *Dehalococcoides* 16S rRNA gene copy number.

The number of target genes per ml of sample was determined with equation 2. The number of gene copies per reaction mix was determined from the appropriate standard curve based on the cycle number at the set threshold fluorescent intensity; this value was multiplied by the volume (μl) of extracted DNA obtained from each sample and divided by both the μl of DNA used per reaction mix (e.g., 3 μl) and the volume of sample from which the DNA was extracted.

$$\text{gene copies per ml sample} = \frac{(\text{gene copies per reaction mix}) \times (\text{volume of DNA [µl]})}{(3\text{ µl DNA per reaction mix}) \times (\text{ml sample used})} \quad (2)$$

Statistical analysis. Duplicate DNA extractions were performed on each sample, and qPCR analysis was performed in triplicate. The symbols and error bars, thus, represent the averages and standard deviations, respectively, of six qPCR reactions per sample. The average slope and *y*-intercept of each standard curve were determined by regression analysis and used to calculate the number of gene copies per ml of sample as described above. Single-factor analysis of variance was used to determine whether the standard curves generated for the five primer/probe combinations were consistent with one another and between replicate experiments.

RESULTS AND DISCUSSION

***Bacteria* and *Dehalococcoides* 16S rRNA gene-targeted primer/probe sets.** qPCR applied to *Dehalococcoides* pure cultures yielded standard curves comparable to standard curves generated with universal primers Bac1055YF/Bac1392R and Bac1115Probe under optimum conditions (Fig. 1; Table 3). Results were similar for cloned 16S rRNA genes of strain BAV1 and strain FL2 or chromosomal DNA of strain BAV1 and strain GT as templates (Table 3), supporting the prediction of a single 16S rRNA gene copy per *Dehalococcoides* genome. The inset for Fig. 1 demonstrates that the optimized universal primer/probe set works

TABLE 3. Primer/probe pair amplification efficiencies of different DNA targets

Target gene	Template	n^a	Amplification efficiency	Slope	*y*-Intercept	R^2	Linear range (copies per μl DNA)
Bacterial 16S rRNA	pBAV1/16S	5	1.90 ± 0.05	−3.58 ± 0.14	42.6 ± 1.0	>0.960	1.7×10^2–1.7×10^8
	BAV1 DNA	5	1.90 ± 0.07	−3.41 ± 0.12	42.5 ± 0.8	>0.985	1.2×10^2–1.2×10^8
	pFL2/16S	3	1.99 ± 0.06	−3.34 ± 0.14	42.7 ± 1.3	>0.990	1.8×10^1–1.8×10^8
	GT DNA	3	1.93 ± 0.02	−3.51 ± 0.05	41.4 ± 0.8	>0.926	4.6×10^2–4.6×10^6
	2CP-C DNA	3	1.97 ± 0.10	−3.39 ± 0.26	40.3 ± 2.0	>0.991	8.9×10^2–8.9×10^6
Dehalococcoides 16S rRNA	pBAV1/16S	13	1.84 ± 0.07	−3.80 ± 0.22	43.3 ± 2.1	>0.985	1.7×10^1–1.7×10^8
	BAV1 DNA	6	1.78 ± 0.10	−3.85 ± 0.33	43.8 ± 2.7	>0.975	1.2×10^1–1.2×10^7
	pFL2/16S	3	1.93 ± 0.07	−3.51 ± 0.21	43.2 ± 1.8	>0.955	1.8×10^1–1.8×10^8
	FL2 DNA	8	1.85 ± 0.07	−3.76 ± 0.24	42.3 ± 1.8	>0.985	3.0×10^0–3.0×10^7
	GT DNA	5	1.97 ± 0.05	−3.41 ± 0.12	42.3 ± 0.8	>0.985	2.1×10^1–2.1×10^6
	195 DNA	3	1.96 ± 0.20	−3.40 ± 0.40	40.6 ± 2.0	>0.985	3.6×10^2–3.6×10^7
	2CP-C DNA	3	NA[b]				
bvcA gene	pBAV1/bvcA	8	1.77 ± 0.04	−4.05 ± 0.18	41.8 ± 2.6	>0.975	1.7×10^1–1.7×10^8
	BAV1 DNA	4	1.86 ± 0.11	−3.74 ± 0.33	45.1 ± 2.8	>0.995	1.2×10^1–1.2×10^8
	GT DNA	3	NA				
	FL2 DNA	3	NA				
	195 DNA	3	NA				
	2CP-C DNA	3	NA				
vcrA gene	BAV1 DNA	3	NA				
	pGT/vcrA	8	2.00 ± 0.04	−3.31 ± 0.09	39.7 ± 0.7	>0.985	1.8×10^1–2.1×10^8
	GT DNA	5	2.00 ± 0.11	−3.33 ± 0.27	40.7 ± 2.6	>0.994	1.6×10^1–1.6×10^6
	FL2 DNA	3	NA				
	195 DNA	3	NA				
	2CP-C DNA	3	NA				
tceA gene	BAV1 DNA	3	NA				
	GT DNA	3	NA				
	pFL2/tceA	8	1.81 ± 0.11	−3.89 ± 0.22	45.1 ± 2.7	>0.995	1.3×10^1–1.3×10^8
	FL2 DNA	3	1.81 ± 0.06	−3.89 ± 0.02	47.3 ± 3.3	>0.975	6.0×10^2–6.0×10^7
	195 DNA	3	1.85 ± 0.1	−3.76 ± 0.31	43.5 ± 3.1	>0.985	3.6×10^2–3.6×10^7
	2CP-C DNA	3	NA				

[a] *n* indicates the number of replicates of independent dilution series.
[b] NA, no amplification.

equally well for *Dehalococcoides* spp. and *Anaeromyxobacter dehalogenans*. Analysis of variance (95% confidence interval, $P > 0.05$) indicated that qPCR standard curves using the *Bacteria* or the *Dehalococcoides* 16S rRNA gene-targeted primer/probe sets were not statistically different under the PCR conditions applied.

Also illustrated in Fig. 1 (circled datum points) are results of qPCR with the bacterial 16S rRNA gene-targeted primer/probe set performed at a higher annealing/elongation temperature of 56°C. The slope of the line describing cycle number versus log gene copy number at 56°C (−4.46 ± 0.59) is statistically different from the slope obtained with reactions carried out at 52°C (−3.58 ± 0.14; $n = 5$, $P < 0.05$), and the *y*-intercept of the regression line is significantly higher in the 56°C reactions (56.02 ± 4.1) than in the 52°C reactions (42.6 ± 1.0; $n = 5$, $P < 0.0001$). A higher *y*-intercept indicates a lower detection limit at a given cycle number. For example, after 30 cycles at 52°C, 1,000 gene copies would be detected, as indicated by the standard curve; however, when run at 56°C, 100,000 16S rRNA gene copies would be required to give the equivalent fluorescence intensity (Fig. 1). Universal bacterial 16S rRNA gene-targeted methods are very sensitive to contamination, and 16S rRNA genes present in commercial reagents may contribute to false-positive results (6, 14). Hence, qPCR approaches require molecular biology reagents free of foreign DNA and the stringent use of good laboratory practice. We reproducibly estimated total copies of bacterial 16S rRNA genes in a sample spanning a concentration range from 1.8×10^1 to 1.8×10^8 copies per μl of PCR mix (i.e., linear standard curves over 8 orders of magnitude were obtained) (Fig. 1; Table 3). The *Dehalococcoides*-targeted probe/primer sets sensitively detected 1 to 20 target gene copies per reaction volume; however, accurate quantification (i.e., reproducible crossing of the threshold value in replicates) required 50 to 100 gene copies per reaction volume. Hence, quantitative estimates obtained with fewer than 50 *Dehalococcoides* gene copies per reaction volume must be interpreted cautiously.

***Dehalococcoides* reductive dehalogenase gene-targeted primer/probe sets.** qPCR with the RDase-specific primer/probe sets applied to plasmids containing *tceA*, *bvcA*, and *vcrA* genes yielded standard curves comparable to the standard curves generated with the *Dehalococcoides* 16S rRNA gene-targeted primer/probe set and the universal primer/probe set (Fig. 2; Table 3). Figure 2 illustrates four standard curves generated using the *Dehalococcoides* probe Dhc1240P and pFL2/16S target DNA and the RDase-specific primer/probe sets targeting cloned *tceA*, *vcrA*, and *bvcA* RDase genes (i.e., pFL2/tceA, pGT/vcrA, and pBAV1/bvcA, respectively) (Table 3). There was no significant difference

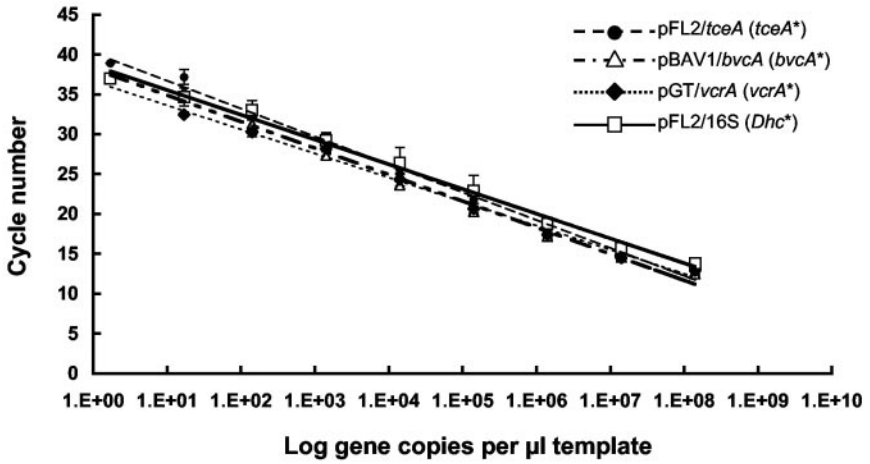

FIG. 2. Standard curves for *Dehalococcoides* 16S rRNA genes and RDase genes. Shown are the *Dehalococcoides*-specific 16S rRNA gene primer/probe set (Dhc*) with pFL2/16S rRNA gene fragment as the template, the *bvcA*-targeted primer/probe set (bvcA*) with pBAV1/bvcA as the template, the *vcrA*-targeted primer/probe set (vcrA*) with pGT/vcrA as the template, and the *tceA*-targeted primer/probe set (tceA*) with pFL2/tceA as the template.

in the slopes, *y*-intercepts, or amplification efficiencies (95% confidence interval, $P > 0.05$) among the standard curves carried out under optimal reaction conditions.

Determining gene copy numbers from standards. The accurate interpretation of qPCR results requires information of the copy number at which genes of interest occur on the genome of the target organism(s). *Dehalococcoides ethenogenes* strains 195 (44) and CBDB1 (24) possess single 16S rRNA and RDase gene copies. qPCR regression lines generated by plotting the cycle number versus gene copies were similar for *Dehalcoccoides* 16S rRNA genes and RDase genes independent of whether chromosomal DNA or plasmids carrying a single cloned gene fragment were used as templates. These findings suggest that the target genes exist as single-copy-number genes on the *Dehalococcoides* genomes. BAV1 pure-culture DNA consistently yielded the same copy number estimates with the *Bacteria*-targeted, *Dehalococcoides* 16S rRNA gene-targeted, and *bvcA*-targeted primers and probes (Fig. 3; Table 3). The amplification efficiencies and standard curves for *Dehalococcoides* sp. strains GT, FL2, and 195 were not statistically different between the *Bacteria* 16S rRNA gene-, *Dehalococcoides* 16S rRNA gene-, and RDase gene-targeted probe and primer sets (Table 3). Fluorescence was not detected with template DNA from *Dehalococcoides* organisms without the target RDase gene or in *Anaeromyxobacter dehalogenans* when targeted with *Dehalococcoides*-specific primers and probes (Table 3).

Application of qPCR to mixed cultures. qPCR analysis of two SZ enrichment cultures showed that different microbial communities resulted from enrichment with VC and PCE. *Dehalococcoides* 16S rRNA genes represented a different fraction of the total 16S rRNA genes, and different *Dehalococcoides* RDase genes were selected by the different enrichment

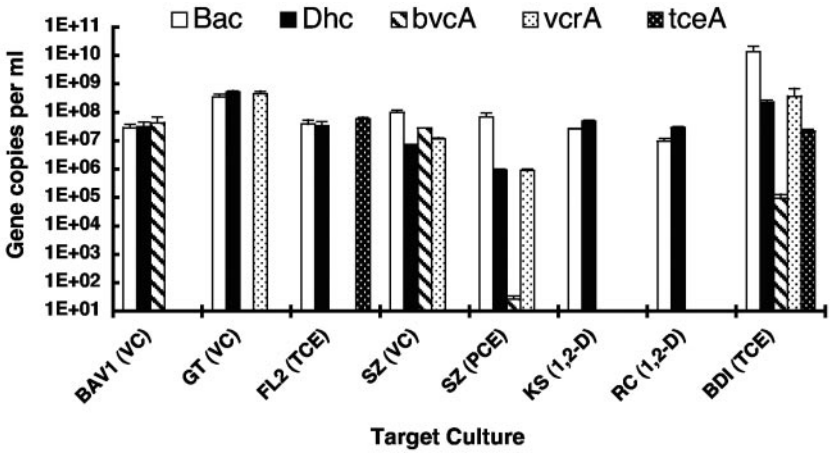

FIG. 3. Analysis of *Dehalococcoides*-containing cultures by use of the specific qPCR primers and probes. The bar graph depicts three pure *Dehalococcoides* (Dhc) cultures (BAV1, GT, and FL2), two SuZi Creek (SZ) sediment-derived consortia (enriched with VC or PCE), two 1,2-D-dechlorinating consortia, and the BDI consortium grown on TCE. The provided chlorinated electron acceptor is designated in parentheses. Each bar represents the average of two DNA extractions, each quantified in triplicate ($n = 6$). Note that the gene copy numbers are given on a log scale; therefore, the heights of the bars are not additive. Bac, *Bacteria*.

procedures (Fig. 3). In both cultures, total bacterial numbers ranged from $6.9 \times 10^7 \pm 2.6 \times 10^7$ for SZ(PCE) to $9.9 \times 10^7 \pm 0.8 \times 10^7$ for SZ(VC). The VC enrichment had a higher total *Dehalococcoides* 16S rRNA gene copy number than the PCE-enriched culture [$7.6 \times 10^6 \pm 0.5 \times 10^6$ for SZ(VC) and $9.0 \times 10^5 \pm 1.0 \times 10^5$ for SZ(PCE)], thus representing a larger fraction of the total bacterial community [7.6% for SZ(VC) versus 1.3% for SZ(PCE)]. These observations are in agreement with the current understanding of the microbiology contributing to chloroethene reductive dechlorination. A number of bacteria dechlorinate PCE, and one such organism, *Geobacter lovleyi* strain SZ, was isolated from the SZ(PCE) enrichment (48). VC dechlorination, in contrast, appears to be limited to a few members of the *Dehalococcoides* cluster (9, 15, 36), and qPCR analysis of culture SZ(VC) corroborated that *Dehalococcoides* organisms are responsible for VC dechlorination in consortium SZ(VC). In the VC-enriched culture, *bvcA*-like and *vcrA*-like genes were present at similar abundances, and their sum matched the total numbers of *Dehalococcoides* cells quantified with 16S rRNA gene-targeted qPCR. This suggests that all *Dehalococcoides* cells present in this culture possess a gene coding for a VC RDase. In contrast, *vcrA*-like genes were present in a majority of the *Dehalococcoides* cells in consortium SZ(PCE), suggesting that the *bvcA*-carrying strain was less competitive than the *vcrA*-carrying strain in the PCE-amended enrichment. *Dehalococcoides* sp. strain GT, an isolate that dechlorinates TCE to ethene with very little intermediate formation of *cis*-DCE and VC (49), possesses *vcrA* but not *bvcA*. The presence of a strain GT-type *Dehalococcoides* organism could explain why *Dehalococcoides* organisms that respire DCEs and VC (i.e., strain BAV1-type organisms) were less abundant in the SZ(PCE) enrichment. *Dehalococcoides* sp. strain BAV1 carries *bvcA* but cannot capture energy from the PCE and TCE dechlorination steps (16).

Figure 3 further illustrates that none of the three chloroethene RDase genes (i.e., *tceA*, *bvcA*, and *vcrA*) were detected in two 1,2-D-dechlorinating cultures, thus supporting previous observations that the *Dehalococcoides* organisms present in these cultures cannot dechlorinate chlorinated ethenes (39). The *Dehalococcoides* 16S rRNA gene-targeted primer/probe set yielded a slightly higher *Dehalococcoides* cell count ($5 \times 10^7 \pm 0.6 \times 10^7$ per ml) than did the *Bacteria* 16S rRNA-gene targeted assay ($1.0 \times 10^7 \pm 0.2 \times 10^7$). This may be due to a procedural difference, emphasizing the sensitivity of the qPCR approach. The *Bacteria*-targeted approach used a 1:1,000 dilution of the template DNA, while undiluted DNA was used for the *Dehalococcoides* assay.

The BDI consortium contains at least three different *Dehalococcoides* organisms, including strains BAV1, FL2, and GT. Only *Dehalococcoides* organisms of the Pinellas group were detected in BDI, excluding the possibility that the *tceA* gene was supplied by a member of the Cornell group (e.g., *Dehalococcoides ethenogenes* strain 195). The *Dehalococcoides* 16S rRNA gene copy number in BDI was greater than $2.5 \times 10^8 \pm 0.4 \times 10^8$ per ml. The *vcrA* gene was most abundant ($3.7 \times 10^8 \pm 3.1 \times 10^8$ copies/ml), contributing to 94% of the *Dehalococcoides* community in the TCE-grown BDI culture. *Dehalococcoides* organisms containing the *tceA* gene represented 6% ($2.2 \times 10^7 \pm 0.3 \times 10^7$ copies/ml TCE) and *Dehalococcoides* containing the *bvcA* gene represented 0.02% ($9.6 \times 10^4 \pm 3.4 \times 10^4$ copies/ml) of the total *Dehalococcoides* community, as determined by the sum of the abundances of all three RDase genes. Continued transfers with TCE as an electron acceptor favored *Dehalococcoides* sp. strain GT, which dechlorinates TCE directly to ethene (49), although strains FL2 and BAV1 were maintained in the TCE-fed consortium.

Site material and microcosm analysis. Site materials and microcosms were analyzed to evaluate if the qPCR approach would detect and quantify changes in the *Dehalococcoides* community following laboratory incubation. qPCR with DNA extracted from the contaminated aquifer material quantified $5.5 \times 10^6 \pm 0.9 \times 10^6$ bacterial 16S rRNA genes per gram of aquifer material; no *Dehalococcoides* genes could be quantified or detected with nested PCR. DNA extracted from 1 liter of GW yielded $3.4 \times 10^4 \pm 1.0 \times 10^4$ bacterial 16S rRNA genes/ml, of which less than 10 copies per ml represented *Dehalococcoides* spp. *bvcA* and *vcrA* genes were detected (one to five copies per μl of template) with qPCR, but the *tceA* gene was not detected (Fig. 4). Total bacterial numbers initiating the microcosms were calculated based on the site sample qPCR results and ranged from 1.0×10^7 to 3.5×10^7 bacteria and 50 to 140 *Dehalococcoides* organisms per microcosm.

The *Dehalococcoides* qPCR data from microcosms established with GW and MM following 206 days of incubation are shown in Fig. 4 and are compared with the qPCR results of the original site groundwater. When sacrificed for analysis, the liquid fraction contained $1.0 \times 10^6 \pm 0.5 \times 10^6$ bacterial 16S rRNA genes per ml. *Dehalococcoides* 16S rRNA genes were too low in numbers to be quantified but were detectable by nested PCR with the decanted liquid fraction (i.e., <10 copies per ml). *Dehalococcoides* 16S rRNA gene copies associated with the solid fraction of the microcosms increased from a nondetectable level at the initiation of the experiment to $6.7 \times 10^6 \pm 0.6 \times 10^6$ and $1.2 \times 10^5 \pm 0.2 \times 10^5$ genes per ml of slurry in the GW and MM microcosms, respectively (Fig. 4). Notably, *Dehalococcoides* 16S rRNA genes increased from a minute fraction of 0.03% of the total bacterial 16S rRNA genes in the original groundwater to greater than 66% in GW microcosms and to 2% in MM microcosms. The fact that more *Dehalococcoides* cells were produced in the microcosms amended with groundwater is likely due to the higher concentrations of PCE and TCE added with the site groundwater or may reflect other nutritional differences between the groundwater and the synthetic mineral salts medium. RDase gene copy numbers also increased from single-digit copies per ml of groundwater (and undetected in the original aquifer material) to easily quantifiable copy numbers (Fig. 4).

Finding the three characterized RDase genes (*tceA*, *vcrA*, and *bvcA*) at the site attests to the distribution of these RDase genes at chloroethene-contaminated sites. There was a marked shift in the proportion of *tceA*, *vcrA*, and *bvcA* RDase genes between the initial groundwater sample and the microcosms. For example, the *tceA* gene copy number increased from a nondetectable level to $6.0 \times 10^5 \pm 0.6 \times 10^5$ copies per ml, whereas the *bvcA* and *vcrA* gene copies increased to $4.0 \times 10^4 \pm 0.1 \times 10^4$ and $1.6 \times 10^4 \pm 0.2 \times 10^4$, respectively (Fig. 4), per ml of GW microcosm slurry. Notably, the total number of the three RDase genes was nearly an order of magnitude less than the estimate for the total *Dehalococcoides* population. This observation suggests the presence of additional endoge-

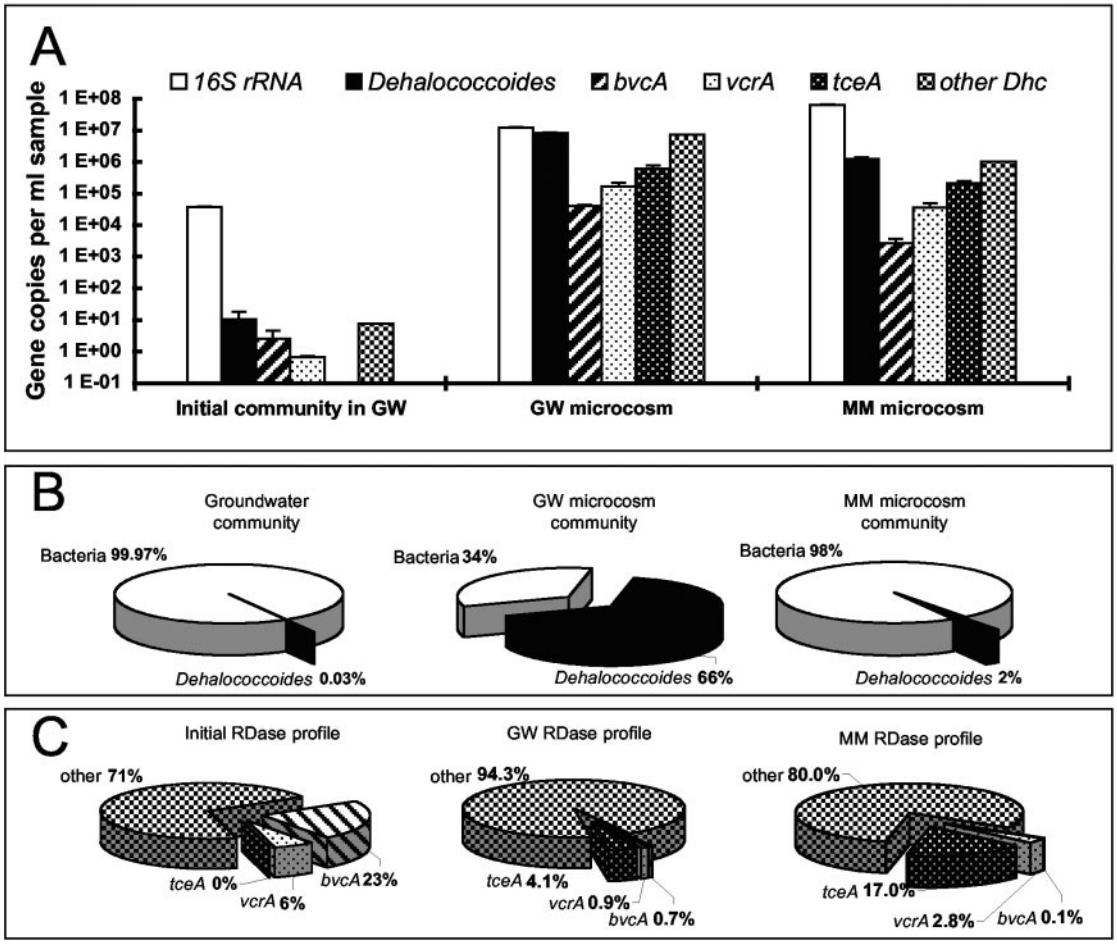

FIG. 4. qPCR analysis of GW samples collected at a site in Michigan contaminated with chloroethenes and other chlorinated compounds. (A) qPCR data from duplicate microcosms established with site aquifer material and site GW or MM following a 206-day incubation period. Each bar represents the average of duplicate DNA extractions from one microcosm per treatment, each quantified in triplicate ($n = 6$). Dhc, *Dehalococcoides*. (B) Pie charts describing the proportions of non-*Dehalococcoides* and *Dehalococcoides* 16S rRNA genes in the initial GW and the enrichment cultures. Panel C highlights the shift in known RDase gene distribution prior to and following enrichment in GW or MM.

nous *Dehalococcoides* organisms that are not detected with the *tceA*-, *vcrA*-, and *bvcA*-targeted primers.

Totals of $2.7 \times 10^7 \pm 1.4 \times 10^7$ and $4.8 \times 10^6 \pm 1.4 \times 10^6$ new *Dehalococcoides* cells were produced in the GW and MM microcosms, respectively. The GW microcosms contained about 12 μmol of organic-bound chlorine (present as C-56, C-58, PCE, and TCE), while the MM microcosms contained about 4.7 μmol of organochlorine (present as C-56 and C-58). Following 206 days of incubation, all parent compounds were consumed and *cis*-DCE and *trans*-DCE were detected (0.46 and 0.16 μmol in the groundwater microcosms, respectively). Additional products, including C-56 and C-58 degradation products, were observed by gas chromatographic analysis but could not be identified or quantified. Assuming growth yields of 10^7 to 10^8 *Dehalococcoides* cells per μmol of chloride released (9, 16) and assuming that 50% of the organochlorine available was released as chloride in a respiratory process supporting growth of *Dehalococcoides* organisms, the amount of chlorinated electron acceptors present exceeded the theoretical amount required to form 2.7×10^6 cells by at least 5- to 50-fold in the GW microcosms and 2- to 20-fold in the MM microcosms. The fact that dechlorination was incomplete may reflect the absence of organisms possessing the appropriate RDase genes or may be due to nutritional (e.g., electron donor) limitations.

Evaluation of the qPCR approach. In TaqMan-based PCR experiments using genomic DNA from *Dehalococcoides* strains 195, FL2, BAV1, and GT as templates, the amplification efficiency of the *Dehalococcoides*-targeted primer pair was high (>1.85), and the regression curves were linear over a range of 10^1 to 10^8 gene copies, with slopes ranging from −3.3 to −3.5 and R^2 values of >0.970. Perfect PCR amplification (e.g., an efficiency of 2) results in two replicate amplicons for each template strand and a slope of −3.33. The TaqMan approach allowed the detection and quantification of *Dehalococcoides* in environmental samples with more than 1,000 *Dehalococcoides* cells per liter of groundwater. For example, 1 liter of groundwater that yields 75 μl of 30 to 90 ng DNA μl^{-1} with 100 to 200 *Dehalococcoides* gene copies per μl of DNA template would contain 500 to 1,000 *Dehalococcoides* gene copies (cells). Theoretically, the ABI real-time PCR system should yield a calibration curve up to 10^{10} copies per μl of template, but under

the experimental conditions applied in this study, saturation occurred when template concentrations exceeded 5×10^8 copies/30 μl reaction volume. If a 10-fold dilution series spanning a concentration range of 10^{11} to 10^1 gene copies yields similar cycle threshold values for the initial three dilution steps (i.e., 10^9 to 10^{11}), then appropriate dilutions of the template DNA must be analyzed to ensure that quantification occurs within the linear portion of the calibration curves. Routinely, 1:10 and 1:100 dilutions (or more) of the target DNA were analyzed to verify that the DNA had not saturated the reaction mix (i.e., $>5 \times 10^8$ copies/30 μl reaction volume).

Compared to a previous study that used a SYBR green-based qPCR approach for similar target genes (47), the TaqMan approach designed in the current study allows more-sensitive detection and quantification. The SYBR green method had a lower dynamic range for the *Dehalococcoides* 16S rRNA gene and was quantitative over a narrower range of 10^5 to 10^7 gene copies per μl of template DNA. The sensitivity of this TaqMan approach allows the detection of *Dehalococcoides* 16S rRNA and RDase genes at environmentally relevant, low concentrations (e.g., 100 to 1,000 genes per liter of groundwater).

Estimates of gene copy numbers that relate to population sizes are subject to biases occurring at all steps of analysis, including biomass collection, cell lysis, and DNA purification. PCR inhibition due to the presence of humic substances or other organic compounds in the DNA preparation is another concern (46, 54, 55). In all cases reported here, we could amplify and quantify 16S rRNA genes. Despite inherent PCR biases, the described qPCR approaches provide valuable information regarding bacterial population size and dynamics. Better resolution of the dechlorinating *Dehalococcoides* community is obtained by integrating quantitative information from both phylogenetic (i.e., 16S rRNA) and functional (i.e., RDase) genes. The combined qPCR approaches for monitoring *Dehalococcoides* organisms demonstrated their applicability as prognostic and diagnostic tools. These tools could also monitor *Dehalococcoides* RDase gene expression, as was recently demonstrated for the *tceA* gene (3, 22). As additional RDase gene targets are identified, expansion of these approaches will describe the *Dehalococcoides* community with even greater resolution and accuracy, thus enhancing the armamentarium of molecular tools available for site assessment and bioremediation monitoring at sites impacted with chlorinated contaminants.

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