Oxygen Effect on
Dehalococcoides Viability and
Biomarker Quantification

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Received December 22, 2007. Revised manuscript received May 16, 2008. Accepted May 22, 2008.

Oxygen-sensitive Dehalococcoides bacteria play crucial roles in detoxification of chlorinated contaminants (e.g., chlorinated ethenes), and bioremediation monitoring relies on quantification of Dehalococcoides DNA and RNA biomarkers. To explore the effects of oxygen on Dehalococcoides activity, viability, and biomarker quantification, batch experiments with a tetrachloroethene-to-ethene dechlorinating consortium (Bio-Dechlor INOCULUM [BDI]) harboring multiple Dehalococcoides strains were performed to quantify the effects of ≤4 mg/L dissolved oxygen. Oxygen inhibited reductive dechlorination, and only incomplete dechlorination to vinyl chloride (VC) occurred following oxygen consumption and extended incubation periods (89 days). Following 30 days of oxygen exposure and subsequent oxygen removal (i.e., reversibility experiments), all trichloroethene- (TCE-) fed cultures dechlorinated TCE to VC, but VC dechlorination to ethene occurred in only one out of fourteen replicates. These results suggest that Dehalococcoides strains respond differently to oxygen exposure, and strains catalyzing the VC-to-ethene dechlorination step are more susceptible to oxygen inhibition. Quantitative real-time PCR (qPCR) analysis detected a 1−1.5 order-of-magnitude decrease in the number of Dehalococcoides biomarker genes (i.e., 16S rRNA gene and the reductive dehalogenase [RDase] genes tceA, vcrA, and bvcA) in the oxygen-amended cultures, but qPCR analysis failed to distinguish viable, dechlorinating from irreversibly inhibited (nonviable) Dehalococcoides cells. Reverse transcriptase qPCR (RT-qPCR) detected Dehalococcoides genes transcripts in the oxygen-amended, non-dechlorinating cultures, and biomarker transcription did not always correlate with dechlorination (in)activity. Enhanced molecular tools that complement existing protocols and provide quantitative information on the viability and activity of the Dehalococcoides population are desirable.

Introduction

Chlorinated ethenes are common groundwater pollutants with stringent regulatory standards that mandate corrective action(s) at many contaminated sites. Traditionally, pump-and-treat systems have been deployed to prevent expansion of contaminant plumes, but this technology is inefficient, has high operating costs, and must be maintained for decades. The need for affordable remedial technologies has driven substantial research investment to identify alternative approaches that yield near- and long-term benefits. Remarkable progress has been made in understanding the microbiology involved in detoxification of chlorinated ethenes (1), and bioremediation has emerged as a viable remedy (2). A milestone for the success of chlorinated ethene bioremediation was the identification of Dehalococcoides organisms as the key catalysts for reductive dechlorination of dichloroethenes (DCEs) and vinyl chloride (VC) to innocuous products (i.e., ethene and inorganic chloride) (3−6). Labora
tory and field-scale investigations demonstrated a cause-and-effect relationship between the presence of Dehalococcoides spp. and ethene formation (7−10). Hence, assessment of chlorinated ethene-impacted sites where bioremediation is considered a treatment option includes one key objective: determining if Dehalococcoides spp. are present or not. To address this question, Dehalococcoides-specific, PCR-based tools targeting the 16S rRNA gene and reductive dehalogenase (RDase) genes (i.e., tceA, bvcA, and vcrA) implicated in chlorinated ethene reductive dechlorination (11−13) are applied to DNA extracted from site samples (e.g., groundwater). These tools allow for sensitive, specific detection and quantification of Dehalococcoides biomarker genes (7, 8, 14−21) and are offered commercially.

Dehalococcoides spp. are native to many contaminated sites but are often heterogeneously distributed throughout the aquifer and/or are present in very low numbers (9, 10). At sites where native Dehalococcoides spp. occur, the lack of substrates and/or suitable redox conditions often limit (i.e., control) reductive dechlorination and detoxification. To overcome the nutritional limitations, biostimulation with organic (e.g., lactate) and inorganic (i.e., N and P) substrates has been successfully implemented at the field scale (9). Although biostimulation has been employed productively at several sites, this approach may be insufficient to sustain desirable dechlorination rates and only works at sites that have native Dehalococcoides spp. capable of efficient ethene formation. As an alternative approach, bioaugmentation with Dehalococcoides-containing consortia has been implemented at numerous sites (9, 10, 22, 23), and bioaugmentation inocula are commercially available.

To accompany biostimulation and bioaugmentation field efforts, detailed laboratory studies with Dehalococcoides pure and mixed cultures have been performed to elucidate the organisms’ nutritional requirements. Dehalococcoides spp. are very difficult to grow and maintain in pure culture (3, 5). The reasons for the intricate growth of Dehalococcoides spp. in pure culture are unclear but may be due to unknown nutritional requirements and/or sensitivity to oxygen (3, 5). The effects of oxygen on Dehalococcoides viability have not been thoroughly explored, but several studies reported that brief exposure of Dehalococcoides cultures to air or oxygen completely and irreversibly inhibited dechlorination (8, 24, 25). Commercial vendors of Dehalococcoides-containing bioaugmentation consortia recognize that proper diligence is required to limit oxygen exposure during inoculum transport to the contaminated site and/or delivery to the subsurface (26). The state-of-the-art practice of bioaugmentation includes techniques (e.g., shipping consortia in sealed and pressurized containers, injecting consortia into the subsurface under pressure with argon or nitrogen) that reduce...
exposure of the bioaugmentation culture to air (26). The ability to manage exposure of subsurface *Dehalococcoides* organisms to oxygen during biostimulation and bioaugmentation field efforts, however, can be challenging due to infiltration of oxygenated surface water (e.g., rain events) or migration of oxygenated groundwater into treatment areas. Such oxygen exposure, if not adequately controlled, may have a profound impact on the success of the biological remedy. Hence, the aim of this study was to explore the effects of oxygen on *Dehalococcoides* viability and dechlorination performance in more detail, and evaluate if the current PCR-based tools are useful to detect oxygen exposure and distinguish viable, dechlorinating cells from inactive, oxygen-exposed cells. To this end, we also explored the use of DNA in addition to DNA biomarkers as useful indicators of oxygen exposure. Information on *Dehalococcoides* survivability (i.e., resistance and resilience) following oxygen exposure and knowledge of the resolution of the current molecular tools to detect oxygen exposure has practical relevance for bioremediation applications.

**Materials and Methods**

**Chemicals.** Trichloroethene (TCE; ≥99.5%) was obtained from Sigma-Aldrich Co. (St. Louis, MO). *cis*-1,2-Dichloroethene (*cis*-DCE; 99.9%) and *trans*-1,2-dichloroethene (*trans*-DCE; 99.9%) were purchased from Supelco Co. (Bellefonte, PA). Gaseous VC (≥99.5%) was purchased from Fluka Chemical Corp. (Ronkonkoma, NY), and gaseous ethene (99.5%) was obtained from Scott Specialty Gases (Durham, NC). All other chemicals used were reagent grade or better unless otherwise specified.

**Culture, Medium Preparation, and Growth Conditions.** Bio-Dechlor INOCULUM (BDI), a tetrachloroethene- (PCE-) to-ethene dechlorinating consortia has been successfully used in bioaugmentation field applications (22) and contains at least three *Dehalococcoides* strains: FL2, GT, and BAV1 (15). Strain FL2 metabolically dechlorinates TCE to VC and cometabolically transforms VC to ethene (4). Strain GT metabolically dechlorinates TCE to ethene (6), while strain BAV1 metabolically dechlorinates all DCE isomers and VC to ethene (3). These *Dehalococcoides* strains can be tracked via quantitative real-time PCR (qPCR) (15) by targeting specific RDase genes: the TCE-to-VC RDase gene (*tceA*) of strain FL2 (11), the VC-to-ethene RDase gene (*vcrA*) of strain GT (6, 13), and the putative VC-to-ethene RDase gene (*bvcA*) of strain BAV1 (12). Reduced anaerobic mineral salts medium was prepared as described (25), except the concentration of Na₂SO₄ × 9H₂O was 0.1 mM. The medium contained resazurin (1 µM; 0.25 mg/L) as a redox indicator (27–30). Resazurin, which can be oxidized by oxygen (27), is useful as a redox indicator in a narrow range of redox potentials (~51 mV ± 60 mV at pH 7 (29)). Solutions containing resazurin are bright pink at redox potentials above +10 mV, clear at redox potentials below ~110 mV, and various intensities of pink at redox potentials between ~110 mV and ~10 mV. The BDI consortium was grown in 160-mL (nominal volume; Wheaton Co, Millville, NJ) glass serum bottles containing 100 mL ± 1 mL of medium and a N₂/CO₂ (80%/20% [vol/vol]) headspace. Bottles were sealed with Teflon-lined, gray butyl-rubber septa (West Pharmaceuticals, Lionville, PA) held in place with aluminum crimp caps (Wheaton). Lactate (5 mM) and hydrogen (10 mL) served as electron donors and were provided in excess (i.e., were not limiting dechlorination activity). Lactate was added by syringe from sterile, anoxic stocks. Acetate, which was produced by lactate fermentation, served as the carbon source for *Dehalococcoides* organisms in BDI. TCE (4 µL of neat liquid) or VC (3 mL of sterile gas) were provided as electron acceptors. TCE was added with a 5-µL gastight syringe (model 95 with a reproducibility adapter; Hamilton Co., Reno, NV); VC was added via 3-mL disposable syringe. Triplicate or duplicate cultures were incubated statically, in an upside-down position, at room temperature.

**Oxygen Exposure.** Sterile oxygen gas was amended to triplicate bottles containing autoclaved mineral salts medium via syringe and 30-gauge needle at an initial amount of 10% (vol/vol) of the headspace. Assuming equilibrium partitioning, the initial dissolved oxygen concentration was approximately 4 mg/L (31), which is in the range of dissolved oxygen concentrations when surface water or oxygenated groundwater migrate into biobarriers. Triplicate positive control cultures did not receive oxygen but instead were amended with equal volumes of sterile nitrogen gas. The bottles received TCE or VC and were allowed to equilibrate for 2 days before inoculation with 5% (vol/vol) of a dechlorinating BDI stock culture maintained with TCE as electron acceptor and lactate as electron donor. Before inoculation of individual bottles, two 100-mL aliquots of the BDI stock culture were dispensed into sterile, N₂-flushed serum bottles. Filter-sterilized streams of N₂/CO₂ (80%/20% [vol/vol]) were bubbled through each cell suspension for 15 min to remove residual chlorinated ethenes and ethene. Three 5-mL samples of each cell suspension were collected for qPCR and reverse transcription qPCR (RT-qPCR) analyses before distribution of the inoculum to the VC-fed or TCE-fed bottles. The oxygen exposure experiment was repeated in an independent experiment with duplicate, VC-fed cultures and the following modifications: the inoculum size was increased to 15% (vol/vol) and oxygen was initially provided at 3.5% (vol/vol) of the headspace. The bottles received additional oxygen (12.6 and 2.1 mL) on Days 10 and 12, respectively, of the 21-day incubation. Liquid samples were taken periodically for qPCR and/or RT-qPCR analyses as described below.

**Oxygen Consumption Experiments.** To determine abiotic and/or biotic oxygen consumption, oxygen concentrations were measured in an independent experiment with triplicate cultures not amended with chlorinated ethenes. The bottles were allowed to equilibrate for 2 days after oxygen addition before inoculation (5%, vol/vol) from a dechlorinating BDI stock culture. Triplicate abiotic control cultures received 5% (vol/vol) of sterile medium 2 days after oxygen addition. Headspace samples were taken for oxygen analysis immediately after oxygen addition and periodically thereafter.

**Reversibility Experiments.** Aqueous 1-mL samples from triplicate VC- and TCE-fed, oxygen-amended cultures were transferred on Day 30 of the incubation to triplicate vessels containing reduced, oxygen-free medium equilibrated with VC and TCE, respectively. Aqueous 1-mL samples were also transferred from the triplicate VC- and TCE-fed positive control cultures (i.e., not exposed to oxygen) to triplicate vessels containing reduced, oxygen-free medium. Separate reversibility experiments were conducted with washed cell suspensions as described by Amos et al. (25) to ensure complete removal of oxygen from the transferred culture suspensions. Briefly, biomass was collected by centrifugation (4300g, 30 min) in an anoxic glovebox (Coy Laboratory Products, Ann Arbor, MI) from 5 mL of triplicate VC- or TCE-fed, oxygen-amended or positive control cultures on Day 30 of the incubation. The resulting cell pellets were washed once with reduced (i.e., oxygen-free) medium and suspended in 1 mL of reduced medium. The washed cell suspensions served as inocula to triplicate (duplicate for the VC-fed, positive control cultures) vessels containing reduced, oxygen-free medium amended with VC or TCE, respectively. In the independent, repeated oxygen-exposure experiment, reversibility experiments were performed with washed cell suspensions collected from 15 mL of culture on Day 21.

**DNA and RNA Extraction.** Biomass was collected periodically from aqueous samples by centrifugation at 16,000g for 10 min. The supernatant was decanted, and the biomass
from five 1-mL samples of culture suspension was collected in the same tube. The resulting pellet was suspended in 500 µL of RNAProtect Bacteria Reagent (Qiagen, Valencia, CA) to stabilize and protect RNA inside intact cells. The suspension was homogenized by vortexing, incubated at room temperature for 5 min, and centrifuged at 16,000g for 10 min. Genomic DNA and total RNA were extracted with the AllPrep DNA/RNA Mini Kit (Qiagen) from previously frozen cell pellets. The cell pellets were suspended in 250 µL of Tris-EDTA (TE) buffer (RNase-free, pH 8; Ambion, Austin, TX) containing 15 mg/mL lysozyme (Sigma). Each sample received 1 µL of 10% SDS solution (20% RNase-free SDS stock [Ambion] diluted with diethyl pyrocarbonate-[DEPC–] treated water) and 20 µL of RTL lysis buffer (supplied with AllPrep DNA/RNA Mini Kit) containing 0.5 M β-mercaptoethanol (Sigma). All samples were then vortexed for 5 min before following the remainder of the Qiagen protocol. DNA was obtained in a final volume of 100 µL of buffer EB (provided with the AllPrep DNA/RNA Mini Kit) and stored at -20 °C until qPCR analysis. RNA was obtained in a final volume of 100 µL of RNase-free water, amended with 1 µL of RNaseOUT Ribonuclease Inhibitor (Invitrogen, Carlsbad, CA), and stored at -80 °C until further processing.

In the independent experiment with VC-fed, oxygen-amended cultures, biomass was collected periodically from 10 mL of culture fluid by centrifugation as described (25). The cell pellet was stored at -20 °C until genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) following a modified protocol (15). DNA was obtained in a final volume of 200 µL of buffer AE (provided with the QIAamp DNA Mini Kit) and stored at -20 °C until qPCR analysis.

**RNA Purification and Reverse Transcription.** To remove contaminating DNA, the RNA was DNase treated with the TURBO DNA-free kit (Ambion) according to the manufacturer’s recommendations. After the DNase treatment, RNA was purified by successive phenol, phenol/chloroform/isoamyl alcohol (24:1 vol/vol), and chloroform/isoamyl alcohol (25:24:1 vol/vol/vol), and chloroform/isoamyl alcohol (24:1 vol/vol) extractions, and recovered by ethanol precipitation with 0.3 M sodium acetate as described (32). The precipitated RNA was dissolved in 20 µL RNase-free water, amended with 1 µL of RNaseOUT Ribonuclease Inhibitor (Invitrogen), and stored at -80 °C until use. Removal of contaminating DNA was confirmed via PCR with universal bacterial 16S rRNA gene-targeted primers (14).

Reverse transcription was performed with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Before reverse transcription, the volumes of the RNA samples were reduced to <8 µL using an SC210A SpeedVac Plus (ThermoSavant, Milford, MA) for 30–40 min at room temperature. Subsequently, each reverse transcription reaction consisted of 1 µL of random hexamer primers (50 ng/µL), 1 µL of 10 mM dNTP mix, the RNA sample, and DEPC-treated water to bring the final reaction volume to 10 µL. Reverse transcription then proceeded according to the manufacturer’s protocol. cDNA was obtained in a final volume of 21 µL and stored at -20 °C until qPCR analysis.

**Quantification of RNA Recovery.** Quantification of RNA recovery during RNA extraction, purification, and reverse transcription to cDNA was performed as described by Johnson et al. (19). Briefly, select cell pellets (4 of 30) received 2 × 10¹¹ transcripts of Luciferase Control RNA (Promega, Madison, WI) before DNA and RNA extraction. The number of transcripts added to each sample was estimated from the RNA concentration (100 ng/µL; a 1:10 dilution of the purchased 1000 ng/µL stock), assuming an average molecular weight of 330 for a nucleotide of single-stranded RNA and a transcript size of 1.8 kb (15, 19). RNA recovery was determined by dividing the total number of luciferase transcripts detected via qPCR analysis in the cDNA by the total number of transcripts added to each cell pellet. The average luciferase transcript recovery was 9.8 ± 0.9% (n = 4; data not shown); this recovery is comparable to the recovery reported previously (19). The numbers reported for the *Dehalococcoides* 16S RNA, *tceA, vcrA*, and *bvcA* transcripts were adjusted to reflect the determined RNA recovery.

**Quantitative Real-Time PCR (qPCR) Analysis.** TaqMan-based qPCR analysis of DNA and cDNA was used to quantify the number of *Dehalococcoides* 16S rRNA, *tceA, vcrA*, and *bvcA* genes and transcripts as described (15). qPCR analysis to quantify the luciferase control RNA utilized a published TaqMan-based protocol (19) except that the probe (Integrated DNA Technologies, Coralville, IA) was modified to include 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein (JOE NHS Ester) as the reporter fluorophore on the 5′ end and a black hole quencher ( BHQ-1) on the 3′ end. Each well of a MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems, Foster, CA) contained 1× TaqMan universal PCR master mix (ABI), 300 nM probe, 300 nM of each primer, and 2 µL of template DNA or cDNA in a total reaction volume of 20 µL. qPCR analysis was carried out in an ABI 7500 Fast Real-Time PCR System with cycle parameters as follows: 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C using the standard 7500 operating mode. Standard curves were generated for the *Dehalococcoides* 16S rRNA, *tceA, vcrA*, and *bvcA* genes as described (15). For the luciferase standard curve, luciferase control RNA transcripts were reverse transcribed as described above. The luciferase cDNA was quantified by spectrophotometry (15), and 10 ng of the luciferase cDNA was serially diluted (10-fold) for use as standards. The number of luciferase cDNA transcripts in each standard was estimated from the cDNA concentration and the size of the cDNA fragment. The terms “gene copies” and “cell numbers” are per mL of culture fluid and are used interchangeably because the known *Dehalococcoides* organisms contain one 16S RNA gene per genome (33, 34) and one target reductive dehalogenase gene per cell (15). Transcript copy numbers are reported on a per cell basis (e.g., *tceA* gene transcript copy numbers divided by *tceA* gene copies).

**Analytical Methods.** Aqueous phase (1 mL) samples were collected periodically for organic acid analysis and for chlorinated ethene and ethene quantification as described (8, 25, 35). Oxygen was analyzed by manually injecting 100-µL headspace samples into a Hewlett-Packard (HP) 6890N gas chromatograph equipped with an HP-Plot Molsieve column (15 m × 0.33 mm; film thickness 25 µm nominal) and a thermal conductivity detector. Helium (6 mL/min) was the carrier gas, and the injector (split at 10:1), oven, and detector were maintained at 90, 40, and 150 °C, respectively. The method detection limit for oxygen was ca. 2% (vol/vol).

**Results**

**Oxygen Consumption Experiments.** Headspace oxygen was initially present at 9.5 ± 0.1% and 9.7 ± 0.4% (vol/vol) for the BDI-inoculated cultures and abiotic controls, respectively (see Figure S1, Supporting Information). The medium turned bright pink within minutes after oxygen amendment, indicating that the redox indicator resazurin was oxidized. For both the inoculated cultures and abiotic controls, the headspace oxygen decreased linearly for the first three days after oxygen amendment, before leveling off at ca. 8% (Figure S1). The initial decrease in oxygen in both the inoculated cultures and abiotic controls is likely due to abiotic reactions of oxygen with the chemical reductants present in the medium. Further oxygen reduction was not observed in the abiotic controls (Figure S1), even after 28 days of incubation, and the medium remained pink. In the inoculated cultures, the medium started to turn clear (i.e., resazurin reduced) on Day 14 and oxygen concentrations began to decrease (Figure S1). By Day 28, the oxygen concentration had declined to 2.6
The presence of oxygen negatively affected the ability of the BDI consortium to dechlorinate VC (Figure 1A) and TCE (Figure 2B). As shown in Figure 1A, oxygen prevented VC dechlorination, whereas control cultures without oxygen produced stoichiometric amounts of ethene. In the oxygen-amended cultures, ethene formation did not occur even after extended incubation periods (89 days; data not shown). The proportion of the Dehalococcoides strain GT, strain FL2, and strain BAV1, respectively, remained quantifiable. Initially, the fractions of the Dehalococcoides strains present in BDI carrying the vcrA, tceA, and bvcA genes were 82 ± 8%, 18 ± 1%, and <0.1%, respectively. Copy numbers of each RDase gene decreased proportionally to the decrease in the total number of Dehalococcoides cells (i.e., about 5-fold reduction), resulting in identical proportions at the end of the incubation. For control cultures incubated without oxygen, growth of the Dehalococcoides population was observed during VC dechlorination. The percentage of the Dehalococcoides population possessing vcrA, increased from 82 ± 8% to 95 ± 4% during VC dechlorination. The percentage of the Dehalococcoides population possessing tceA declined from 18 ± 1% to 5.3 ± 0.5%. The copy number of bvcA increased by approximately 3-fold during the incubation, but the proportion of Dehalococcoides organisms containing bvcA still represented <0.1% of the total Dehalococcoides population. An independent experiment corroborated these findings.

The TCE-fed BDI cultures dechlorinated TCE to stoichiometric amounts of ethene in the absence of oxygen (Figure 2A). Initially, amendment of oxygen inhibited TCE dechlorination, but detection of cis-DCE on Day 30 indicated resumed dechlorination activity (Figure 2B). In fact, TCE...
TABLE 1. Summary of Reverse Transcription (RT) qPCR Analysis of Dehalococcoides 16S rRNA Molecules and tceA and vcrA Transcripts in the Oxygen-Amended and Positive Control BDI Cultures Relative to the Inoculum

<table>
<thead>
<tr>
<th>time (d)</th>
<th>16S rRNA</th>
<th>tceA</th>
<th>vcrA</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxygen-amended</td>
<td>VC-fed Cultures</td>
<td>log change in transcripts*</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>-0.5 ± 0.2</td>
<td>-1.4 ± 0.7</td>
<td>-0.8 ± 0.2</td>
</tr>
<tr>
<td>30</td>
<td>-0.05 ± 0.21</td>
<td>-0.6 ± 0.93</td>
<td>-0.93 ± 0.3</td>
</tr>
<tr>
<td>positive control</td>
<td>VC-fed Cultures</td>
<td>log change in transcripts*</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>0.2 ± 0.2</td>
<td>-1.2 ± 0.2</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>30</td>
<td>-0.01 ± 0.29</td>
<td>2.0 ± 0.04</td>
<td>0.66 ± 0.18</td>
</tr>
<tr>
<td>oxygen-amended</td>
<td>TCE-fed Cultures</td>
<td>log change in transcripts*</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.3 ± 0.2</td>
<td>0.8 ± 0.4</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>30</td>
<td>0.6 ± 0.1</td>
<td>ND*</td>
<td>-0.05 ± 0.40</td>
</tr>
<tr>
<td>positive control</td>
<td>TCE-fed Cultures</td>
<td>log change in transcripts*</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.3 ± 0.3</td>
<td>2.1 ± 0.4</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>30</td>
<td>0.9 ± 0.2</td>
<td>0.2 ± 0.4</td>
<td>2.0 ± 0.02</td>
</tr>
</tbody>
</table>

* Log change in transcripts per cell (e.g., tceA gene transcripts divided by tceA gene copies) relative to the number of transcripts per cell in the inoculum. ND, not detected.

dehlorination to VC occurred by Day 61, and by Day 89, a small amount of ethene (5 mol% of the total chlorinated ethenes and ethene) was detected in the oxygen-amended cultures (Figure 2B). When accounting for mass loss due to sampling, the mass balance was 105 ± 3% at Day 89 for the oxygen-amended cultures, indicating stoichiometric dechlorination of TCE to VC. As observed in the VC-fed cultures, the medium was initially pink in the TCE-fed, oxygen-amended vials; the medium turned clear and then slightly turbid by Day 30. The concentrations of lactate fermentation products (e.g., acetate and propionate) were similar to those described above for the VC-fed cultures. Although Dehalococcoides activity was initially inhibited, Dehalococcoides cell numbers were easily quantified in the oxygen-amended cultures but rather a significant downregulation of transcription in the oxygen-amended cultures (i.e., 0.4 orders of magnitude at Day 30; Table 1). As with the number of transcript copies per cell in the control cultures (Figure 3, Day 30). This finding does not suggest increased tceA transcription in the oxygen-amended cultures but rather a significant downregulation of tceA transcription in the VC-fed control cultures (i.e., 2.0 ± 0.4 orders of magnitude at Day 30; Table 1).

Detection of Gene Transcripts during Oxygen Exposure. Dehalococcoides 16S rRNA and tceA and vcrA transcripts were detected in samples of the BDI stock culture used as inoculum. bvcA gene transcripts were not detected, a likely consequence of the small Dehalococcoides sp. strain BAV1 population carrying bvcA in BDI maintained with TCE as electron acceptor. Substrate-dependent transcription of the tceA and vcrA genes was observed in the oxygen-free, VC- and TCE-fed control cultures (Table 1). Table 1 presents the relative change in the number of transcripts per cell with time (i.e., the ratio of transcript copy numbers per cell to the number of transcript copies per cell in the inoculum). A ratio near unity (represented as 0 on the log scale in Table 1) indicates an insignificant change in the number of transcripts per cell. A ratio above unity (a positive number on the log scale) represents upregulation of gene transcription, while a ratio below unity (a negative number on the log scale) indicates downregulation of gene transcription. Normalizing gene expression to cell numbers may introduce a bias toward downregulation of gene transcription since the total number of cells, both viable and nonviable, was likely higher than the number of cells actively producing transcripts. As shown in Table 1, transcription of the tceA gene was downregulated by 1.2 ± 0.2 to 2.0 ± 0.4 orders of magnitude in the VC-fed control cultures (Days 13 and 30, respectively). In contrast, the number of vcrA gene transcripts per cell in the VC-fed control cultures increased by 0.7 ± 0.2 orders of magnitude (Table 1, Day 13) before returning to levels similar to those observed initially. In the TCE-fed control cultures, transcription of the tceA gene was initially upregulated (2.1 ± 0.4 orders of magnitude; Table 1, Day 7) during TCE conversion to cis-DCE and VC (Figure 2A, Day 7). By the end of the incubation, the number of tceA gene transcripts per cell returned to levels similar to those observed at initially (Table 1). Transcription of the vcrA gene in the TCE-fed control cultures was upregulated at the onset of ethene production (0.5 ± 0.2 orders of magnitude; Figure 2A and Table 1, Day 7) and remained elevated in the presence of VC (2.0 ± 0.2 orders of magnitude; Figure 2A and Table 1, Day 30). As with the stock culture of BDI, bvcA gene transcripts were not detected in the control cultures. Dehalococcoides 16S rRNA and tceA and vcrA transcripts were also detected in the oxygen-amended VC- and TCE-fed cultures (Table 1). In contrast to the control (i.e., no oxygen cultures), transcription of the tceA and vcrA genes did not correlate with dechlorination activity and seemed independent of the presence and/or absence of specific chlorinated electron acceptors (Table 1). In the oxygen-amended, VC-fed cultures, transcription of the target genes was downregulated (0.05 ± 0.21 to 1.4 ± 0.7 orders of magnitude; Table 1). The number of 16S rRNA molecules and tceA and vcrA transcripts per cell initially increased in the oxygen-amended, TCE-fed cultures (Table 1, Day 7), although tceA gene transcripts were not detected on Day 30. Figure 3 demonstrates that the ratios of gene transcripts per cell in the inactive (i.e., oxygen-amended) and actively dechlorinating control cultures declined or were indistinguishable. In the VC-fed cultures, tceA gene transcripts per cell were actually detected at significantly higher levels per cell (1.8 ± 0.7 orders of magnitude) in the oxygen-amended as compared to the control cultures (Figure 3, Day 30). This finding does not suggest increased tceA transcription in the oxygen-amended cultures but rather a significant downregulation of tceA transcription in the VC-fed control cultures (i.e., 2.0 ± 0.4 orders of magnitude at Day 30; Table 1).

Reversibility Experiments. Washed cell suspensions from oxygen-amended, VC-fed BDI cultures failed to dechlorinate VC in oxygen-free medium even after 161 days of incubation (Figure 4A). Similar observations were made in two of three cultures initiated by transferring 1-mL inocula from oxygen-amended, VC-fed cultures to oxygen-free medium (Figure 4B). In one replicate culture, however, slow VC-to-ethene dechlorination occurred after Day 40 (Figure 4B). Additional
samples taken on Day 262 (data not shown) revealed complete conversion of VC to ethene in this culture. With washed cell suspensions (Figure 4A) or aqueous phase transfers (Figure 4B) from oxygen-free control cultures, dechlorination activity was observed by Day 10 and resulted in stoichiometric VC conversion to ethene. No VC dechlorination occurred in an independent reversibility experiment with washed cell suspensions from the second set of oxygen-amended, VC-fed cultures whereas washed cell suspensions from the oxygen-free control cultures produced ethene.

In oxygen-free medium, washed cell suspensions derived from TCE-fed control cultures (no oxygen exposure) dechlorinated TCE to stoichiometric amounts of ethene with intermediate formation of cis-DCE and VC (Figure 5A). In contrast, washed cell suspensions from oxygen-amended cultures dechlorinated TCE only to VC (Figure 5B). Slow dechlorination of VC began on Day 31, after complete conversion of TCE and cis-DCE, resulting in gradual accumulation of ethene (22 and 34 mol% of the total chlorinated ethenes and ethene on Days 161 and 262, respectively). Similar results were observed in the reversibility experiments initiated with aqueous phase transfers from control cultures or oxygen-amended cultures to oxygen-free medium (data not shown).

Discussion
Dissolved oxygen (≤4 mg/L) significantly, and sometimes irreversibly, inhibited the ability of Dehalococcoides organisms in the PCE-to-ethene-dechlorinating BDI consortium to reductively dechlorinate chlorinated ethenes. Our experimental setup exposed Dehalococcoides to oxygen for up to 30 days, but the results are consistent with previous pure culture studies that demonstrated irreversible inhibition after short exposure (∼5 s) to air (24). Our results suggest a strain-specific response to oxygen, and several lines of evidence imply that, of the three Dehalococcoides strains present in BDI, only strain FL2 survived. For example, incomplete dechlorination of VC to ethene in the TCE-fed reversibility cultures is consistent with slow, cometabolic production of ethene, as observed in FL2 pure cultures (4). In contrast to strain FL2, isolates GT and BAV1 metabolically dechlorinate VC to ethene (3, 6). Our results demonstrate that these strains were not only inactive in the oxygen-amended BDI cultures but were also irreversibly inhibited by the 30-day oxygen exposure. Only in one of eight VC-fed reversibility cultures was VC slowly dechlorinated to ethene after a significant (40 day) lag time. VC dechlorination rates characteristic for the VC-respiring strains GT and BAV1 were not observed in any of the other VC- or TCE-fed, oxygen-exposed cultures, even after extended incubation periods (up to 262 days), suggesting that the observed recovery in the single culture was likely an experimental artifact (e.g., cross-contamination following oxygen removal). These observations suggest that strain FL2-like organisms may be more robust and less sensitive to environmental stressors (e.g., oxygen) than VC-dechlorinating Dehalococcoides strains (e.g., strains GT and BAV1). Similar results were observed with Dehalococcoides-containing dechlorinating consortia exposed to elevated temperatures (36), corroborating that strain FL2-like organisms, which cannot resile VC, are more tolerant of environmental stressors. Survival of strain FL2-like organisms, but not VC-

![FIGURE 4](image4.png)

**FIGURE 4.** Reversibility experiments for the VC-fed BDI consortium with (A) washed cell suspensions or (B) 1% (vol/vol) transfers in identical sets of fresh, oxygen-free medium. The inocula came from oxygen-amended (open symbols, dashed lines) or positive control (closed symbols, solid lines) cultures. Symbols: ■, VC; ○, ethene. The closed symbols in (A) represent average values from duplicate cultures. The open symbols in (B) represent average values from two of three triplicate cultures, while the open, crossed symbols in (B) represent values from the third triplicate culture. All other data points represent average values from triplicate cultures. Error bars represent one standard deviation.

![FIGURE 5](image5.png)

**FIGURE 5.** Reversibility experiments in identical sets of fresh, oxygen-free medium inoculated with washed cell suspensions of the TCE-fed BDI consortium from (A) positive control cultures or (B) oxygen-amended cultures. Symbols: ▲, TCE; ●, cis-DCE; ■, VC; ○, ethene. All data points represent average values from triplicate cultures. Error bars represent one standard deviation. When error bars are not visible, they are smaller than and therefore hidden behind the data symbols.
respiring Dehalococcoides strains, might lead to undesirable accumulation of VC during natural attenuation and bioremediation of contaminated sites (37).

The observed sensitivity of Dehalococcoides organisms to oxygen does not imply that infiltration of oxygenated surface water or migration of oxygenated groundwater into treatment zones would be detrimental to the success of bioremediation, but the oxygen-sensitivity of Dehalococcoides does indicate that proper diligence is required to manage and control such exposure. Biobarriers, if properly designed and maintained, may prevent exposure of bioactive remedial zones to dissolved oxygen. Interactions of Dehalococcoides cells with other bacteria (e.g., formation of cell aggregates or biofilms) may also limit exposure of Dehalococcoides organisms to oxygen. Although brief contact with oxygen irreversibly inhibits Dehalococcoides pure cultures (24), future work is needed to evaluate the kinetics of Dehalococcoides inactivation after various durations of oxygen exposure in the presence of a complex microbial community and in continuous-flow systems (e.g., columns) that closely mimic biostimulation and bioaugmentation field efforts.

Although the present study suggests Dehalococcoides strain-specific differences in the ability to tolerate and recover from oxygen exposure, current PCR-based tools used to detect and quantify Dehalococcoides DNA and RNA biomarkers did not prove useful in distinguishing viable, dechlorinating cells from inactive and irreversibly inhibited cells. Although decreases in the abundance of Dehalococcoides DNA biomarkers (i.e., RDase and 16S rRNA genes) were observed in the experiments with oxygen exposed cultures, the biomarkers remained easily quantifiable. Use of qPCR for detection of Dehalococcoides DNA biomarkers, therefore, has limitations in determining cell viability and predicting dechlorination activity, and results from such analyses should be interpreted accordingly.

Unlike DNA biomarkers, use of RNA biomarkers is generally considered to be a direct prognostic assessment of microbial activity as opposed to metabolic potential (2, 38). Our findings indicate that detection of Dehalococcoides RNA biomarkers (i.e., gene transcripts) via RT-qPCR does not necessarily indicate active, dechlorinating cells. Dehalococcoides biomarker gene transcripts were detected in both the control and oxygen-amended cultures. In the control cultures, RDase gene transcription correlated to dechlorination activity and available chlorinated ethene(s). These results demonstrate that measurable mRNA turnover occurred in active cells, which is consistent with previous studies (39–41). In the oxygen-amended cultures, detection of Dehalococcoides biomarkers did not always correlate with observed dechlorination (in)activity. Biomarker transcripts were detected in the oxygen-amended cultures, often at levels similar to those in the control cultures, even when dechlorination was not occurring. These results suggest that mRNA turnover (i.e., degradation) in oxygen-exposed, inactive cells is slow; therefore, detection of RNA biomarkers may not directly indicate activity but instead represent “ghost” signals without correlation to metabolic activity. The longevity of Dehalococcoides mRNA, particularly in inactive cells, represents a limitation in using mRNA as a biomarker to infer in situ Dehalococcoides activity. Mining available Dehalococcoides genome information suggests that these organisms possess enzyme systems that protect against reactive oxygen species ((42); unpublished results). Hence, oxygen may have limited effects on the cells’ transcriptional apparatus and primarily affect protein biosynthesis or inactivate RDases associated with the cell membrane. Future work should investigate mRNA levels and turnover rates in actively dechlorinating, starved, and oxygen-inhibited cultures to establish limits for the application of RNA biomarker analysis to estimate in situ activity.

The use of PCR-based tools to assess and monitor bioremediation at chlorinated solvent sites is gaining increased acceptance with regulators and environmental practitioners (2, 38). The results of this study suggest potential shortcomings in the resolution of these tools. New techniques (or improvements to existing ones) are desirable to complement the current site assessment and bioremediation monitoring technologies. For example, recent studies in medical and food microbiology quantitatively distinguished viable and nonviable (dead) cells by pretreating samples with ethidium monoazide (EMA) or propidium monoazide (PMA) before DNA extraction and qPCR analysis (43–47). EMA and PMA penetrate cells with compromised cell membranes and cell wall systems (i.e., dead cells), and covalent cross-linking of EMA or PMA with DNA by photoactivation prevents DNA extraction/purification and PCR amplification. Therefore, only DNA from viable cells is detected after sample pretreatment. Such techniques, if applicable to Dehalococcoides organisms and Dehalococcoides-targeted DNA and RNA biomarker analysis, could easily be incorporated into existing protocols and commercially available diagnostic services. At present, the application of molecular biological tools to assess and monitor bioremediation has greatly improved the ability of scientists and engineers to establish cause-and-effect relationships between anaerobic microbial processes and contaminant detoxification (2, 9, 21, 38). The potential limitations of molecular tools described herein do not imply these approaches are ineffective, and the results of the present study should not be used to discount these valuable diagnostic technologies. Additional gains in refining Dehalococcoides-targeted biomarker analysis are likely to overcome such shortcomings, further enhancing the power of molecular approaches as prognostic and diagnostic tools for assessing chlorinated ethene-impacted sites and implementing cost-effective biological remedies.

Acknowledgments

We thank R. C. Daprato, K. E. Fletcher, and N. Lee for helpful discussions, J. Waddell for maintaining the BD stock culture, and U. Tezel and S. H. Thomas for assistance with oxygen analysis. This research was funded by the Strategic Environmental Research and Development Program (project ER-1586), and in part by Regenesis and the NSF under Grant 0090496 (CAREER Award to F. E. L.). B.K.A. is a recipient of an NSF graduate research fellowship and an EPA STAR fellowship (1916150). E.P.-C. acknowledges partial support through an NSF IGERT fellowship and is a recipient of an NSF graduate research fellowship.

Supporting Information Available

Data from the oxygen consumption experiments (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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ES703227G