Acetate versus Hydrogen as Direct Electron Donors To Stimulate the Microbial Reductive Dechlorination Process at Chloroethene-Contaminated Sites†

JIAN ZHONG HE, YOULBOONG SUNG, MIKE E. DOLLHOPF, BABU Z. FATHEPURE, JAMES M. TIEDJE, AND FRANK E. LÖFFLER.*†

A study to evaluate the dechlorination end points and the most promising electron donors to stimulate the reductive dechlorination process at the chloroethene-contaminated Bachman Road site in Oscoda, MI, was conducted. Aquifer materials were collected from inside the plume and used to establish microcosms under a variety of electron donor conditions using chlorinated ethenes as electron acceptors. All microcosms that received an electron donor showed dechlorination activity, but the end points depended on the sampling location, indicating a heterogeneous distribution of the dechlorinating populations in the aquifer. Interestingly, several microcosms that received acetate as the only electron donor completely dechlorinated PCE to ethene. All acetate-amended microcosms rapidly converted PCE to cis-DCE, whereas PCE dechlorination in H2-fed microcosms only occurred after a pronounced lag time and after acetate had accumulated by H2/CO2 acetogenic activity. The microcosm experiments were corroborated by defined co-culture experiments, which demonstrated that H2 sustained PCE to cis-DCE dechlorination by acetotrophic populations in the presence of H2/CO2 acetogens. In sediment-free nonmethanogenic enrichment cultures derived from ethene-producing microcosms, acetate alone supported complete reductive dechlorination of chloroethenes to ethene, although the addition of H2 resulted in higher cis-DCE and VC dechlorination rates. Measurements of H2 production and consumption suggested that syntrophic acetate-oxidizing population(s) were active in the enrichment cultures. These findings demonstrated that either acetate or H2 alone can be sufficient to promote complete reductive dechlorination to ethene, provided syntrophic acetate-oxidizing population(s) and H2/CO2 acetogenic population(s) are present, respectively. Approaches that result in increased fluxes of both electron donors (e.g., by addition of fermentable substrates) seem most promising to sustain complete high rate reductive dechlorination to ethene in the contaminated zone of the Bachman aquifer, although acetate or H2 alone may be sufficient to drive the dechlorination process to completion.

Introduction

Chlorinated ethenes are abundant groundwater pollutants of environmental concern (1, 2), and the stimulation of microbial degradation is often the most feasible remediation strategy. Respiratory organochlorine-reducing bacteria use chlorinated compounds, such as chloroethenes, as terminal electron acceptors in their energy metabolism (a process known as chlororespiration or dechlororespiration) (3–5). Reductive dechlorination is an electron-consuming process and is often limited by the availability of suitable electron donors in contaminated subsurface environments. The majority of tetrachloroethene (PCE)-respiring populations available in pure culture, including several Desulfitobacterium species, Dehalospirillum multivorans, Dehalobacter restrictus, and Dehalococcoides species use H2 but not acetate as the electron donor for the reduction of PCE (summarized in refs 5 and 6). H2 is generally considered to be the ultimate electron donor for the reductive dechlorination process (7–19). Consequently, current efforts to stimulate dechlorination at chloroethene-contaminated sites focus on stimulating H2-consuming (hydrogenotrophic) dechlorinators by increasing the H2 concentrations in the impacted zones. This can be achieved by different approaches including H2 sparging, H2-generating electrodes, zerovalent iron walls, or addition of fermentable substrates such as ethanol, lactate, propionate, butyrate, or complex organic materials such as molasses, corn cobs, wood chips, newprint, or microbial biomass (11, 15, 16, 18, 19).

Hydrogenotrophic dechlorinators are efficient H2 utilizers; however, they have to compete for H2 with other microbial groups that consume H2 in terminal electron-accepting processes, i.e., methanogenesis and acetogenesis. Microbial fermentation of lactate, for instance, proceeds quickly and generates high concentrations of H2 (11, 15). Consequently, a considerable amount of H2 is consumed in alternate terminal electron-accepting processes, thus limiting the efficiency of reducing equivalent consumption in the reductive dechlorination process. In contrast, the fermentation of propionate or butyrate proceeds slowly and only at low H2 partial pressures. Because of thermodynamic constraints, fatty acid fermentation is a syntrophic process and depends on the activity of hydrogenotrophic populations (20). The fermentation of such electron donors provides a slow and steady release of H2 at levels that favor the reductive dechlorination process over methanogenic and acetogenic activity (4, 15). Table 1 shows relevant H2-releasing fermentations and H2-consuming processes and indicates the free energy changes associated with these reactions under standard conditions. Also indicated are ΔG* values, which were calculated at concentrations of reactants that are more likely to be encountered at contaminated subsurface environments. The positive ΔG* values of reactions 7 and 8 in Table 1 explain why hydrogenotrophic reduction of CO2 to...
ethane are in the gaseous state.

\[ \text{acetate} + 4\text{H}_2\text{O} \rightarrow 2\text{HCO}_3^- + 4\text{H}_2 + \text{H}^+ \]

+104.55

\[ \text{propionate} + 3\text{H}_2\text{O} \rightarrow \text{acetate} + \text{HCO}_3^- + \text{H}^+ + 3\text{H}_2 \]

+76.48

\[ \text{butyrate} + 2\text{H}_2\text{O} \rightarrow 2\text{acetate} + \text{H}^+ + 2\text{H}_2 \]

+48.30

\[ \text{ethanol} + 2\text{H}_2\text{O} \rightarrow \text{acetate} + \text{H}^+ + 2\text{H}_2 \]

+9.65

\[ \text{methanol} + 2\text{H}_2\text{O} \rightarrow \text{HCO}_3^- + \text{H}^+ + 3\text{H}_2 \]

+23.03

\[ \text{lactate} + 2\text{H}_2\text{O} \rightarrow \text{acetate} + \text{HCO}_3^- + \text{H}^+ + 2\text{H}_2 \]

−3.96

\[ \text{H}_2\text{-Releasing Reactions} \]

\[ \Delta G^\circ \] (kJ/rxn)

−49.57

−57.56

−65.67

−87.23

−94.12

−109.52

\[ \Delta G^\circ \] (kJ/rxn)

−49.57

+9.88

−191.48

−175.43

−183.36

Acetate or methane will not occur at low H₂ partial pressures. Hydrogenotrophic dechlorinators, in contrast, exhibit H₂ consumption threshold concentrations that are at least an order of magnitude below the minimum H₂ concentration required to sustain methanogenesis, and reductive dechlorination remains an energetically favorable process under low H₂ conditions. Therefore, reductively dechlorinating populations outcompete methanogens at low H₂ partial pressures (4, 10, 11, 15). Such slow H₂-release substrates are desired in bioremediation of chlorinated solvents because they ensure the specific stimulation of the process of interest at high efficiency (e.g., the majority of reducing equivalents provided by H₂ oxidation are consumed in reductive dechlorination).

Acetate, although a major intermediate in the anaerobic decomposition of organic matter and also a byproduct in the syntrophic fermentation of propionate and butyrate, is generally not regarded as a relevant electron donor to sustain the reductive dechlorination process. Krumholz and co-workers (21, 22) described Desulfuromonas chloroethenica that reduced PCE to cis-DCE with acetate as electron donor. Another PCE-dechlorinating, acetate-oxidizing Desulfuromonas isolate was obtained since then (23). This type of dechlorinator cannot use H₂ as an electron donor. A recent study detected PCE to cis-DCE-dechlorinating activity in microcosms established with Bachman Road site aquifer material in acetate-amended microcosms but not in H₂-fed microcosms. The presence of one or more populations related to D. chloroethenica was confirmed using dechlorinator-targeted 16S rDNA primers (6). Following a prolonged incubation period, both acetate- and H₂-amended microcosms completely dechlorinated chloroethenes to the environmentally benign product ethene. This was a surprising observation because the current dogma is that the reductive dechlorination process is best stimulated by H₂. In particular, reductive dechlorination past cis-DCE with acetate as the only available electron donor has not been observed previously. Hence, the goals of this study were to precisely determine the dechlorination end points in microcosms established with Bachman Road site material using a variety of electron donors and to test whether H₂ or acetate was the relevant direct electron donor for the reductive dechlorination process. In addition, aquifer material that became available from a chloroethene-contaminated site at Cape Canaveral Air Station was included in this study (24).

**Table 1. Catabolic H₂-Releasing Reactions of Substrates Relevant in Biostimulation of the Microbial Reductive Dechlorination Process and Relevant H₂-Consuming Reactions**

<table>
<thead>
<tr>
<th>H₂-Releasing Reactions</th>
<th>ΔG° ‡ (kJ/rxn)</th>
<th>ΔG° † (kJ/rxn)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) acetate + 4H₂O → 2HCO₃⁻ + 4H₂ + H⁺</td>
<td>+104.55</td>
<td>−49.57</td>
</tr>
<tr>
<td>(2) propionate + 3H₂O → acetate + HCO₃⁻ + H⁺ + 3H₂</td>
<td>+76.48</td>
<td>−57.56</td>
</tr>
<tr>
<td>(3) butyrate + 2H₂O → 2acetate + H⁺ + 2H₂</td>
<td>+48.30</td>
<td>−65.67</td>
</tr>
<tr>
<td>(4) ethanol + 2H₂O → acetate + H⁺ + 2H₂</td>
<td>+9.65</td>
<td>−87.23</td>
</tr>
<tr>
<td>(5) methanol + 2H₂O → HCO₃⁻ + H⁺ + 3H₂</td>
<td>+23.03</td>
<td>−94.12</td>
</tr>
<tr>
<td>(6) lactate + 2H₂O → acetate + HCO₃⁻ + H⁺ + 2H₂</td>
<td>−3.96</td>
<td>−109.52</td>
</tr>
</tbody>
</table>

\[ \Delta G = \Delta G^\circ + R T \ln (n_{\text{products}}/n_{\text{reactants}}) \]

a Gibbs free energy changes under standard conditions (25 °C, 1 M concentrations of reactants at 1 atm) at pH 7.0. b Calculations of free energy changes at 25 °C and pH 7.0 according to equation \[ \Delta G = \Delta G^\circ + R T \ln (n_{\text{products}}/n_{\text{reactants}}) \] and based on the following concentrations: organic substrates, 1 mM; HCO₃⁻, 30 mM; CH₄, 1000 ppmv; H₂, 10 ppmv; chloroethenes, 5 ppm; ethene, 5 ppmv; chloride, 1 mM; CH₄, H₂, CO₂, VC, and ethene are in the gaseous state.

**Materials and Methods**

**Chemicals.** Chemicals were of the highest purity available and purchased from Aldrich (Milwaukee, WI) and Sigma Chemical Co. (St. Louis, MO). Vinyl chloride (VC) was obtained from Fluka Chemical. Corp. (Ronkonkoma, NY).

**Microcosm Setup and Incubation Conditions.** Aquifer materials from the Bachman Road site in Oscoda, MI, were retrieved inside the chloroethene plume with a Geoprime at two depths, 12–14 ft below ground surface (bgs) (shallow zone) and 18–20 ft bgs (deep zone). Procedures for sampling and microcosm setup were essentially performed as described by Fennell et al. (24), unless indicated otherwise. The cores were capped and immediately stored at 4 °C. Reductive dechlorination potential of indigenous microorganisms and dechlorination end points were assessed in duplicate microcosms with a single chloroethene added as electron acceptor and a variety of substrates including H₂, formate, acetate, pyruvate, lactate, fumarate, glycerol, glucose, molasses, or whey. Microcosms were established by placing 2 g (wt wt) of well-mixed Bachman aquifer material into sterile 24-mL (nominal capacity) glass vials. Anoxic groundwater collected from the sampling location, anoxic potassium phosphate buffer (10 mM, pH 7.2), or reduced anaerobic mineral salts medium was added to a total volume of 10 mL. PCE, TCE, or cis-DCE were added from anoxic aqueous stock solutions to initial aqueous concentrations of 20 μM. Gaseous VC was added by syringe at 50 μM. Soluble electron donors were added to final concentrations of 2.5 mM, and molasses and whey were added at 1 mg/mL. H₂ was added at initial concentrations of 350 000 ppmv. H₂ concentrations are expressed in parts per million by volume (1 ppmv = ca. 0.1 Pa = ca. 10⁻⁶ atm). Aqueous H₂ concentrations can be calculated using the Nernst equation (e.g., 1 ppmv = ca. 0.78 nM at 25 °C) (4). The vials were sealed with sterilized Teflon-lined rubber stoppers and incubated in the dark at 25 °C with the stopper down. All manipulations were performed under a stream of sterile N₂ gas.

To monitor electron donor consumption and dechlorination more carefully, additional aquifer material was collected at the Bachman site from an intermediate depth of 15–17 ft bgs. Aquifer material from the Cape Canaveral site was kindly provided by Dr. D. Fennell and was included in this study. Microcosm setup was essentially performed as described by Fennell et al. (24). Inside an anaerobic chamber (Coy, Ann Arbor, MI) filled with H₂/N₂ [3%/97% (vol/vol)], 40 g of aquifer material (wet weight) was placed into sterile...
160-mL serum bottles. Sterile, O₂-free phosphate buffer (10 mM, pH 7.2) with or without 5 mM acetate was added to the bottles before they were sealed with butyl rubber stoppers (Geo-Microbial Technologies, Inc., Ochelata, OK). All bottles were purged with H₂-free N₂ to remove any residual H₂ prior to the addition of 50 μL of neat PCE dissolved in 5 mL of hexadecane, resulting in an initial aqueous PCE concentration of ca. 0.12 mM. Three bottles contained 5 mM acetate, three bottles received 170 000 ppmv of H₂, and three bottles had no electron donor additions. Two additional microcosms of each treatment were autoclaved twice for 60 min and served as killed controls. H₂ concentrations in the H₂-amended microcosms were periodically monitored, and additional H₂ (45 μmol) was added to maintain H₂ concentrations above 20 000 ppmv. To prevent contamination, aseptic technique was applied to the greatest extent possible during sample collection, material handling, and microcosm setup inside the anaerobic chamber. Aquifer materials from different sites were never handled simultaneously to avoid cross contamination. Microcosms were incubated stationary in horizontal position at 25 °C in the dark.

To test whether both aquifer materials were conducive for the activity of hydrogenotrophic PCE dechlorinators without the addition of an organic substrate, microcosms were established as described above. Then, PCE dissolved in hexadecane was added, and microcosms received a 1% (vol/vol) inoculum (ca. 10⁸ cells) from a Deh. multivorans (DSM 12446) culture grown on formate and PCE or a 2% (ca. 2 x 10⁹ cells) inoculum from a Dehalococcoides-containing mixed culture grown with PCE and H₂ (6). No analytically detectable formate or acetate was transferred with the inocula. H₂ (170 000 ppmv) was added to the microcosms as the electron donor.

Ethene-Producing Enrichment Cultures. To study the dechlorination of cis-DCE to ethene in more detail, the acetate-amended and ethene-producing microcosms established after the initial sampling event were used to derive enrichment cultures. Repeated (12 times) 1% (vol/vol) transfers to bicarbonate-buffered defined mineral salts medium amended with acetate as the only electron donor and 0.24 mM cis-DCE or 0.37 mM VC as the electron acceptor yielded sediment-free dechlorinating enrichment cultures. Methanogens were completely inhibited by the chloroethenes present in the cultures and were diluted out after three sequential transfers.

Organisms and Growth Conditions in Defined Cultures Experiments. The PCE to cis-DCE dechlorinator Desulfitomomas sp. strain BB1 was isolated from a pristine river sediment (23). Cultures of strain BB1 were grown with 0.2–2 mM acetate and 10–30 μL of neat PCE dissolved in 200 μL of hexadecane (resulting in initial aqueous PCE concentrations of approximately 0.3–0.9 mM) at 25 °C in 160-mL serum bottles with a total volume of 100 mL. Anaerobic medium was prepared as previously described except that 0.4 mM Na₂S·9H₂O was added as the only reductant (4, 25). The acetogens Sporomusa ovata and Clostridium acetidicum were obtained from the American Type Culture Collection (ATCC 35899) and the German Collection of Microorganisms and Cell Cultures (DSM 1496), respectively, and grown in the same defined bicarbonate-buffered medium used for strain BB1, except that the headspace consisted of a H₂/CO₂ (80/20 vol/vol) atmosphere.

The co-culture experiments were performed in 160-mL serum bottles containing 100 mL of acetate-free bicarbonate-buffered medium amended with 300 μmol of PCE dissolved in 0.2 mL of hexadecane under a H₂/CO₂ (80/20) headspace. The experiments were initiated by adding 1 mL of a S. ovata (or C. aceticum) culture, grown with H₂/CO₂, and 10 mL of a BB1 culture, grown with 100 μmol of PCE and 1 mM acetate. The BB1 culture used as the inoculum had consumed all the PCE and depleted the acetate concentration to 0.43 mM. Hence, each 10 mL of BB1 inoculum used in the co-culture experiments contained 10 μmol of cis-DCE and 4.3 μmol of acetate. Each 1-mL S. ovata inoculum contained 3.5 μmol of acetate. Controls included cultures that received no S. ovata inoculum and cultures that received a heat-inactivated (autoclaved) S. ovata inoculum.

Analytical Methods. Chloroethenes were measured with a Hewlett-Packard model 6890 gas chromatograph equipped with a HP-624 column (60 m length, 0.32 mm diameter, 1.8 μm film thickness) and a flame ionization detector (FID). Headspace samples (100 or 200 μL) were withdrawn with gastight 250-μL glass syringes (model 1725, Hamilton Co., Reno, NV) and manually injected into a split injector operated at a split ratio of 2:1. In the co-culture experiments, chloroethenes were analyzed using a HP 7694 headspace sampler. Aqueous samples (1 mL) were withdrawn from the cultures by syringe, placed in 20-mL autosampler vials, and immediately closed with Teflon-lined rubber septa. The vials were equilibrated at 60 °C for 20 min prior to automated headspace injection (injection volume was 100 μL). Chlorinated ethenes were separated using the following temperature program: initial hold at 50 °C for 3.5 min, increase to 200 °C at a rate of 50 °C per min, final hold at 200 °C for 2.5 min, and quantified (4, 26). Ultra-high-purity nitrogen (AGA Specialty Gas, Maumee, OH) was used as the carrier gas at a flow rate of 3 mL min⁻¹. Chloroethene concentrations are reported as aqueous concentrations or as total mass per 160-mL serum bottle. Ethene, methane, and H₂ were quantified by gas chromatography as described previously (4). H₂ concentrations were measured in the headspace of cultures incubated at 25 °C and are expressed in ppmv. The instrument detection limit for H₂ analysis was 0.00223 nmol. For measurement of volatile fatty acids (VFAs) and chloride release, aqueous samples (1 mL) were periodically withdrawn from the microcosms by syringe and spun down in a microcentrifuge to remove solids, and the supernatants were frozen at −20 °C. For VFA analysis, 570 μL of sample was added to 30 μL of 1.25 M H₃PO₄ in a 1.5-mL autosampler vial and analyzed using a Shimadzu HPLC (27). The instrument detection limit was 5 nmol of acetate. For the defined culture experiments, acetate concentrations were determined by gas chromatography (28). Chloride release was measured using a modified protocol of the colorimetric assay described by Bergman and Sanik (29). Chloride concentrations were calculated from spectrophotometer readings at 460 nm on a Hewlett-Packard 8452A diode array spectrophotometer using a standard curve (0–2 mM NaCl). For chloride and VFA determinations, the variability among triplicate microcosms and replicate measurements was generally less than 20% and 5%, respectively.

Results

Microcosm Studies. Microcosms established with material collected from the deep zone of the Bachman aquifer dechlorinated PCE, TCE, cis-DCE, and VC completely to ethene within 14 weeks, independent of the type of the organic electron donor added. The addition of electron donor, however, was essential to achieve complete dechlorination of the initial amount of chloroethene added. Without substrate addition, no more than 20% of the initially added chloroethene was reduced, indicating that electron donors associated with the aquifer material allowed some limited dechlorination but were insufficient to drive the dechlorination process to completion. No differences in dechlorinating activity were observed in microcosms that were established with groundwater, phosphate buffer, or bicarbonate-buffered mineral salts medium. Ethene was also found as the dechlorination end product in microcosms amended with acetate as the only electron donor (Table 2).
This was an unexpected finding because $H_2$ is generally regarded as the ultimate electron donor for reductive dechlorination, and dechlorination beyond cis-DCE has not been reported with acetate as the electron donor. PCE- and TCE-containing microcosms that received $H_2$ as the electron donor also accumulated ethene; however, PCE and TCE dechlorination were delayed, and it took more than 4 months before all PCE or TCE was dechlorinated to ethene (Table 2). Microcosms established with material collected from the shallow zone of the Bachman aquifer showed similar PCE to cis-DCE dechlorinating activity; however, dechlorination beyond cis-DCE only occurred in 50% of the microcosms and after an extended incubation period of at least 14 weeks. In addition, acetate did not support dechlorination beyond cis-DCE in the microcosms established with material from the shallow zone.

To further investigate these observations, additional microcosms were established with materials from the Bachman Road site (collected at an intermediate depth of 15–17 ft bgs) and the Cape Canaveral site, and electron donor consumption and dechlorination were carefully monitored. Reductive dechlorination of PCE to cis-DCE occurred in microcosms established with Bachman Road site (Figure 1) and the Cape Canaveral site (Figure 2) materials supplemented with acetate or $H_2$ as electron donor. The lag time before dechlorination started was shorter in the acetate-amended microcosms (Figures 1A and 2A) as compared to the $H_2$-fed microcosms (Figures 1B and 2B). Dechlorination in the $H_2$-amended microcosms started only after acetate had accumulated, most likely through $H_2$/CO$_2$ acetogenesis. The only other possible source of acetate is through the fermentation of organic matter associated with the aquifer material. No acetate formation, however, was observed in live microcosms that were not amended with $H_2$; hence, this possibility was ruled out. Another indication that no fermentable substrates were transferred with the aquifer materials is that insignificant dechlorination occurred in the microcosms that were not amended with $H_2$.

<table>
<thead>
<tr>
<th>Electron donor added</th>
<th>PCE$^{a,b}$</th>
<th>cis-DCE$^a$</th>
<th>VC$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetate$^d$</td>
<td>cis</td>
<td>cisVC</td>
<td>ETH</td>
</tr>
<tr>
<td>$H_2$</td>
<td>PCE</td>
<td>PCE/cis</td>
<td>cisVC</td>
</tr>
<tr>
<td>acetate, $H_2$</td>
<td>cisVC</td>
<td>VC/ETH</td>
<td>ETH</td>
</tr>
</tbody>
</table>

* Chlorinated electron acceptor added. $^a$ Similar results were observed in the TCE-amended microcosms. $^b$ Indicated are the dechlorination products that constituted for greater than 90% of the initial mass of chlorinated electron acceptor added. $^d$ All 16 microcosms established with Bachman aquifer material from the deep zone and amended with acetate dechlorinated PCE and TCE to cis-DCE. Twelve of them showed complete dechlorination to ethene.
was observed in the killed control microcosms. The total amount of cis-DCE formed after 222 days was approximately 5-fold higher in the acetate-amended microcosms as compared to the H2-amended microcosms established with the Bachman material (Table 3). In the Cape Canaveral microcosms, cis-DCE formation was nearly an order of magnitude higher in the acetate-amended microcosms as compared to the H2-amended microcosms. At the end of the experiment, all microcosms still contained PCE, and more than 2 mM acetate was present in the acetate-amended microcosms. Until day 150, H2 concentrations were maintained above 20 000 ppmv in the H2-amended microcosms to ensure that hydrogenotrophic processes (e.g., H2/CO2 acetogenesis, methanogenesis, and reductive dechlorination) never became electron donor limited. At the end of the experiment, H2 concentrations dropped below 1000 ppmv in the H2-amended microcosms, and acetate production from H2/CO2 acetogenesis ceased, which may explain why dechlorination slowed in the H2-amended microcosms after about 200 days (Figures 1B and 2B). However, even after 300 days, H2 was not consumed below 100 ppmv in the H2-fed microcosms. Since H2 consumption thresholds for hydrogenotrophic dechlorinators are below 0.5 ppmv, H2-dependent dechlorination was not electron donor limited throughout the experiment (4, 15). These findings suggest that PCE to cis-DCE dechlorination in microcosms established with aquifer material from both sites was carried out by acetotrophic dechlorinating populations rather than hydrogenotrophic dechlorinators.

Bioaugmentation of Aquifer Materials. Additional microcosms were established with live or heat-sterilized aquifer materials to investigate whether the activity of hydrogenotrophic dechlorinators in the H2-amended microcosms was limited by the availability of a suitable carbon source. This was deemed necessary because only H2 and no organic carbon was added to the microcosms, and growth and activity of hydrogenotrophic dechlorinating populations might have been limited by the availability of a suitable carbon source. Microcosms were bioaugmented with Deh. multivorans or a defined Dehalococcoides sp. containing strict hydrogenotrophic, PCE-dechlorinating mixed culture (6). In microcosms that received a small Deh. multivorans inoculum, the initial amount of PCE was completely dechlorinated to cis-DCE after a 4-week incubation period. PCE was also completely consumed after 6 weeks in the microcosms that received the Dehalococcoides sp. inoculum, and VC accumulated. These results indicated that dechlorination by hydrogenotrophic dechlorinating populations was not limited by the availability of a carbon source in the microcosms that only received H2 and no organic substrate, and an experimental bias favoring acetotrophic dechlorinators was ruled out.

Defined Co-culture Experiments. To explain the microcosm observations and to corroborate the relevance of H2/CO2 acetonogens in the H2-fed microcosms, co-culture experiments with an acetotrophic dechlorinating pure culture were performed. Desulfituromonas sp. strain BB1 couples PCE reduction to acetate oxidation but is unable to use H2 as an electron donor. H2 supported the reductive dechlorination process only in co-cultures of strain BB1 with a H2/CO2 acetogen Sporomusa ovata (Figure 3A). Growth of S. ovata in the bicarbonate-buffered medium in the presence of H2 as the electron donor resulted in the production of acetate, which was then used by the acetate-oxidizing dechlorinator for the reduction of PCE. Although no acetate accumulation could be measured in the co-cultures in the presence of PCE, acetate production was evident by the continued dechlorination of PCE with the concomitant production of cis-DCE. Acetate accumulation became apparent only after PCE had been consumed, and 311.6 ± 30.1 μmol of cis-DCE accumulated (Figure 3A). Acetate production continued, and after day 28, 1887 ± 268.3 μmol of H2 was consumed and 378 ± 35.1 μmol of acetate accumulated. No growth inhibition of S. ovata with H2/CO2 was observed at the chloroethene concentrations used in the co-culture experiments. Similar results were obtained in co-culture experiment with strain BB1 and C. aceticum (not shown). The cometabolic reductive dechlorination of PCE to TCE by acetogens (e.g., S. ovata) with an induced Wood–Ljungdahl pathway (acetyl-CoA pathway) has been reported (30); however, dechlorination by S. ovata and C. aceticum under the growth conditions employed in this study was not detected. As expected, no

### Table 3. PCE Dechlorination Products Present in the Microcosms Established with Bachman Road (15–17 ft bgs) and Cape Canaveral Aquifer Materials after 222 Days

<table>
<thead>
<tr>
<th>Site Material</th>
<th>Electron Donor</th>
<th>PCE Dechlorination Products Formed (μmol/microcosm)</th>
<th>Chloridea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bachman</td>
<td>Acetate</td>
<td>TCE: 34.6 ± 8.5, cis-DCE: 166.9 ± 47.0, trans-DCE: 0.8 ± 0.2, VC: &lt;1.3</td>
<td>350 ± 50</td>
</tr>
<tr>
<td>Cape Canaveral</td>
<td>H2</td>
<td>TCE: 10.5 ± 3.0, cis-DCE: 32.0 ± 2.1, trans-DCE: 0.0, VC: 0</td>
<td>75 ± 32</td>
</tr>
<tr>
<td>Bachman</td>
<td>H2</td>
<td>TCE: 4.9 ± 0.8, cis-DCE: 370.8 ± 22.5, trans-DCE: 1.6 ± 0.1, VC: 6.4 ± 5.4</td>
<td>790</td>
</tr>
<tr>
<td>Cape Canaveral</td>
<td>H2</td>
<td>TCE: 14.4 ± 6.0, cis-DCE: 44.0 ± 13.4, trans-DCE: 0, VC: &lt;1.0</td>
<td>69</td>
</tr>
</tbody>
</table>

* Shown are mean values from triplicate cultures ± standard deviation. Values reported without standard deviation were averaged from duplicate cultures. * Corrected for the chloride background concentrations measured at the start of the experiments.

**FIGURE 3.** Reductive dechlorination of PCE to cis-DCE (A) in co-cultures of Desulfuromonas strain BB1 and the H2/CO2 acetogen Sporomusa ovata; (B) in cultures that were only inoculated with strain BB1; (C) in cultures that received a strain BB1 inoculum and an autoclaved Sporomusa ovata inoculum. All cultures contained H2 as the only electron donor. The data shown were corrected for the small amounts of cis-DCE transferred with the strain BB1 inoculum. All data points represent average values from triplicate cultures, and standard deviations are included. If no error bars are shown, the standard deviations were too small to be illustrated.
dechlorination occurred in H2-fed cultures of strain BB1 in the absence of an acetogenic population (Figure 3B). The small amounts of cis-DCE and TCE produced are due to the small amount of acetate (4.3 μmol) transferred with the inoculum. Some dechlorination was detected in H2-amended cultures of strain BB1 that received autoclaved S. ovata inoculum (Figure 3C). The likely sources of electron equivalents allowing the limited dechlorination to TCE and cis-DCE in these cultures are the small amounts of acetate transferred with the inoculum and electron donors released from the autoclaved S. ovata cells.

**Reductive Dechlorination of cis-DCE and VC.** The sediment-free nonmethanogenic enrichment cultures derived from ethene-producing microcosms sustained complete reductive dechlorination with acetate as the only available electron donor. Figure 4 shows the reductive dechlorination of VC to ethene by the acetate/VC-enriched culture with acetate as the only electron donor. After 187 days, about 50 ± 12 μmol of VC was consumed, and 33 ± 7 μmol of ethene was formed. Less than expected reduced product was measured because VC concentrations were overestimated in the initial analysis, (e.g., the measurements were performed before the system had reached equilibrium). In addition, a total of 7 mL of headspace gas containing ca. 10 μmol of VC was removed for analyses, and some minor loss of VC (<2% of the total amount of VC added) occurred in all cultures, presumably due to absorption onto the stopper. The H2 concentration at the start of the experiment was below 1.5 ppmv but increased in all of the triplicate cultures before the concentrations fell below 0.5 ppmv (Figure 4). In cultures that had consumed all electron acceptor (VC was completely converted to ethene), H2 accumulated to 46 ± 16 ppmv (determined in six cultures in two independent experiments). An increase to similar H2 concentrations was measured in cultures that were transferred to acetate-amended medium in the absence of cis-DCE or VC. No ethene formation and no change in H2 concentrations were observed in control cultures that received filter-sterilized inocula or contained no acetate. As demonstrated in Figure 4, the dechlorination rates in the acetate-fed cultures increased notably after the addition of 223 μmol of H2 (ca. 85 000 ppmv) as another source of reducing equivalents. After 140 days, the amount of H2 added was consumed below 0.5 ppmv; however, acetate was still present, and VC reduction to ethene continued at lower rates.

**Discussion**

The microcosm studies demonstrated the presence of bacteria that completely dechlorinated chloroethenes to the innocuous end product ethene at the Bachman Road site. Microcosms established with material collected from different depths exhibited distinct dechlorination patterns and end points. Obviously, the Bachman aquifer contained multiple dechlorinating populations exhibiting different dechlorination activities, and at least the population(s) dechlorinating beyond cis-DCE were not uniformly distributed throughout the contaminated zone. Evidence for a heterogeneous distribution of the PCE to cis-DCE dechlorinating population(s) in the Bachman aquifer was provided earlier in a 16S rDNA-based study (6). Fennell et al. recently reported that bacteria related to Dec. ethenogenes were present at the Cape Canaveral site; however, they also noted that the dechlorinating populations were heterogeneously distributed in the aquifer and did not rule out the presence of other dechlorinators (24). Our observations corroborate a heterogeneous distribution of the dechlorinating populations in the contaminant plume of the Cape Canaveral aquifer. No evidence for the presence of a hydrogenotrophic Dehalococcoides-type population was obtained; however, our results indicated the presence of another type of dechlorinator that used acetate and not H2 as a direct electron donor for PCE to cis-DCE dechlorination. Apparently, multiple and heterogeneously distributed dechlorinating populations were present at both sites. These observations are relevant for practitioners and suggest that the analysis of a sufficient number of samples is a prerequisite in order to make any meaningful predictions on the potential of natural attenuation at a given site.

An increased flux of H2 is currently viewed as the method of choice to stimulate reductively dechlorinating populations. Unfortunately, H2 is a preferred electron donor for different microbial groups, which compete with the dechlorinating populations for this substrate. To favor hydrogenotrophic dechlorinators, propionate and butyrate were suggested as slow-release H2 donors to specifically stimulate the reductive dechlorination process. Other than H2, the anaerobic oxidation of propionate and butyrate (reactions 2 and 3 in Table 1) also yields acetate, and direct electron donors for hydrogenotrophic and acetotrophic dechlorinators become available. Generally, all approaches that increase H2 concentrations in the field will also result in increased acetate concentrations, either by fermentation processes or H2/CO2 acetogenesis. H2/CO2 acetogens are commonly found in anaerobic aquifers and were detected in the Wurtsmith Air Force Base aquifer located in the vicinity of the Bachman Road site (31). H2/CO2 acetogenic activity and acetate production will occur in most anaerobic aquifers provided that sufficient H2 is available. The careful measurements of electron donor consumption, dechlorination, and acetogenesis in the microcosms and the co-culture experiments with Desulfuromonas sp. strain BB1 and S. ovata or C. acetobutylicum demonstrated that PCE dechlorination by non-hydrogenotrophic, acetate-oxidizing PCE dechlorinators can be stimulated by H2 in the presence of H2/CO2 acetogenic populations. Consequently, the apparent lag time prior to the onset of dechlorination activity in the H2-amended microcosms does not reflect the acclimation period of the dechlorinating population(s). Rather, the lag time is attributed to distinct microbial groups, H2/CO2 acetogenic bacteria, and acetotrophic dechlorinator(s). These findings also demonstrate that the stimulation of the reductive dechlorination process after increasing H2 fluxes cannot automatically be attributed to the activity of hydrogenotrophic dechlorinators unless a careful evaluation of the process is performed. In the acetogen/dechlorinator co-cultures, no significant accumulation of acetate from H2/CO2 acetogenesis occurred as long as PCE was present due to the immediate consumption of acetate by the acetate-oxidizing dechlorinator. This observation indicates that acetate can be present in low concentrations at contaminated sites and not detectable by
standard analytical methods but still be the important electron donor for reductively dechlorinating populations. Recent findings suggested that acetate is also a relevant direct electron donor to stimulate the reductive dechlorination of other chlorinated environmental pollutants such as polychlorinated biphenyls. For instance, the reductive dechlorination of 2,3,5,6-tetrachlorobiphenyl in a sediment-free enrichment culture was supported by acetate but not by H₂ (32).

Information about microbial populations that reductively dechlorinate cis-DCE and VC is scarce. The dechlorinating enrichment cultures that have been described require H₂ as the direct electron donor, and reductive dechlorination of cis-DCE and VC coupled to the oxidation of acetate has not been reported (9, 33, 34). We were surprised to see that acetate was sufficient to drive the dechlorination of cis-DCE and VC to ethene in several microcosms and the enrichment cultures derived from the Bachman aquifer. The addition of H₂, however, increased the dechlorination rates, suggesting that H₂ is a better direct electron donor than acetate. Another possible explanation for the observations is a syntrophic association in these cultures, and acetate served as an indirect electron donor. Like butyrate and propionate oxidation, the oxidation of acetate is thermodynamically feasible at low H₂ partial pressures (reactions 1–3 in Table 1). Interpreting the results of a study by Yang and McCarty, Dolfing already speculated that acetate could be a relevant source of reducing equivalents for cis-DCE- and VC-dechlorinating populations under low H₂ conditions (35). Yang and McCarty, however, conclusively demonstrated that their culture did not derive reducing equivalents for reductive dechlorination from acetate (17).

A few syntrophic acetate-oxidizing bacteria have been described, but only limited information is available, especially on mesophilic populations (36–41). Schnürer et al. described Clostridium ultunense, a mesophilic organism that oxidized acetate in the presence of a H₂-consuming methanogenic population (38). Nüsslein et al. obtained circumstantial evidence for syntrophic acetate oxidation in methanogenic lake sediment at 15°C (39). Hydrogenotrophic cis-DCE and VC dechlorinators should be excellent partners for acetate-oxidizing syntrophic populations because these dechlorinators maintain even lower H₂ partial pressures than methanogens (4). The H₂ concentrations in Bachman enrichment cultures increased to about 46 ppmv after all chlorinated electron acceptor, i.e., cis-DCE or VC, had been consumed. Because acetate was the only possible source of H₂ in these cultures, the increase in H₂ concentration indicated the presence of an acetate-oxidizing population. At a H₂ concentration of 46 ppmv and under the experimental conditions chosen, the ΔG° for acetate oxidation (reaction 1 in Table 1) is −34.7 kJ/mol. This value is consistent with earlier observations on the minimum free energy change required to support the energy metabolism of a syntrophic acetate-oxidizing population (41). An important fact to consider is that the change in free energy decreases with temperature and that the temperatures in contaminated aquifers are typically below 25°C. At a temperature of 10°C, syntrophic acetate oxidation requires H₂ concentrations below 10 ppmv to yield a change in free energy of −34.7 kJ/mol of acetate oxidized (calculated according to ref 41 using the ThermoDyn program [www.microeco.unizh.ch/therm/thermodyn.html]). H₂ concentrations in contaminated aquifers are typically in the low nanomolar range, indicating that syntrophic acetate oxidation can be a viable process at ambient temperatures. These considerations suggest that syntrophic acetate oxidation can provide H₂ to hydrogenotrophic chloroethene-dechlorinating populations in contaminated subsurface environments. Because syntrophic acetate-oxidizing populations make a living on the small amount of energy available from acetate fermentation, they grow very slowly and are difficult to isolate and to maintain in pure culture. Consequently, the distribution of these populations in the environment is currently unclear as is their competitive fitness with other acetate-fermenting populations (i.e., acetoclastic methanogens). Schnürer et al. (37) suggested that the inhibition of acetoclastic methanogens is required to allow syntrophic acetate oxidation at mesophilic temperatures. Acetoclastic methanogenesis was observed in Bachman microcosms; however, this activity was readily inhibited by chloroethenes. Several other cis-DCE- and VC-reducing enrichment cultures were unable to use acetate as a source of reducing equivalents for reductive dechlorination. For instance, dechlorination was not supported by acetate in the culture described by Yang and McCarty and in three cis-DCE to ethene dechlorinating enrichment cultures derived from river sediments (17, 34).

Because of the oligotrophic nature of many chloroethene-contaminated subsurface environments, electron donor addition is a promising approach to enhance the microbial reductive dechlorination process. The current focus is on electron donors that ultimately release H₂ to stimulate the reductive dechlorination process. At some sites, however, acetate and not H₂ is the relevant direct electron donor for PCE to cis-DCE dechlorination, and acetate alone is sufficient to sustain complete dechlorination to ethene. A careful evaluation of the relevant dechlorinating populations is warranted in order to decipher whether hydrogenotrophic or acetotrophic dechlorinators are responsible for the dechlorination reactions when they occur. At the chloroethene-contaminated Bachman Road site, approaches that increase fluxes of both electron donors, acetate and H₂, seem most promising to sustain complete high rate reductive dechlorination of PCE to ethene. In the deep zone of the Bachman aquifer where a microbial community consisting of dechlorinating populations, H₂/CO₂ acetogens, and syntrophic acetate oxidizers exists, either electron donor may be sufficient to drive the reductive dechlorination process to completion.

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Page 3946. The $\Delta G'$ values presented in Table 1 are incorrect because the proton concentration was accounted for twice. The revised Table 1 below shows the correct $\Delta G'$ values.

Page 3951. The $\Delta G'$ for acetate oxidation at a hydrogen concentration of 46 ppmv is $-1.12$ kJ/mol. The overall conclusions of the paper remain unchanged. Jackson and McInerney recently demonstrated that metabolism by syntrophic associations occurs at values close to the thermodynamic equilibrium (Jackson, B. E.; McInerney, M. J. Anaerobic microbial metabolism can proceed close to thermodynamic limits. Nature 2002, 415, 454–456).

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