Distribution of a dechlorinating community in relation to the distance from a trichloroethene dense nonaqueous phase liquid in a model aquifer

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Abstract

The toxicity of trichloroethene (TCE) likely restricts microbial activity in close vicinity of a TCE dense nonaqueous phase liquid (DNAPL). This study examined the distribution of a dechlorinating community in relation to the distance from a TCE DNAPL using a diffusion-cell set-up. Subcultures of the KB-1™ culture dechlorinating TCE to cis-dichloroethene and grown with either formate or lactate as electron donor were used to inoculate the diffusion-cells. 16S rRNA gene clone library analysis showed that both inocula consisted of dechlorinating bacteria similar to Geobacter lovleyi SZ and fermentative microorganisms related to Clostridium and Clostridiales. qPCR and RFLP analyses of pore water and sand samples showed a stratified microbial community composition in the diffusion-cells. Geobacter dominated where TCE was present, that is, in the lower 3 cm of the 5.5-cm-thick sand layer. Even at 0.5 cm distance from the DNAPL layer, Geobacter densities were two orders of magnitude higher than at inoculation, despite the expected TCE toxicity. In the upper 2.5 cm of the sand layer, where TCE was depleted, apparently fermenting populations prevailed, corresponding to Clostridium in some diffusion-cells. This analysis demonstrates that the microbial community composition in a source zone is related to the distance from the DNAPL.

Introduction

Leakage and spillage of widely used solvents like trichloroethene (TCE) led to numerous groundwater contaminations throughout the world. In the subsoil, the high density and relatively low solubility of this contaminant cause the formation of dense nonaqueous phase liquids (DNAPL). Such DNAPLs are sources of long-term groundwater contamination and pose high challenges to remediation. Besides physical and chemical remediation technologies, bioremediation through reductive dechlorination by halorespiring bacteria is being examined. Past research focused on bioremediation of the contaminant plumes. However, recent focus has shifted towards bioremediation of the source zone, that is, the zone containing DNAPL, because dechlorination in the vicinity of a DNAPL enhances the DNAPL dissolution and, as such, reduces the remediation time (Carr et al., 2000; Yang & McCarty, 2000).

Several bacterial strains belonging to diverse genera such as Desulfitobacterium, Dehalobacter and Geobacter have been reported to gain energy from the conversion of TCE to cis-dichloroethene (cis-DCE) (Bradley, 2003; Sung et al., 2006). In contrast, the subsequent reduction of cis-DCE to vinyl chloride (VC) and the harmless ethene appears restricted to bacteria belonging to the genus Dehalococcoides (Bradley, 2003). Dechlorinating microorganisms require mostly hydrogen or acetate as electron donor (Aulenta et al., 2006), which can be provided through the conversion of more complex substrates by fermentative microorganisms. As such, dechlorinating as well as fermentative bacteria are essential for the biological removal of TCE. The high TCE concentrations in the vicinity of a TCE DNAPL, however, impose toxic effects on both dechlorinating and fermenting microorganisms (Duhamel et al., 2002; Yu & Semprini, 2004; Bowman et al., 2009). Consequently, bio-enhanced dissolution of TCE DNAPL is limited by the toxicity of TCE, because
this toxicity restricts how close to the DNAPL dechlorinating and fermentative bacteria can be active and, as such, determines the TCE concentration gradient and the DNAPL dissolution rate (Chu et al., 2004). Knowledge about the microbial community composition in relation to the distance of a TCE DNAPL will, as such, contribute to a better understanding of how TCE toxicity affects bio-enhanced DNAPL dissolution.

Several laboratory studies previously quantified cell densities of dechlorinating microorganisms in source zones of perchloroethene (PCE) DNAPL (Sleep et al., 2006; Amos et al., 2008, 2009). However, the set-ups used in these studies did not allow to determine to what distance dechlorinating bacteria approach the DNAPL, neither did these studies examine the distribution of the fermentative bacteria. Philips et al. (2011) recently developed a three-layer diffusion-cell set-up to measure abiotic and biotic conditions in the vicinity of a DNAPL with a 5-mm resolution. This diffusion-cell set-up was used in this study to examine the distribution of both dechlorinating and fermentative microorganisms in relation to the distance from a TCE DNAPL. Diffusion-cells were inoculated with subcultures of the TCE dechlorinating KB-1 culture and were fed with either formate or lactate as electron donor. Samples of the pore water, as well of the sand, were analysed.

**Materials and methods**

**Microbial cultures**

Subcultures of the KB-1 culture (SiREM, Canada) were grown in anaerobic defined medium. The composition of this medium was described by Haest et al. (2011), except that the concentration of yeast extract was lowered to 10 mg L$^{-1}$. Subcultures were amended with 1.6 mM TCE. This concentration was selected to stimulate acclimatization of the KB-1 culture to high TCE concentrations. Either formate or lactate was added as electron donor in an amount equivalent for the conversion of TCE to cis-DCE. Every week, the aqueous phase of the culture bottles was flushed with N$_2$ gas, and fresh TCE and electron donor were added. As such, only dechlorination of TCE to cis-DCE was stimulated, and high TCE degradation rates were obtained. Both subcultures showed dechlorination up to a maximum TCE concentration of about 2.5 mM (data not shown).

**Diffusion-cell experiment**

The three-layer diffusion-cell set-up previously described by Philips et al. (2011) was used to study the microbial distribution in relation to the distance from a TCE DNAPL. This set-up consists of three layers, these are, a bottom DNAPL layer, a central sand layer and a top aqueous layer. The DNAPL layer maintains the saturated TCE concentration at the lower boundary of the sand layer, whereas the top aqueous layer is frequently refreshed to provide electron donor and to remove TCE and its degradation products at the upper boundary. In this study, an experiment with two diffusion-cells, fed with either formate or lactate as electron donor, was performed. Anaerobic medium identical to that used for growing the cultures was applied and the same type of sand was used as described by Philips et al. (2011). The bottom DNAPL layers contained 1 mL of pure TCE droplets. The formate-fed diffusion-cell was filled with medium inoculated with 0.6 volume% of the KB-1 subculture grown on formate, while medium inoculated with 5.9 volume% of the KB-1 subculture grown on lactate was used to fill the lactate-fed diffusion-cell. The top aqueous layers consisted of medium with 4 mM formate and 2 mM lactate in the formate- and lactate-fed diffusion-cell, respectively, and were refreshed twice a week. Incubation was performed at 20 ± 1 °C. The processes in the diffusion-cells were expected to be in steady state after about 20 days (Philips et al., 2011). At that time, pore water samples were taken from the sampling ports of the diffusion-cells for DNA analysis. In addition, pore water was sampled at day 27 for analysis of the chloroethene and methane concentrations, which were measured by GC-FID as described by Haest et al. (2010).

In addition, sand samples from diffusion-cell BIO1 of the experiment previously described by Philips et al. (2011) were analysed. This diffusion-cell was similar to the formate-fed diffusion-cell described above, but was inoculated with 5.9% of the KB-1 subculture grown on formate. At the end of the experiment (after 35 days), the sand layer of this diffusion-cell was sliced as described by Philips et al. (2011), and layers of about 5 mm were sampled for DNA analysis.

**DNA extraction**

The procedure of Uyttebroek et al. (2006) was used to extract DNA from the KB-1 subcultures for 16S rRNA gene clone library preparation. This extraction procedure resulted in low and variable extraction efficiencies. For this reason, other procedures were optimized and used to extract DNA prior to qPCR and RFLP analyses. DNA from aqueous samples was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer’s instructions, except that an extra mechanical lysis step in a Precellys 24 bead beater was included. DNA from sand samples was extracted using the UltraClean Soil DNA Isolation Kit (MOBIO), according to the
manufacturer’s instructions for maximum yields and including an additional mechanical lysis step.

16S rRNA gene clone library preparation
A 16S rRNA gene clone library was prepared for each KB-1 subculture. The 16S rRNA gene fragments were amplified by PCR using the bacterial primers P63F and P518R (El Fantroussi et al., 1999). The PCR products were ligated in the pCR<sup>TM</sup> 2.1-TOPO vector (Invitrogen) and transformed into TransforMax<sup>TM</sup> EC100<sup>TM</sup> electrocompetent Escherichia coli cells (Epicentre). A PCR using primers M13F and M13R (Invitrogen) was performed on white colonies to recover the insert. Subsequently, the insert was amplified using bacterial 16S rRNA gene primers P63F and P518R and analysed by amplified ribosomal DNA restriction analysis (ARDRA) using the restriction enzymes Hin6I and BsuRI (Fermentas). The ARDRA profiles of the clones were used to identify different operational taxonomic units (OTUs). Sequences were determined using the BigDye<sup>TM</sup> Terminator Cycle Sequencing Kit (Applied Biosystems) and a thermocycler UNOII (Biometra). The obtained sequences were corrected with the BioEdit Sequence Alignment Editor software (www.mbio.ncsu.edu/bioedit) and were aligned against known sequences in the GenBank database (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov) with BLAST.

Restriction length fragment polymorphism (RFLP)
16S rRNA gene fragments from DNA extracts of the KB-1 subcultures and the diffusion-cells were amplified using a nested PCR approach. The first PCR used the bacterial primers 27F and 1492R (Polz & Cavanaugh, 1998), while the second PCR was performed using the bacterial primers P63F and P518R (El Fantroussi et al., 1999). The resulting PCR product was digested with the restriction enzyme Hin6I (Fermentas), and RFLP profiles were visualized on a 1.5% agarose gel by applying 90 V during 50 min.

Real-time quantitative PCR (qPCR)
The qPCR analysis was performed on extracted DNA from samples of the KB-1 subcultures and from pore water and sand samples of the diffusion-cells. The 16S rRNA gene copy numbers of Dehalococcoides were determined according to Dijk et al. (2008), while Geobacter and bacterial 16S rRNA gene copy numbers were quantified as described by Haest et al. (2011).

An aqueous or sand control sample was included in every combined DNA extraction and qPCR analysis to reduce the variability between different runs. Hereto, total cell numbers in a formate-fed KB-1 subculture were microscopically counted. Aqueous control samples containing 2.2 ± 0.4 10<sup>7</sup> cells mL<sup>–1</sup> were created by dividing a part of this culture, whereas sand control samples containing 4.0 ± 0.3 10<sup>7</sup> cells g<sup>–1</sup> dry sand were created by saturating sand with another part of this culture. Control samples were stored at −20 °C until use. Aqueous and sand control samples were handled together with the respective experimental samples during DNA extraction and qPCR. After each qPCR run, the copy numbers measured in the control sample were used to calculate a correction factor. The copy numbers of the experimental samples measured in the same run were then multiplied with this correction factor. For bacterial 16S rRNA gene copy numbers, this correction factor was the ratio of the microscopically counted total cell numbers to the bacterial 16S rRNA gene copy numbers measured in the control sample. For Geobacter or Dehalococcoides 16S rRNA gene copy numbers, the correction factor was calculated as the ratio of the average of respective 16S rRNA gene copy numbers measured in control samples over different runs to the copy numbers measured in the control sample in the particular run. The bacterial correction factor directly accounts for the multiple 16S rRNA gene copies per cell, because this correction factor was based on microscopically counted cell numbers. For Dehalococcoides and Geobacter, respectively, one and two 16S rRNA gene copies per cell have been reported (Klappenbach et al., 2001). Geobacter 16S rRNA gene copy numbers were not converted to cell numbers, because the variability of the qPCR quantification is at least a factor two. Bacterial 16S rRNA gene copy numbers of aqueous control samples measured in different qPCR runs differed maximally 11 times (n = 15), whereas bacterial copy numbers for different aqueous control samples measured in one qPCR run differed maximally 1.8 times (n = 6). As such, the variability between different qPCR runs is clearly higher than the variability caused by the DNA extraction.

Results

Microbial composition of the formate- and lactate-fed KB-1 subcultures
The 16S rRNA gene clone library of the formate-fed KB-1 subculture consisted of 61 clones. Based on ARDRA profiles, twelve OTUs were recognized. Sequencing (Table 1) showed that five OTUs (19 clones) corresponded with Geobacter loyleyi SZ and seven OTUs (42 clones) corresponded with Clostridium species. None of the OTUs corresponded with Dehalococcoides or other known dechlorinating species. The 16S rRNA gene fragment
<table>
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<th>OTU name</th>
<th>Accession no.</th>
<th>Number of clones formate subculture</th>
<th>Number of clones lactate subculture</th>
<th>Identity* (%)</th>
<th>Closest GenBank match</th>
<th>Origin</th>
<th>Accession no. match</th>
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<tr>
<td>Geobacter1</td>
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<td>7/15</td>
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<td>JQ309831</td>
<td>1/61</td>
<td>7/15</td>
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<td>1/61</td>
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<td>JQ309834</td>
<td>19/61</td>
<td>99</td>
<td>94</td>
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*Results are based on sequences with lengths between 219 and 499 bp, but for Clostridium7 only a sequence of 56 bp was obtained. The latter sequence was too short to obtain a GenBank accession number.*
sequences of the five Geobacter OTUs were all highly identical to the corresponding sequence of Geobacter lovleyi SZ (Table 1). Nevertheless, only the ARDRA profiles of the two most abundant Geobacter OTUs (Geobacter1 and Geobacter2) corresponded with the ARDRA profile of G. lovleyi SZ (Fig. 1). In silico analysis of the sequences of the Geobacter OTUs showed that differences in the ARDRA profiles were attributed to small differences in sequence (results not shown). The two most abundant Clostridium OTUs had a similar ARDRA profile, whereas the ARDRA profiles of the other Clostridium OTUs were different (Fig. 1). Sequence alignment demonstrated that the Clostridium OTUs were highly similar, but that differences in sequence explained the differences in ARDRA profiles (results not shown). As such, different, but related Clostridium species were present in the formate-fed KB-1 subculture.

Subsequently, samples from different culture bottles containing the formate- or lactate-fed KB-1 subculture were analysed with qPCR and RFLP to examine the representativity of the microbial community composition obtained from the 16S rRNA gene clone libraries. Similar 16S rRNA gene copy numbers were found for all samples (Fig. 2). The Geobacter 16S rRNA gene copy numbers were of the same order of magnitude as the bacterial 16S rRNA gene copy numbers, but were always lower. On average, Geobacter densities were $32 \pm 8\%$ of the bacterial densities. Dehalococcoides 16S rRNA gene copy numbers were at least three orders of magnitude lower than bacterial numbers. In addition, the RFLP profiles for all the samples of the formate- and lactate-fed KB-1 subcultures resembled each other (Fig. 2), showing correspondence with the ARDRA profiles of G. lovleyi SZ and the Clostridium1 and Clostridium7 OTU. However, no correspondence with the Clostridiales OTU of the 16S rRNA gene clone library of the lactate-fed KB-1 subculture was found, even not for the samples of the lactate-fed KB-1 subculture. As such, the RFLP results and the 16S rRNA gene clone library analysis partially disagree regarding the microbial composition of the lactate-fed KB-1 subculture, which might be due to the low number of clones recovered for this KB-1 subculture. Nevertheless, it can be concluded based on the qPCR and RFLP analyses for both KB-1 subcultures that the microbial community in the cultures mainly consisted of bacteria belonging to the genera Geobacter and Clostridium and that the microbial community composition was stable and independent of the used electron donor.

**Microbial distribution in the formate- and lactate-fed diffusion-cell based on pore water samples**

The TCE and cis-DCE concentration profiles measured in the formate- and lactate-fed diffusion-cell (Fig. 3) were similar to those reported by Philips et al. (2011) (Fig. 4). In the formate-fed diffusion-cell, the TCE concentration dropped from about 9 mM, that is, the saturated TCE concentration, in the DNAPL layer to a zero concentration at 3 cm distance from the DNAPL (Fig. 3a). The cis-DCE concentration was maximal at about 2.5 cm distance from the DNAPL. The TCE concentration profile in the lactate-fed diffusion-cell was slightly steeper than in the formate-fed diffusion-cell, while the cis-DCE concentration in the lactate-fed diffusion-cell became maximal at a shorter distance from the DNAPL than in the formate-fed diffusion-cell (Fig. 3b), likely due to a faster TCE dechlorination. However, in other diffusion-cells fed with lactate as electron donor, TCE and cis-DCE concentration profiles similar to those observed in the formate-fed diffusion-cell were recorded (results not shown). These results suggest that the dechlorination rate in the diffusion-cells can be slightly variable. In both the formate- and the lactate-fed diffusion-cell, the methane concentrations were below the detection limit (results not shown), illustrating that methanogenesis was negligible in the diffusion-cells.
In the pore water samples of both the formate- and the lactate-fed diffusion-cell, the highest numbers of Geobacter 16S rRNA gene copies were observed in the lower part of the sand layer (Fig. 3). Even at a distance of 0.5 cm from the DNAPL, where elevated TCE concentrations were measured, Geobacter densities were two orders of magnitude higher than the inoculated Geobacter cell density. Only in the DNAPL layer, the numbers of Geobacter were below the inoculation density (Fig. 3). Geobacter cell densities in the pore water were high throughout the part of the sand layer where TCE was present, but dropped at least one order of magnitude beyond the location where TCE was completely dechlorinated to cis-DCE. In the upper part of the sand layer of the lactate-fed diffusion-cell (Fig. 3b), Geobacter 16S rRNA gene copy numbers corresponded with the inoculation density. Similar Geobacter densities were observed in the upper part of the formate-fed diffusion-cell (Fig. 3a), even though this diffusion-cell received a ten times lower inoculation density. Bacterial 16S rRNA gene copies in the pore water were high throughout the sand layer in both diffusion-cells (Fig. 3) and corresponded with a two to three orders of magnitude increase in comparison to the inoculation density. In the lower sand layer part, where TCE was present, bacterial numbers were comparable to those of Geobacter. In contrast, in the upper part of the sand layer, beyond the location where the TCE concentration became zero, bacterial densities were several orders of magnitude higher than those of Geobacter. These results show that Geobacter was dominant where TCE was present, whereas in the upper sand layer part other bacteria were most abundant.

In the lactate-fed diffusion-cell, Geobacter and bacterial numbers in the pore water were higher compared to those in the formate-fed diffusion-cell (Fig. 3a and b), which might be explained by the higher dechlorination rate in the lactate-fed diffusion-cell.

The microbial composition of the pore water of the formate- and the lactate-fed diffusion-cell was further analysed with RFLP. The resulting RFLP profiles were clearly less complex than those obtained for the KB-1 subcultures, although those cultures were used to inoculate the diffusion-cells (Figs 2 and 3). For the lower part of the sand layer, the RFLP profiles corresponded with the ARDRA profile of G. lovleyi SZ (Fig. 3), confirming that Geobacter was dominant in this part of the sand layer. In the upper part of the sand layer, a still unknown RFLP profile was observed for both diffusion-cells (Fig. 3). In the lactate-fed diffusion-cell, the RFLP profiles also corresponded with that of the Clostridium7 OTU obtained from the formate-fed KB-1 subculture (Fig. 3b). However, none of the RFLP profiles showed correspondence with the RFLP profile of the Clostridium1 and Clostridium2 OTU, although these OTUs were abundant in the formate- and the lactate-fed KB-1 subculture (Fig. 2).

**Microbial distribution in the diffusion-cell BIO1 based on sand samples**

Quantification of 16S rRNA gene copy numbers originating from pore water samples excludes bacteria attached to the solid phase and, as such, maybe underestimates the
actual cell numbers in a porous system. For this reason, sand samples from diffusion-cell BIO1 of the experiment described by Philips et al. (2011) were additionally analysed. Philips et al. (2011) reported similar TCE and cis-DCE concentration profiles (Fig. 4) for diffusion-cell BIO1 as for the formate-fed diffusion-cell of the current study (Fig. 3a). The 16S rRNA gene copy numbers in sand samples (Fig. 4) were expressed mL$^{-1}$ of pore water to enable direct comparison with copy numbers in pore water samples. The *Geobacter* and bacterial numbers in the sand samples from diffusion-cell BIO1 (Fig. 4) were higher than those in the pore water samples of the formate-fed diffusion-cell at corresponding distances from the DNAPL layer (Fig. 3a). Geobacter numbers in the lower part of the sand layer of diffusion-cell BIO1 were one order of magnitude lower than bacterial numbers (Fig. 4), whereas *Geobacter* and bacterial copy numbers for this part of the sand layer were comparable in pore water samples.

![Figure 3](image-url)  
**Fig. 3.** Pore water concentrations of TCE and cis-DCE, number of *Geobacter* and bacterial 16S rRNA gene copies mL$^{-1}$ of pore water and RFLP profiles in relation to the distance from the DNAPL in the formate-fed diffusion-cell (a) and the lactate-fed diffusion-cell (b). The qPCR and RFLP analyses were performed on DNA extracted from pore water samples. The RFLP profiles are given in the order of the corresponding 16S rRNA gene copy number bars in the graph above. The initial *Geobacter* 16S rRNA gene copy numbers are indicated by the dotted line. Error bars on the 16S rRNA gene copy numbers indicate the standard deviation of duplicate qPCR measurements on the same DNA extract. The detection limit of the qPCR analysis was $1 \times 10^4$ 16S rRNA gene copies mL$^{-1}$. Numbered lanes correspond to the ARDRA profiles of known organisms and identified OTUs as follows: 1, *Geobacter lovleyi* SZ; 2, Clostridium1 OTU; 3, Clostridium7 OTU; 4, Clostridiales1 OTU.
water samples (Fig. 3). The RFLP profiles for the sand samples of diffusion-cell BIO1, however, showed that *Geobacter* was dominant in the lower part of the sand layer (Fig. 4). This discrepancy between qPCR and RFLP results for the lower part of the sand layer of diffusion-cell BIO1 might be due to an artefact in the nested PCR of the RFLP analysis. However, for pore water samples, RFLP profiles agreed with the qPCR results. As such, the difference between *Geobacter* and bacterial numbers in the lower part of the sand layer of diffusion-cell BIO1 is likely explained by a less accurate qPCR quantification of DNA originating from sand samples. In the upper part of the sand layer, RFLP profiles for diffusion-cell BIO1 clearly corresponded with the ARDRA profiles of the Clostridium1 OTU, obtained from the formate-fed KB-1 subculture (Fig. 4). This contrasts with the RFLP profiles found for the formate- and lactate-fed diffusion-cell (Fig. 3a), demonstrating that variable communities were present in the upper sand layer part of the diffusion-cells.

**Discussion**

**Geobacter as dominant dechlorinator**

The OTUs corresponding with *G. lovleyi* SZ were the only OTUs in the 16S rRNA gene clone libraries of the formate- and lactate-fed KB-1 subcultures that could be related to known dechlorinating species (Table 1). This species was abundant in both KB-1 subcultures (Fig. 2) and also dominated in the lower part of the sand layer of the diffusion-cells (Figs 3 and 4). Duhamel & Edwards (2006) previously demonstrated that a bacterium related to *G. lovleyi* SZ was associated with TCE dechlorination in the KB-1 culture. They calculated that in their KB-1 subcultures, *Geobacter* was responsible for 80% of the conversion of TCE to cis-DCE, while the other 20% was attributed to *Dehalococcoides* species (Duhamel & Edwards, 2007). In the present study, none of the 16S rRNA gene clones corresponded with *Dehalococcoides* (Table 1, Fig. 1). Moreover, very low *Dehalococcoides* numbers were found in both KB-1 subcultures (Fig. 2). These results suggest that the conversion of TCE to cis-DCE in the KB-1 subcultures of the current study is performed by *Geobacter* only. This discrepancy with the earlier findings of Duhamel & Edwards (2007) could be due to differences in imposed growth conditions. Duhamel *et al.* (2002) stimulated the full conversion of TCE to ethene and amended only 0.15 mM TCE, whereas in this study only the conversion to cis-DCE was stimulated, and 1.6 mM TCE was used. As such, the outcompition of *Dehalococcoides* in the KB-1 subcultures of the current study could be due to a better adaptation to...
high TCE concentrations of *Geobacter* compared to *Dehalococcoides*. Alternatively, the *Dehalococcoides* species in the KB-1 culture might convert TCE only cometabolically and maybe use primarily cis-DCE and VC as electron acceptors. The latter hypothesis is supported by the observation of high *Dehalococcoides* numbers in KB-1 subcultures, grown under similar conditions as described for the formate-fed KB-1 subculture, but stimulated to dechlorinate till ethene (results not shown).

**Role of the Clostridium species**

Species related to *Clostridium* or *Clostridiales* were abundant in the 16S rRNA gene clone libraries of the formate- and lactate-fed KB-1 subcultures (Table 1). In addition, RFLP demonstrated the presence of *Clostridium* in both KB-1 subcultures (Fig. 2), as well as in the upper sand layer part of the lactate-fed diffusion-cell and the diffusion-cell BIO1 (Figs 3b and 4). *Clostridium* and related species are known for their fermenting diversity (Cato et al., 1986). In the KB-1 subcultures, these species potentially played an important role in converting the amended electron donors. Formate and lactate cannot directly support dechlorination by *G. lovleyi* SZ, which requires H₂, acetate or pyruvate as electron donor (Sung et al., 2006). In the KB-1 culture, however, acetate does not support dechlorination (Duhamel et al., 2002; Haest et al., 2011). Therefore, dechlorination in the examined KB-1 subcultures requires the conversion of formate or lactate likely to hydrogen. Lactate is a common growth substrate for fermentative *Clostridia* (Cato et al., 1986) and in the lactate-fed KB-1 subculture and diffusion-cells, the fermentation of lactate to H₂, acetate and bicarbonate (McCarty et al., 2007) likely provided *Geobacter* with hydrogen. In addition, formate is easily converted to H₂ and bicarbonate, but this dehydrogenation reaction rarely supports microbial growth (Ferry, 1990; Kim et al., 2010). As such, this conversion cannot explain the recorded dominance of *Clostridium* in the formate-fed KB-1 subculture and in some of the formate-fed diffusion-cells. Therefore, we hypothesize that the *Clostridium* species grew on the fermentation of yeast extract and cysteine, two constituents of the anaerobic medium which were found to serve as electron donors (J. Philips, unpublished results). This hypothesis is supported by the finding that RFLP profiles corresponding with *Clostridium* had a low intensity, if formate-fed diffusion-cells used anaerobic medium with strongly reduced yeast extract and cysteine concentrations (J. Philips, unpublished results). During the cultivation of the KB-1 subcultures, however, no additional doses of yeast extract and cysteine were applied. As such, the initial growth of *Clostridium* on yeast extract and cysteine was likely sufficient to support long-term conversion of formate. Moreover, *Clostridium* cells have on average nine 16S rRNA gene copies per cell (Klappenbach et al., 2001), and, as such, the lack of sustained growth does not explicitly disagree with the high number of *Clostridium* 16S rRNA gene clones obtained from the formate-fed KB-1 subculture (Fig. 2). Alternatively, the presence of *Clostridium* species in the KB-1 subcultures could be explained by acetogenesis, in which *Clostridium* produce acetate from hydrogen resulting from the formate dehydrogenation (Drake et al., 2008). *Clostridium* would then compete with *Geobacter* for H₂. However, none of the *Clostridium* OTUs were phylogenetically related to known acetogenic *Clostridia* (Table 1) (Drake et al., 2008). In addition, little to no acetate was produced in other formate-fed KB-1 subcultures, in which high H₂ concentrations were measured (L. Paul, unpublished results). The role of *Clostridium* in the examined KB-1 subcultures, however, remains uncertain, because neither acetate, nor formate, lactate or H₂ were monitored in the current study. As such, extended experiments are required to clarify the recorded dominance of *Clostridium*.

Several other studies previously identified *Clostridium* as dominant fermentative species in dechlorinating enrichment cultures (Fletcher et al., 2008; Ise et al., 2011). In contrast, Duhamel & Edwards (2006) did not identify any *Clostridium* species in their KB-1 subcultures, maybe because they used a different electron donor, that is, methanol, than in the current study. Alternatively, the presence of *Clostridium* species in the examined KB-1 subcultures, in contrast to those of Duhamel & Edwards (2006), could be due to the high TCE concentration amended in the current study. Bowman et al. (2009) demonstrated that many *Clostridium* species are tolerant to chlorinated solvents and are able to produce hydrogen even in the presence of PCE concentrations close to saturation. In addition, two studies on the microbial community in DNAPL source zones, where elevated chlorinated solvent concentrations are expected, showed the dominance of *Clostridium* (Macbeth et al., 2004) or of members of the order *Clostridiales* (Bowman et al., 2006).

In the diffusion-cells, differences in the apparent fermentative population were observed (Figs 3 and 4), which could not be related to the amended electron donor and did not affect the dechlorination rate. Freeborn et al. (2005) also found a constant dechlorination rate for subcultures differing in fermentative population, which for their study was related to different electron donors used. In addition, several other studies described fermentative communities, which were functionally stable despite their highly dynamical community composition (Fernandez et al., 1999, 2000; Carballa et al., 2011; Pycke et al., 2011).
Geobacter dominates adjacent to the TCE DNAPL

The qPCR and RFLP results for both pore water and sand samples showed two zones in the sand layer of all diffusion-cells. Apparently, fermentative species prevailed in the upper 2.5 cm of the sand layer, while the lower 3 cm was dominated by Geobacter (Figs 3 and 4). The position of Geobacter close to the TCE DNAPL resulted in a DNAPL dissolution enhancement of about 2.4 times compared to abiotic dissolution (Philips et al., 2011). Philips et al. (2011) discussed that TCE was likely degraded only between 1.5 and 3 cm distance from the DNAPL layer, as the maximum TCE concentration for dechlorination by the inoculated KB-1 subcultures was 2.5 mM. The current study, however, showed that at 0.5 cm of the DNAPL layer, the Geobacter copy numbers were several orders of magnitude higher than the inoculation density (Figs 3 and 4). The qPCR and RFLP analyses were performed on pore water samples taken at the first sampling of the diffusion-cells to ensure that high copy numbers in the vicinity of the DNAPL were not the result of displacement of Geobacter cells towards the DNAPL layer by earlier samplings. The high Geobacter cell densities adjacent to the DNAPL may reflect growth of Geobacter in the sand layer at TCE concentrations exceeding the toxicity threshold observed in aqueous systems (2.5 mM). Biofilm formation on the sand could have increased the resistance against TCE compared to that of planktonic cells (Harrison et al., 2007; Schaefer et al., 2010). However, close to the DNAPL layer, the TCE and cis-DCE concentration profiles were linear (Figs 3 and 4), suggesting that the conditions were abiotic in that part of the sand layer (Philips et al., 2011). In addition, the cis-DCE concentration was maximum around 2 cm of the DNAPL, demonstrating that the largest dechlorinating activity occurred in the zone of the sand layer where the TCE concentrations were below 2.5 mM (Figs 3 and 4). This suggests that the Geobacter cells at the DNAPL sand layer interface were inactive. Potentially, these inactive Geobacter cells resulted from growth before the local toxic TCE concentrations were attained in the vicinity of the DNAPL layer. Alternatively, the presence of Geobacter at the interface of the DNAPL and sand layer could be due to migration, as G. lovleyi SZ exhibits flagellar motility (Sung et al., 2006). The Geobacter cells in the studied KB-1 subcultures are not chemotactically oriented towards or away from a TCE DNAPL (J. Philips, unpublished results). As such, random migration maybe explains the presence of Geobacter adjacent to the DNAPL layer.

The upper part of the sand layer in the diffusion-cells was dominated by other species including Clostridium (Figs 3 and 4). Concentrations of formate and lactate were not measured in the diffusion-cells of the current study. However, other diffusion-cell experiments, including the experiment of Philips et al. (2011), demonstrated that the pore water formate and lactate concentrations were below the detection limit throughout the sand layer. As the electron donor was not rate limiting (Philips et al., 2011), the amended electron donors were likely rapidly converted and, subsequently, the generated H₂ likely diffused towards the dechlorination zone, where it served as direct electron donor. The qPCR and RFLP analyses performed in the present study suggest that the conversion of the amended electron donors occurred in the whole upper part of the sand layer and not only in the top layer, as was suggested by Philips et al. (2011). No methane was produced in the formate- and lactate-fed diffusion-cells of the current study, probably because the elevated chloroethene concentrations in the diffusion-cells inhibited methanogenesis (Carr et al., 2000; Yang & McCarty, 2000).

The Geobacter and bacterial 16S rRNA gene copy numbers in the sand samples of diffusion-cell BIO1 (Fig. 4) were higher than those in the pore water samples of the formate-fed diffusion-cell at corresponding distances from the DNAPL layer (Fig. 3a). This finding could be due to microbial attachment to the sand. Amos et al. (2009) previously reported that G. lovleyi SZ was associated with the solid phase in a source zone of PCE, whereas Haest et al. (2011) did not find significantly different Geobacter cell densities in pore water and sand samples of a flow-through column. However, the quantification of copy numbers originating from sand samples was potentially inaccurate in the current study. Therefore, it is unclear whether the difference between cell densities in the pore water and the sand samples was attributed to attachment to the sand or to an incorrect qPCR analysis for the sand samples.

Conclusion

This study demonstrated that the composition of a TCE-dechlorinating community was related to the distance from a TCE DNAPL. Geobacter dechlorinators dominated in the lower sand layer part of the diffusion-cells (model aquifers), where TCE was present and became dechlorinated. High Geobacter cell densities were even present adjacent to TCE DNAPL, although the TCE toxicity likely inactivated those Geobacter cells. Apparent fermentative Clostridia prevailed in the upper sand layer part, which bordered to the top layer providing electron donor. The close association of Geobacter with the TCE DNAPL explains the complete dechlorination of TCE to cis-DCE at less than 3.0 cm distance of the DNAPL, which resulted in a significant DNAPL dissolution.
enhancement (factor 2.4 in comparison with abiotic dissolution). Consequently, these results demonstrate the high potential of source zone bioremediation.

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