

Frequent concomitant presence of *Desulfitobacterium* spp. and “*Dehalococcoides*” spp. in chloroethene-dechlorinating microbial communities

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Abstract The presence of chloroethene dechlorination activity as well as several bacterial genera containing mainly organohalide-respiring members was investigated in 34 environmental samples from 18 different sites. Cultures inoculated with these environmental samples on tetrachloroethene and amended weekly with a seven organic electron donor mixture resulted in 11 enrichments with *cis*-DCE, ten with VC, and 11 with ethene as dechlorination end product, and only two where no dechlorination was observed. “*Dehalococcoides*” spp. and *Desulfitobacterium* spp. were detected in the majority of the environmental samples independently of the dechlorination end product formed. The concomitant presence of *Dehalococcoides* spp. and *Desulfitobacterium* spp. in the majority of the enrichments suggested that chloroethene dechlorination was probably the result of catalysis by at least two organohalide-respiring genera either in parallel or by stepwise catalysis. A more detailed study of one enrichment on *cis*-DCE suggested that in this culture *Desulfitobacterium* spp. as well as *Dehalococcoides* spp. dechlorinated *cis*-DCE whereas dechlorination of VC was only catalyzed by the latter.

Keywords Organohalide respiration · Dehalorespiration · Halorespiration · Reductive dechlorination · Bioremediation

Introduction

Since decades chloroethenes, such as tetrachloroethene (PCE) and trichloroethene (TCE), are produced in large quantities worldwide and as a result of inappropriate handling or storage, they are very frequently encountered as contaminants in groundwater, soils, and sediments (McCarty 1997; Fetzner 1998; Comber and Gardner 1999). Under anaerobic conditions, these compounds can be reductively dechlorinated via dichloroethene (DCE) and vinyl chloride (VC) to harmless ethene by organohalide-respiring bacteria for which chloroethenes act as electron acceptors in the energy metabolism (Holliger et al. 1999). While *cis*-1,2-DCE has been mainly reported as dechlorination intermediate, some studies have also shown that *trans*-DCE is sometimes formed (Gerritse et al. 1999; Griffin et al. 2004; Miller et al. 2005; Cheng et al. 2010).

The question was, after isolation of several organohalide-respiring bacteria with different metabolic properties, whether these bacteria are also relevant in the environment where natural attenuation of chloroethenes occurs. A survey including samples from the United States of America as well as Europe has shown that “*Dehalococcoides*” spp. seemed to be widespread and often detected where ethene formation has been observed (Hendrickson et al. 2002). Another study has shown that the genus *Desulfitobacterium* could be detected at many different locations in Quebec, Canada (Lanthier et al. 2001). Other organohalide-respiring bacteria such as *G. lovleyi* and *Dehalobacter* spp. have also been observed in groundwater systems and enrichments cultures thereof (Davis et al. 2002; Grostern and Edwards 2006; Amos et

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al. 2007; Daprato et al. 2007; Nijenhuis et al. 2007; Dowideit et al. 2010).

The goal of this study was to evaluate the presence of bacterial genera such as *Desulfitobacterium*, *Dehalobacter*, and *Dehalococcoides* among which most isolates are organohalide-respiring bacteria in numerous environmental samples and enrichment cultures thereof and to link the presence or absence of these genera with the dechlorination end product formed.

Material and methods

Chemicals All chemicals were analytical grade and used without purification. PCE (99%) and n-hexadecane (99%) were purchased from Acros Organics, Geel, Belgium. *Cis*-1,2-dichloroethene (*cis*-DCE) (97%) was obtained from Sigma-Aldrich, Chemie GmbH, Steinheim, Germany. VC (99.5%) and ethene (99.95%) were procured from Fluka AG, Buchs, Switzerland. All gasses (N₂, CO₂, H₂) were purchased from SLGas, Sauerstoffwerk Lenzburg, Switzerland. Due to the possible presence of inhibiting compounds in synthetic *cis*-DCE, also biogenic *cis*-DCE was used prepared as described by Maymo-Gatell et al. (2001), however *Desulfitobacterium hafniense* strain TCE1 was used here as PCE-dechlorinating bacterium.

Source of inocula and enrichment cultures The 34 inocula used in this study were from 18 different sites and comprised the following: 19 wet aquifer samples, eight groundwater samples, two sludge samples from bioreactors treating chlorinated compound-contaminated waters, and five non-contaminated samples (one mix of different digester sludge from municipal wastewater treatment plants, two river and two lake sediment samples) (see Online Resource 1—Table S1).

The enrichment cultures in 100 or 500 ml serum bottles (VWR international AG, Merck, Dietikon, Switzerland) containing 50 or 250 ml of anaerobic medium were performed with a two-liquid phase system that has been described previously (Holliger et al. 1998). In an anaerobic glove box (Anaerobic System model 1,024, Forma Scientific, Brouwer, Luzern, Switzerland), the 100-ml bottles containing freshly prepared anaerobic mineral medium were inoculated by the addition of approximately 1 g of solid material or 10 ml liquid (water or sludge), depending on the inoculum. Oxygen was removed from the anaerobic glove box with R-20 palladium catalyst provided by BASF, Wädenswil, Switzerland. The bottles were closed in the glove box and the gas phase of the bottles was aseptically replaced with a N₂/CO₂ mixture (4:1, v/v, 1.0 bar over pressure) using a gas exchange system (Druva Sonderventie GmbH, Eppelheim, German)

and sterile hydrophobic 0.2 μm filters (Sarstedt AG, Sevelen, Switzerland). PCE, TCE, and DCE were dissolved in hexadecane at a concentration of 100, 80, and 50 mM, respectively. The hexadecane solutions were added to serum bottles that were sealed with Viton rubber stoppers. After changing the gas phase with pure nitrogen, the hexadecane solutions were sterilized by heat (121 °C, 20 min). The volumes added to inoculated culture bottles by syringe were (per 50 ml of medium); PCE 5 ml, TCE 6 ml, DCE 10 ml. Vinyl chloride was injected as pure gas directly to the culture bottles in amounts of approximately 1 mmol (25 ml) per 50 ml of medium. These latter cultures did not contain any hexadecane.

Electron donors were added once a week from 50 times concentrated anoxic stock solutions. A mixture of seven electron donors (acetate-formate-propionate-butyrate-ethanol-lactate-pyruvate) was mainly used, each added at a concentration of 0.285 mM resulting in a total concentration of 2 mM total electron donor per week. For the *cis*-DCE dechlorinating AQ1 culture, an electron donor mixture containing propionate, butyrate and ethanol (0.67 mM each) was used. The enrichment cultures were incubated stationary at 30 °C in the dark.

Analytical methods The dechlorination activity of the enrichments was followed by measuring the concentration of chloride in the aqueous phase by silver ion titration with a Chlor-o-counter (Flohr Instrument, Nieuwegein, Netherlands). Chloroethenes and methane were analyzed by gas chromatography as previously described (Maillard et al. 2003). The volatile fatty acids were analyzed using a HPLC (Varian Star 9,100, Varian AG, Zug, Switzerland) with a refractive index detector (ERC-7,415 A, Varian). Samples of 1.2 ml were deproteinated by adding 150 μl Ba(OH)₂ 0.15 M and 150 μl ZnSO₄ 0.15 M, centrifuging 10 min at 11,000×g, and filtering with a 0.2-μm filter (Sarstedt, Sevelen, Switzerland). The HPLC was equipped with a guard column connected to an organic acid column maintained at 60 °C (ORH-801, InterAction INC, Varian). The solvent was 50 mM H₂SO₄ with a flow rate of 0.61 ml/min.

DNA extraction The Kit UltraClean™ Soil DNA Isolation from MoBio Laboratories, Inc. (BIOzym, Landgraaf, Netherlands) was used for the DNA extraction. Aliquots of 0.5 g of soil and aquifer and 10 ml of environmental water sample, and 15 ml of the enrichment cultures were extracted. Liquid samples were centrifuged at 29,000×g for 5 min at 4 °C, the pellet resuspended in 300 μl 0.1 M sodium phosphate buffer (pH 8.0), and the suspension transferred in the extraction tubes provided by MoBio. Bead beating was performed two times 30 s at 1,000 rpm with a Mikrodismembrator S

(B. Braun Biotech International GmbH, Melsungen, Germany). The DNA was extracted according to the supplier's protocol and recovered finally in 50- μ l samples.

End-point PCR detection and quantitative real-time PCR Specific primers (Online Resource 1—Table S2) used for the detection of known PCE-dechlorinating bacterial species and genera were designed together with people from Bioclear BV (Groningen, Netherlands) in the framework of the EU FP5 project MAROC. Primers for the detection of *bvcA* and *vcrA* genes were taken from (Behrens et al. 2008). All primers were purchased at Microsynth AG (Balgach, Switzerland). Amplification reactions were performed with a thermocycler T3 (Biometra, Biolabo, Châtel-St-Denis, Switzerland). The 10 μ l PCR mixture contained 1 \times PCR Buffer B, 0.2 mM dNTPs, 0.5 μ M of each primer, 0.1 μ l (5 U/ μ l) of Taq polymerase (Promega, Catalys AG, Wallisellen, Switzerland), and 1 μ l of the extracted DNA. The following thermocycling program was used for all primer sets except for *Desulfitobacterium* spp.; 94 °C for 3 min (one cycle); 94 °C for 30 s, annealing temperature (Table S2) for 30 s, 72 °C for 1 min 30 s (25 of 35 cycles); 72 °C for 10 min (one cycle). For *Desulfitobacterium* spp., the elongation was only 30 s instead of 1 min 30 s. For DNA isolated from the inocula, the PCR comprised 35 cycles whereas only 25 cycles were used for enrichment DNA.

Quantitative real-time PCR was carried out as described previously with the primers specifically developed for this purpose (Smits et al. 2004). Here, the 16 S rRNA gene of *Desulfitobacterium* spp. and *Dehalococcoides* spp. were targeted.

T-RFLP analysis The 16 S rRNA genes of the microbial communities present in the samples were amplified with general bacterial primers Eub 8 F-HexTM fluorescently labeled at the 5' position and Eub 534R (Online Resource 1—Table S2). Two tubes of 50 μ l PCR mixture for each sample contained 1 \times PCR buffer B (Promega), 0.2 mM dNTPs, 0.24 μ M of each primers (forward and reverse primers), 0.4 μ l (5 U/ μ l) of Taq polymerase, and 2 ng of the extracted DNA. The following thermocycling program was used; 94 °C for 5 min (one cycle); 94 °C for 30 s, 56 °C for 45 s, 72 °C for 2 min (35 cycles); 72 °C for 10 min (one cycle). The two tubes of each sample were pooled, purified with the QIAquick PCR purification Kit (Qiagen AG, Basel, Switzerland) and resuspended in 30 μ l. The purified PCR products were quantified with a fluorometer (TD 700 Tuner Designs, Witec AG, Littau, Switzerland) using the PicoGreen kit (PicoGreen[®] dsDNA quantification Kit, Molecular Probes Europe B.V., Leiden, Netherlands) and 50 ng were digested with the enzyme *Hae*III (Promega) during 4 h at 37 °C. An aliquot of 1 μ l of the digested

product was prepared according to the supplier ABI (Applied Biosystems, Rotkreuz, Switzerland) and loaded in the ABI Prism[®] 3,100 capillary array (50 cm) and ABI Prism[®] 3,100 POP-6TM polymer. The results were analyzed with the package ABI Prism[®] 3,100 GeneScan Software. Each peak of the electropherogram represented a molecular operational taxonomic unit (OTU) which can originate from one strain, a species, different members of a genus, or any other unit of classification. The results were analyzed with the online software TAP T-RFLP from Ribosomal Database Project II (Maidak et al. 1999). To estimate the relative abundance of an OTU, its peak area was expressed as the percentile of the total of all peak areas of the electropherogram.

Cloning and sequencing The detail procedure is described in the supplementary material (Online Resource 1). Briefly, PCR products targeting bacterial 16 S rRNA genes were purified, ligated into pGEM-T Easy vector (Promega), and transformed into competent *Escherichia coli* DH5 α cells by heat shock. Colony PCR was done with T7 and SP6 promoter primers. Clones containing DNA fragments of expected length were sequenced with T7 and SP6 promoter primers using the BigDye Terminator v3.0 Ready Reaction Kit (ABI Applied Biosystems) according to the manufacturer's instructions. Samples were run on the ABI Prism 3,100 Genetic Analyzer.

Sequences were analyzed with the software package Lasergene (DNASTAR, Inc, Madison, WI, USA) and aligned with ClustalX (Thompson et al. 1994). The phylogenetic trees were designed using the software package MEGA4 (Tamura et al. 2007). The sequences were compared with databases using the BLAST software (Altschul et al. 1990).

Nucleotide sequences The obtained sequences for the AQ1 clones were submitted to Genbank with the following reference numbers: HM63539–HM635368.

Results

Presence of known organohalide-respiring bacteria in numerous inocula and cultures thereof In the framework of different projects, a total of 34 environmental samples were collected from 18 different sites. The majority of the samples originated from sites contaminated with chlorinated compounds (Online Resource 1—Table S1). The environmental samples were used as inocula for enrichment cultures amended with PCE as electron acceptor and a substrate mixture composed of seven organic compounds (acetate-formate-propionate-butyrate-

ethanol-lactate-pyruvate) that was added once a week. Chloride production started in all cultures quite rapidly without considerable lag phases (Fig. 1). PCE dechlorination to *cis*-DCE was typically achieved after 7–9 weeks of incubation and no formation of *trans*-DCE was observed. Chloride continued to be formed in cultures producing VC and ethene but at lower rates (Fig. 1). However, formation of VC and ethene was not observed in all enrichments. In 11 cultures, *cis*-DCE was the dechlorination end product, VC in another ten, and in 11 it was ethene (Table 1). Only in two cultures no dechlorination was observed.

End-point PCR used for the detection of genera *Dehalococcoides*, *Desulfitobacterium*, and *Dehalobacter* showed the presence of *Dehalococcoides* in 23 inocula, *Desulfitobacterium* in 21, and *Dehalobacter* only in seven (Table 1). *Dehalococcoides* was also detected in most enrichments, even in those where dechlorination did not go beyond *cis*-DCE, but was below the detection limit in three enrichments where ethene was formed (Table 1). *Desulfitobacterium* was detected in all enrichments but one where dechlorination occurred whereas *Dehalobacter* was only detected in ten (Table 1).

Analysis of an enrichment culture dechlorinating DCE to ethene One of the cultures obtained from the inoculum AQ1 with *cis*-DCE as electron acceptor instead of PCE was analyzed in more detail. It was fed with a propionate-butyrate-ethanol electron donor mixture and transferred approximately once in every 6–7 weeks.

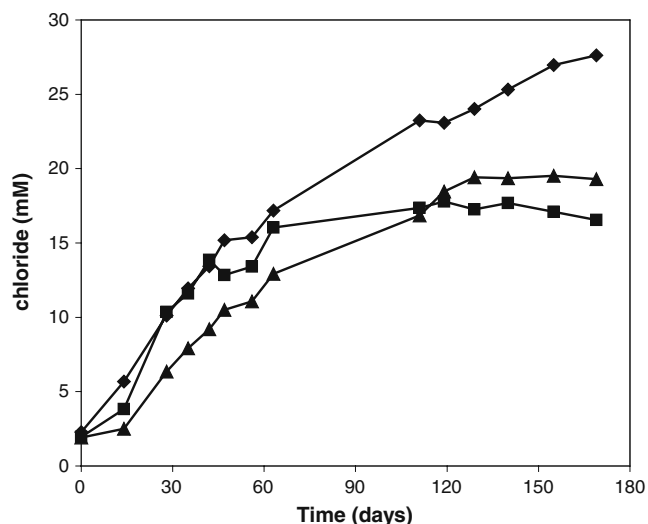


Fig. 1 Chloride production in cultures inoculated with different environmental samples amended with a seven electron donor mixture once a week and with hexadecane containing 100 mM PCE (nominal PCE concentration in water phase ca. 8 mM), and producing different dechlorination end products. Culture from sample GW3 (filled square) producing DCE, from sample GW5 (filled triangle) producing VC, and from sample AQ5 (filled diamond) producing ethene

After more than ten transfers and up-scaling from 50 to 250 ml cultures, cultures were inoculated containing either PCE or *cis*-DCE as chlorinated electron acceptor. *Cis*-DCE was dechlorinated in a two-step process with an initial accumulation of VC and no formation of ethene until almost all *cis*-DCE was consumed followed by the dechlorination of VC to ethene (Fig. 2). Ethanol was completely consumed between two weekly electron donor additions whereas propionate and butyrate were only partially consumed. Hydrogen and acetate balances indicated that all hydrogen formed upon syntrophic electron donor oxidation was consumed by reductive dechlorination and methanogenesis and that no or almost no homoacetogenesis took place in this enrichment culture (Online Resource 1—Fig. S1). PCE was also dechlorinated by this culture line but slowly, and the formed *cis*-DCE was further dechlorinated to VC and some ethene although large amounts of PCE were still present (Online Resource 1—Fig. S2).

Cloning and sequencing of bacterial 16 S rRNA genes amplified from DNA isolated from the initial AQ1 culture on the propionate-butyrate-ethanol mixture and *cis*-DCE, showed the presence of *Desulfitobacterium* spp. and *Dehalococcoides* spp. in this microbial consortium. Twelve of the 30 clones classified with the bacterial genus *Dehalococcoides* and five with *Desulfitobacterium* spp. (Online Resource 1—Fig. S3). The closest relatives of the *Dehalococcoides*-affiliated clones were the strains GT, CBDB1, FL2, and BAV1 with 99% sequence identity. The closest relative to the *Desulfitobacterium*-affiliated clones was the first 16 S rRNA gene copy of strain DCB-2 (Dhaf_R0006, 98% identity), with all of these five clones corresponding to the shortest version of *Desulfitobacterium* 16 S rRNA genes (Online Resource 1—Fig. S3). The other clones affiliated with *Wolinella* spp. (seven clones, 100%), *Azovibrio* spp. (two clones, 100%), or *Bacteroidetes* (one clone, 100%). Three additional clones were only distantly related to *Treponema* spp. (75%).

The analysis of samples taken during 6 weeks of incubation suggested that *cis*-DCE was dechlorinated by *Desulfitobacterium* spp. and *Dehalococcoides* spp. whereas VC was dechlorinated by *Dehalococcoides* spp. (Fig. 2). On successive transfers, *Desulfitobacterium* spp. was systematically and abundantly present during the first 2 weeks of incubation whereas *Dehalococcoides* dominated the consortium after 4–6 weeks of incubation.

Discussion

The concomitant presence of *Dehalococcoides* spp. and *Desulfitobacterium* spp. in the majority of the samples

Table 1 End-point PCR detection of *Dehalococcoides*, *Desulfitobacterium*, and *Dehalobacter* in inocula and enrichments thereof amended with a seven electron donor mixture added weekly and containing PCE dissolved in hexadecane

End product ^a	Site ^b	Sample ^b	<i>Dehalococcoides</i>		<i>Desulfitobacterium</i>		<i>Dehalobacter</i>	
			Inoculum	Enrichment	Inoculum	Enrichment	Inoculum	Enrichment
DCE	S3	GW1	X	X	X	X	X	
	S4	AQ8	X	X	X	X		X
	S4	GW2	X	X	X	X		
	S5	AQ9	X	X	X	X		
	S5	GW3	X		X	X		
	S7	GW6	X	X	X	X	X	
	S7	GW7	X	X	X	X		
	S13	AQ14	X		X	X	X	X
	S14	AQ16		X		X		
	S14	AQ17		X	X	X		
	S14	AQ18				X		
	No. of samples	11	8	8	9	11	3	2
VC	S1	AQ3	X	X		X		
	S3	AQ6	X			X		
	S4	AQ4	X	X	X	X		
	S5	GW4	X	X	X	X		
	S6	GW5	X	X	X	X		
	S7	AQ12	X	X	X	X		
	S10	SL1	X	X	X	X		X
	S15	RS1		X		X		
	S14	AQ19	X	X	X	X		X
	S18	LS2		X		X	X	X
	No. of samples	10	8	9	6	10	1	3
Ethene	S1	AQ1	X	X	X	X		X
	S2	AQ2		X		X		
	S3	AQ5	X			X	X	
	S4	AQ7				X		
	S6	AQ10	X	X		X		
	S7	AQ11	X		X	X		
	S9	AQ13	X	X	X	X		X
	S8	GW8	X	X	X	X	X	X
	S11	SL2	X	X	X	X	X	X
	S12	SL3		X				
	S14	AQ15		X	X	X		X
	No. of samples	11	7	8	6	10	3	5

^a End product observed in enrichment culture after several months of incubation

^b Information about the sites and samples is given in Online Resource ×Table S1

indicated that either both populations dechlorinated chloroethenes in parallel or that there was a stepwise catalysis where one population dechlorinated the higher chlorinated ethenes (PCE, TCE) and the other the lower chlorinated ones (DCE, VC). Several studies have presented evidence that PCE and TCE dechlorination to ethene has been the result of the activity of different populations successively catalyzing the different dechlorination steps, either by

multiple *Dehalococcoides* spp. populations with different metabolic properties (Duhamel et al. 2002; Waller et al. 2005; Holmes et al. 200; Behrens et al. 2008) or by populations of two or three different genera (Yang et al. 2005; Grostern and Edwards 2006; Amos et al. 2009). In analogy, it is postulated that in the numerous enrichments obtained in this study the dechlorination of PCE was catalyzed first by *Desulfitobacterium* spp. followed by

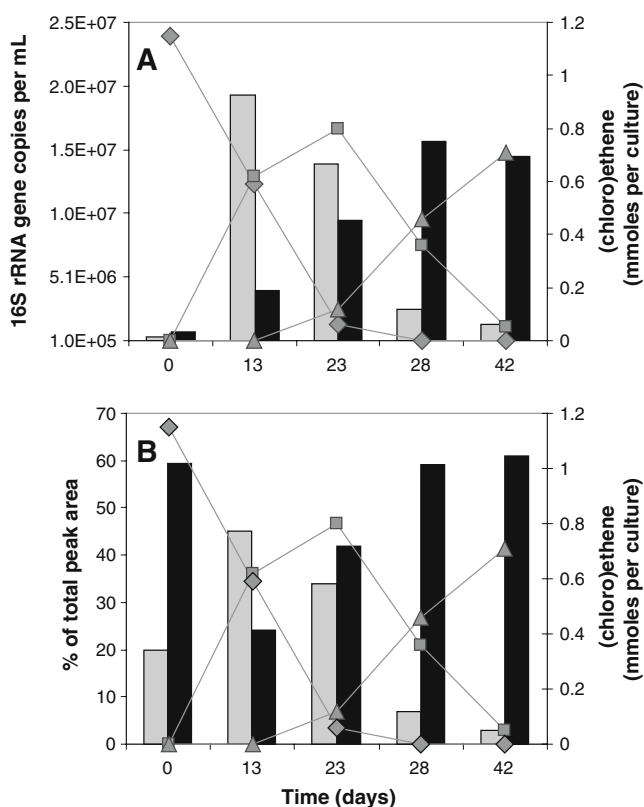


Fig. 2 Number of 16 S rRNA gene copies per mL of culture as determined by qPCR (a) and relative population sizes as determined by T-RFLP (b) of *Desulfitobacterium* spp. and *Dehalococcoides* spp. in an AQ1 enrichment culture dechlorinating *cis*-DCE to ethene with propionate-butyrate-ethanol as electron donors. For *Dehalococcoides* spp. the relative peak area of T-RF 168 is depicted, for *Desulfitobacterium* spp. the peak area represents the sum of the peaks of T-RF 212 and T-RF 329. Gray bars, *Desulfitobacterium* spp.; black bars, *Dehalococcoides* spp.; filled diamond, *cis*-DCE; filled square, VC; filled triangle, ethene

dechlorination of the lower chlorinated ethenes catalyzed by *Dehalococcoides* spp. However, dechlorination by *Desulfitobacterium* spp. was perhaps not only restricted to PCE and TCE.

In all but one enrichment cultures where dechlorination was observed, *Desulfitobacterium* spp. was detected as very abundant population. This genus was also detected in more than 50% of the inoculum samples that originated from different European countries (Germany, The Netherlands, and Switzerland). The possible ubiquity of this bacterial genus has already been suggested in a study of soil samples from the Canadian province of Quebec (Lanthier et al. 2001) and presence of *Desulfitobacterium* spp. in chloroethene-dechlorinating microbial communities has also been reported in several other studies (Davis et al. 2002; Yang et al. 2005; Yoshida et al. 2007; Dowideit et al. 2010).

Besides *Desulfitobacterium* spp., mainly *Dehalococcoides* spp. was detected in the enrichment cultures on PCE independently of the dechlorination end product observed.

The first survey on the presence of *Dehalococcoides* spp. at chloroethene-contaminated sites has shown that this bacterial genus is widely distributed and that it has not been detected in samples from sites at which only incomplete dechlorination occurred (Hendrickson et al. 2002). A link between complete chloroethene dechlorination and the presence of *Dehalococcoides* spp. has also been presented in numerous other studies investigating enrichment cultures and field sites (Duhamel et al. 2002; Freeborn et al. 2005; Yang et al. 2005; Grostern and Edwards 2006; Holmes et al. 2006; Daprato et al. 2007; Lee et al. 2008; Dowideit et al. 2010). In many enrichment cultures of the present study where *Dehalococcoides* spp. was detected in the inoculation material, dechlorination was incomplete and stopped at DCE or VC. This could be explained by the fact that in several *Dehalococcoides* spp.-containing consortia different strains of this bacterial genus seemed to be present with different metabolic properties (Duhamel et al. 2002; Waller et al. 2005; Holmes et al. 2006; Daprato et al. 2007; Lee et al. 2008). Hence, it seems that the mere detection of the presence of *Dehalococcoides* spp. in a microbial community does not guarantee that dechlorination of chloroethenes will be complete.

In three of the 11 enrichment cultures producing ethene, *Dehalococcoides* spp. was not detected and also not in two inocula. This suggested that there are other bacteria not affiliated with this genus that are capable to dechlorinate chloroethene to ethene. The identification of a novel group of bacteria within the phylum *Chloroflexi* as responsible organism for PCE dechlorination (Kittelmann and Friedrich 2008) and of *Dehalogenimonas lykanthroporepellens* as novel genus and species using polychlorinated alkanes as electron acceptor (Moe et al. 2009) indicates that the phylum *Chloroflexi* might contain other yet unidentified organohalide-respiring bacteria producing ethene from chloroethenes. Another possible explanation for not detecting *Dehalococcoides* spp. is, however, that members of this genus were present but not detected by the specific primers used.

The analysis of the AQ1 enrichment culture on *cis*-1,2-DCE suggested based on relative abundances of *Desulfitobacterium* spp. and *Dehalococcoides* spp. during dechlorination that *cis*-DCE was first reduced to VC by both genera, followed by VC dechlorination by the latter population. This was rather surprising since all described isolates of the genus *Desulfitobacterium* that dechlorinate PCE and/or TCE produce exclusively *cis*-DCE (Smid and de Vos 2004; Villemur et al. 2006). Moreover and regarding the *Dehalococcoides* spp. population, the VC reductive dehalogenase genes *vcrA* and *bvcA* could not be detected in this enrichment (data not shown). Attempts to isolate the different populations present in the AQ1 enrichment on *cis*-DCE did unfortunately fail so far but will be continued.

Future efforts will in addition concentrate on the identification of the reductive dehalogenase genes involved in complete chloroethene dechlorination by this enrichment.

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