Dehalogenation of diverse halogenated substrates by a highly enriched Dehalococcoides-containing culture derived from the contaminated mega-site in Bitterfeld

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Abstract
An enrichment culture dominated by one type of Dehalococcoides sp. (83% of clones) was characterised. This culture, originally derived from contaminated groundwater from the area of Bitterfeld-Wolfen (Saxony-Anhalt, Germany), dehalogenates chlorinated ethenes to ethene. Further, the culture also dehalogenated vinyl bromide (VB) and 1,2-dichloroethane (DCA) to ethene, 1,2,3,4- and 1,2,3,5-tetrachlorobenzene (TeCB), penta- and hexachlorobenzene (PeCB and HCB) to trichlorobenzenes (TCB), lindane to monochlorobenzene (MCB) and pentachlorophenol (PCP) to 2,3,4,6-tetrachlorophenol (TeCP). Growth was proven by quantitative PCR for all active cultures, except for those with TeCB, lindane and PCP. The growth yields obtained ranged from \((2.9 \pm 0.7) \times 10^7 \text{ cells mol}^{-1} \text{Br}^{-}\) released on VB to \((34.8 \pm 5.4) \times 10^7 \text{ cells mol}^{-1} \text{Cl}^{-}\) released on VC. Genes coding for nine putative reductive dehalogenases, the enzymes that mediate the respiratory process of dehalogenation, were identified. Phylogenetic analysis revealed eight reductive dehalogenases with similar sequences in other Dehalococcoides strains and one unique sequence.

Introduction
Halogenated organic compounds have been and are extensively used in industry and agriculture. They serve as solvents, degreasers, agents, biocides, intermediates for chemical synthesis and have functions in many other applications and, as a result of their usage, are common groundwater contaminants (McCarty, 2010). Under anoxic conditions, microbial reductive dehalogenation of halogenated solvents has been found to be an important means of removal from the environment (Holliger et al., 2002; Smidt & de Vos, 2004; Taş et al., 2009). Field studies have so far shown the importance of the genus Dehalococcoides, capable of the dehalogenation of groundwater contaminants such as the chlorinated ethenes, in bioremediation applications (Ellis et al., 2000; Major et al., 2002).

However, to date, the substrate range has been investigated for only a few Dehalococcoides strains (Taş et al., 2009; Löffler et al., in press). Chlorinated ethenes are utilised by several identified Dehalococcoides strains leading mainly (but not exclusively) to the nontoxic end product ethene. In some strains, not all of the catalysed dechlorination steps yield energy. For example, Dehalococcoides mccartyi strain 195 (formerly D. ethenogenes) dechlorinates tetrachloroethene (PCE) to vinyl chloride (VC) in a relatively fast metabolic
manner, while the dehalogenation from VC to ethene is significantly slower and cometabolic (Maymo-Gatell et al., 1999; Löfler et al., in press). Chlorobenzenes, chlorophenols, chlorinated dioxins and some other halogenated substrates have been found to be reductively dechlorinated by strains 195 and CBDB1 (Adrian et al., 2000; Bunge et al., 2003; Fennell et al., 2004; Adrian et al., 2007b). Other \textit{D. mccartyi} sp. strains, such as strain BAV1 and VS, were capable of growing with dehalogenation of VC to ethene (He et al., 2003; Müller et al., 2004).

A further means to explore the bioremediation potential of specific strains is their molecular characterisation. In this context, the reductive dehalogenases, the enzymes catalysing reductive dehalogenation, are of special interest also as the different \textit{Dehalococcoides} sp. were found to contain up to 36 copies of putative reductive dehalogenase genes. The genome of \textit{D. mccartyi} strain 195 contains 17 reductive dehalogenase homologous (\textit{rdh}) genes (Seshadri et al., 2005), whereas in strain CBDB1, 32 different \textit{rdh} genes have been found (Kube et al., 2005). Strain VS even contained 36 \textit{rdh} genes, while in strain BAV1, only 11 \textit{rdh} genes were detected (McMurdie et al., 2009). Currently, only a few of the \textit{rdh} genes are characterised for their function. In \textit{D. mccartyi} strain 195 a tetrachloroethene reductive dehalogenase (\textit{pceA}) and a trichloroethene reductive dehalogenase (\textit{tceA}) were found and their substrate ranges characterised (Magnuson et al., 1998, 2000; Fung et al., 2007). In strain VS (Müller et al., 2004) and BAV1 (Krajmalnik-Brown et al., 2004), vinyl chloride reductive dehalogenases (\textit{vcrA} and \textit{bvcA}, respectively) were identified. A chlorobenzene reductive dehalogenase (\textit{cbrA}) was identified in strain CBDB1 (Adrian et al., 2007a). The \textit{rdh} loci in the genomes are typically composed of two genes, \textit{rdhA} and \textit{rdhB}. While the protein encoded by \textit{rdhA} contributes to the enzymatic function, \textit{rdhB} encodes a small hydrophobic protein that serves most likely as a membrane anchor (Kube et al., 2005). Previously, an enrichment culture dominated by \textit{Dehalococcoides} originating from Bitterfeld capable of the dehalogenation of PCE to ethene was investigated (Cichocka et al., 2010). Only minor (< 5%) populations related to \textit{Sulfurospirillum}, another putative PCE to cis-DCE dehalogenating organism, could be observed in only a subset of cultures. Growth of the \textit{Dehalococcoides} ribotype was coupled to PCE, 1,1-dichloroethene (1,1-DCE), cis-DCE and vinyl chloride (VC) dehalogenation (Cichocka et al., 2010). Hydrogen and acetate were used as electron donor and carbon source, respectively.

In this study, the substrate range of the \textit{Dehalococcoides}-dominated enrichment culture was investigated and growth of \textit{Dehalococcoides} was tested. Additionally, the diversity of the community and \textit{rdh} genes present in this \textit{Dehalococcoides}-dominated culture was assessed.

### Materials and methods

#### Chemicals

Chemicals were purchased from Sigma-Aldrich Chemie [including Fluka, Supelco and Riedel de Haën (Seelze, Germany)], Merck (Darmstadt, Germany), or Linde Gas AG (Pullach, Germany) at the highest purity available. Gases were purchased from Airproducts (Hattingen, Germany). Monochlorobenzene (MCB), trichlorobenzene (TCB), 1,2,3,5-tetrachlorobenzene (TeCB), hexachlorobenzene (HCB), pentachlorophenol (PCP), 1,1,2-trichloroethane (TCA) and 1,1,2,2-tetrachloroethane (TeCA) were obtained from Fluka; 1,2-dichlorobenzene (DCB) and 1,4-DCB from Supelco; 1,3-DCB, 1,2,3,4-TeCB, 1,2,4,5-TeCB, 2,3-dichlorophenol (DCP), 1,2-dichloroethane (DCA), 2,4-dichlorotoluene (DCT) and PCE from Merck; pentachlorobenzene (PeCB) and 1,2-dichloropropane (D) from Riedel de Haën; vinyl bromide (VB) from Aldrich; and VC from Linde (see also Supporting Information, Table S1).

#### Growth medium and culture conditions

Cultures were prepared as described previously in Cichocka et al. (2010) in 50-mL glass bottles, filled with 25 mL of medium (Zinder, 1998) with a headspace of 70% N\textsubscript{2} and 30% CO\textsubscript{2}, closed with a Teflon\textsuperscript{®}-lined grey butyl rubber stopper and crimped. All vials were amended with NaHCO\textsubscript{3} (1 g L\textsuperscript{-1}), Na\textsubscript{2}S (25 mg L\textsuperscript{-1}) and vitamins (Zinder, 1998). Electron acceptors were added either as neat solution, gas, solid salt crystals or dissolved in acetone as indicated in Table S1.

For each compound, five vials were prepared, three inoculated and two negative, abiotic controls. Triplicate cultures were inoculated with 1 mL (4% v/v) of the \textit{Dehalococcoides}-dominated enrichment culture described in Cichocka et al. (2010) grown with PCE as the electron acceptor. The original culture used as inoculum was analysed for its actual microbial diversity (see below). Additionally, three vials were inoculated with the \textit{Dehalococcoides} enrichment culture but did not receive any electron acceptor to serve as a reference for the determination of growth compared to sets amended with the halogenated electron acceptors. H\textsubscript{2} was added as an overpressure (c. 50 kPa). Vials were incubated upside-down at 20 °C in the dark without shaking.

#### Analytical methods

For analysis of chlorinated ethenes, vinyl bromide, chloroethanes and 1,2-dichloropropane, 0.5 mL headspace samples were taken and analysed as described in Cichocka et al. (2010) using a gas chromatograph (Varian Chrompack...
The programme used was as follows: initial temperature of 40 °C for 5 min, increased with 6 °C min⁻¹ to 225 °C, held for 2.5 min. The FID was operated at 250 °C, and helium was used as carrier gas (0.69 × 10⁵ Pa; 11.5 mL min⁻¹). This method allowed the separation of ethene, vinyl chloride, 1,1-, trans- and cis-DCE, trichloroethene (TCE) and PCE. The sampling was automated using an HP 7694 headspace autosampler (Hewlett Packard, Palo Alto, CA), adding 0.5 mL headspace samples to 10 mL autosampler vials flushed with helium, which were closed with a Teflon®-coated butyl rubber septum and crimped.

For the analysis of mono- to trichlorobenzenes and 2,4-dichlorotoluene, 0.1 mL liquid culture samples were taken and added to 10 mL vials containing 0.1 mL of a saturated Na₂SO₄ solution (pH 1, H₂SO₄) to inhibit bacterial activity. Samples were analysed using gas chromatography (Agilent 6890; Agilent Technologies, Palo Alto, CA) with flame ionisation detection and an Rtx-VMS column (Restek, Bad Homburg, Germany) with a length of 30 m, an inner diameter of 0.25 mm and a film thickness of 1.4 μm. The temperature programme was as follows: initial temperature of 35 °C for 2 min, increased with 4 °C min⁻¹ to a temperature of 60 °C and with 20 °C min⁻¹ to a final temperature of 225 °C and held for 1 min. Helium was used as carrier gas with an initial pressure of 137 kPa, held for 3 min and then increased to 237 kPa with 8 kPa min⁻¹ and held for 2 min.

The liquid sampling for tetra- to hexachlorobenzenes and lindane was done as described for lower chlorinated benzenes, and 0.1 mL liquid samples were added to 10 mL sampling vials containing 0.1 mL of a saturated Na₂SO₄ solution (pH 1, H₂SO₄). Solid phase microextraction (SPME) was used for the extraction of higher chlorinated benzenes and lindane from the gas phase of the sampling vials. A 65 μm polydimethylsiloxane–divinylbenzene (PDMS-DVB, Sigma-Aldrich, Steinheim, Germany)-coated fibre was exposed to the gas phase within the sampling vial for 45 min at room temperature without shaking. The injection into the GC inlet was done manually. Desorption occurred at 250 °C for 5 min. The samples were analysed using gas chromatography and mass spectroscopy (GC-MS) (Agilent 7890A GC System, Agilent 5975C Series GC/MSD) equipped with a HP-5 ms column (Agilent J&W) with a length of 30 m, an inner diameter of 0.25 mm and a film thickness of 0.25 μm. The temperature programme used was as follows: initial temperature of 40 °C for 5 min, increased with 6 °C min⁻¹ to a temperature of 200 °C, then with 20 °C min⁻¹ to a final temperature of 300 °C and held for 5 min. Helium was used as carrier gas with a pressure of 38 kPa.

For the analysis of chlorinated phenols, 85 μm polycrylate (PA)-coated SPME fibres (Sigma-Aldrich, Steinheim, Germany) were selected. For extraction, the fibre was immersed in the sample. The extraction time was 3 h at ambient temperature without shaking. The needle was injected into the GC inlet manually, and the fibre was exposed for desorption for 5 min at 280 °C. GC-MS, column, temperature programme and carrier gas were the same as described above for higher chlorinated benzenes and lindane.

**DNA extraction, PCR amplification and quantitative real-time PCR**

From the original culture inoculum and triplicate bottles of each treatment, bacterial cells were harvested (different electron acceptors) in duplicate (1 mL each) by centrifugation at 16 100 g for 30 min. DNA was extracted from the cell pellet with the DNeasy Tissue kit (QIAGEN, Hilden, Germany) following the manufacturer’s instructions for Gram-positive cells and was eluted in 50 μL elution buffer provided in the kit. Reductive dehalogenase homologous genes were then amplified with the forward primer RR2 (5′-SHMGBMGWGATTYYATGAARR-3′) and the reverse primer B1R (5′-CHADHAGCCAYTCR TACCA-3′) (Krajmalnik-Brown et al., 2004). The detailed composition of the PCR and PCR temperature programme used is described in Tables S2 and S3. PCR products were purified using the QIAquick PCR Purification kit (QIAGEN). 16S rRNA gene copies of *Dehalococcoides* were quantified for each subsample in triplicate by qPCR using StepOnePlus™ Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) and SensiMix™ Low_ROX kit (Bioline, Luckenwalde, Germany) following the manufacturer’s instructions (Bioline, Luckenwalde, Germany) with *Dehalococcoides*-specific primers 539f (5′-AGGAAGCAAGCGTTATCC-3′) and 731r (5′-GACAACCTAGAAAACCGC-3′) (Ewald et al., 2007). The reaction mixture was prepared according to the SensiMix Low ROX kit manual including the following modifications. Each 12.5 μL reaction contained 6.25 μL 2× SensiMix, 1.5 μL ROX (final conc. 300 nM), 3 μL molecular grade water and 10 pmol of each primer, and 1 μL DNA served as template. The thermocycler programme applied is shown in Table S4. The qPCRs were performed in 96-well microtiter plates (0.2 mL volume) in triplicate. 16S rRNA gene copy numbers were quantified by comparison with a 16S rRNA gene standard curve (10⁵–10⁷ copies mL⁻¹) using previously cloned 16S rRNA gene amplicons of *Dehalococcoides* strain DCMB5 (Bunge et al., 2008) as template (for details, see Supporting Information). Negative controls (without DNA) and samples from treatments without electron acceptor addition were added in each run. The significance of bacterial growth was confirmed by performing the t-test. The probability was
calculated using the mean of cell number of each treatment (triplicate samples of each duplicate subsample) and compared to the control sets. Significance of growth was considered confirmed at a \( P < 0.05 \). The respective treatment was not considered to have grown significantly if the cell number did not increase, or a \( P \geq 0.05 \) was obtained.

The growth yield in active treatments was subsequently calculated using the increase in 16S rRNA gene copies relative to the treatments without electron acceptor addition and normalised to the concentration of substrate dehalogenated and product formation expressed as halogen \((\text{Cl}^-, \text{Br}^-)\) released.

**Cloning and screening of the clones**

LB medium and plates with ampicillin, IPTG and X-gal were prepared as described in the technical manual for pGEM-T and pGEM-T Easy Vector Systems (Promega, Mannheim, Germany). Ligation and transformation of the PCR-amplified \( rdh \) genes was done according to the protocol, with incubation of the ligation reaction at 4 °C overnight, followed by transformation to \( E. coli \) JM109 High Efficiency Competent Cells (Promega). The cloned \( rdh \) genes were re-amplified from DNA isolated from single clones using the same reaction mixture, primers and temperature programme as described above.

The restriction digestion of purified PCR products from clones was done with the restriction enzymes HindIII (Fermentas) and BsuRI (Fermentas) in separate reactions according to the manufacturer’s protocol to obtain the characteristic restriction pattern, which was visualised in a 1.6% agarose gel after electrophoretic separation and staining with ethidium bromide.

**Sequencing and primer design**

The sequencing reactions were performed using the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and an ABI PRISM 3130 × 1 Genetic Analyser (Applied Biosystems). To obtain a full sequence of the gene loci, the sequencing was performed from both ends of the \( rdh \) genes with the PCR primers RRF2 and B1R and with additional internal primers designed in this study (Table 1). The obtained sequences were assembled, and the different \( rdh \) genes were aligned with MEGA version 4 (Tamura et al., 2007). They were compared to published sequences with the Nucleotide Basic Local Alignment Search Tool [BLASTN, (Altschul et al., 1997)] and database from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). MEGA version 4 was also used to generate phylogenetic trees (neighbour-joining) from the newly identified putative reductive dehalogenases and known sequences showing highest similarity on the BLASTN search results. The determined \( rdh \) gene sequences were submitted to the EMBL database under the accession numbers HE659349 to HE659357.

| Table 1. Sequencing primers designed for this study |
|---------------------------------|----------------|----------------|----------------|
| **Sequence**                     | **Length (bases)** | **GC content (%)** | **Basic melting temperature (°C)** |
| **Forward primers**              |                 |                 |                             |
| Dhc_seqA_01                      | AAGAGGCTTCAGTATGCTCAGG | 23              | 52  | 57  |
| Dhc_seqB_01                      | TACTCGGAGGCCCGGATATAAAG | 22              | 55  | 57  |
| Dhc_seqC_01                      | GGTCCTCGAGTATTTGGCA | 21              | 52  | 54  |
| Dhc_seqC_03                      | GCAGGTTCGAGATTTGGCAG | 21              | 52  | 54  |
| Dhc_seqD_01                      | GGCAACAGCAGATTGGTCATTG  | 23              | 48  | 55  |
| Dhc_seqE_01                      | TCGAAGATGCTGCTATGCA | 22              | 55  | 57  |
| Dhc_seqF_01                      | CTACATTTACTCCTGCTTG  | 21              | 52  | 54  |
| Dhc_seqH_01                      | CCTGACTATGTTGGCCGACA | 21              | 52  | 54  |
| Dhc_seqL_01                      | ATGGGAACCTCGCCTTCACTT | 21              | 57  | 56  |
| **Reverse primers**             |                 |                 |                             |
| Dhc_seqA_02                      | TTCTGCTAGGCAGGAAATTCCAT | 22              | 55  | 57  |
| Dhc_seqB_02                      | TTGGCCGAGCGCGCATATTCCATA | 22              | 55  | 57  |
| Dhc_seqC_02                      | AGAATACGCGCTATCCTCCA | 21              | 52  | 54  |
| Dhc_seqD_02                      | CAGTCTGAGCGGAAAGCTTTT | 22              | 55  | 57  |
| Dhc_seqE_02                      | ATACAGTGTTGCCGGCGCG | 22              | 55  | 57  |
| Dhc_seqF_02                      | AGAAGTCGCCACCAATACCGCA | 22              | 55  | 57  |
| Dhc_seqF_04                      | AAATGCGGTATTGGTTGTCG | 21              | 52  | 54  |
| Dhc_seqH_02                      | TGCTCAAAGGACACGCTGTGT | 21              | 57  | 56  |
| Dhc_seqL_02                      | GCTGCTGAGATTCCCAACATT | 21              | 52  | 54  |
| Dhc_seqN_02                      | GGACATTCATCGGACACTT | 21              | 52  | 54  |
16S rRNA gene clone library
The diversity of the Dehalococcoides highly enriched culture BTF08 was investigated by establishing a 16S rRNA gene clone library. PCR, cloning of the PCR products and screening of the clones by amplified ribosomal DNA restriction analysis (ARDRA) were performed as described by Cichocka et al. (2010). The primer 27f, 1378r and 907r were used to obtain almost the entire sequence of the 16S rRNA gene. Examination of phylogenetic relationships and taxonomic assignments at a confidence threshold of 80% was performed using the naïve Bayesian rRNA Classifier (Version 2.2) and the Sequence Match tools of the RDP-II (release 10, http://rdp.cme.msu.edu/), respectively (Wang et al., 2007). 16S rRNA gene sequences were deposited in EMBL public database under the accession numbers from HE652867 to HE652876.

Results
Substrate range
To test the substrate range of the enrichment culture, parallel sets were prepared to test the ability to dehalogenate chlorinated benzenes, lindane, di- or pentachlorophenol, 1,2-DCA, 1,2,3,4-TeCB, 1,2-D, 2,4-DCT and VB. During cultivation, the increase in cell number of Dehalococcoides spp. was monitored. As positive controls, sets with PCE or VC were prepared. PCE and VC were completely dechlorinated to ethene as shown previously by Cichocka et al. (2010), and between 820 and 1750 µM, ethene was produced within 187 days (Fig. S1 for VC). Further substrates for dehalogenation were found to be VB, 1,2-DCA, 1,2,3,4-TeCB, 1,2,3,5-TeCB, PeCB, HCB, lindane and PCP. Several doses of VB were dehalogenated to ethene, but considerably slower than VC (Fig. 1a; Fig. S1). The triplicate cultures produced about 250 µM ethene during the incubation of 187 days. 1,2-DCA (Fig. 1b) was dehalogenated completely to ethene. The first dose of 1,2-DCA (about 500 µM) was completely depleted after about 100 days of incubation in the triplicate cultures. Subsequent doses of 1,2-DCA were dehalogenated faster in all sets. 1,2-DCA was always fully dehalogenated when sampling (the shortest interval being 10 days). At the same time, ethene was produced. The average final concentration of ethene (day 209) of the triplicate cultures was 2200 µM (Fig. 1b).

All higher chlorinated benzenes except 1,2,4,5-TeCB were dehalogenated to lower chlorinated benzenes. HCB (Fig. 1c) and PeCB were dehalogenated to 1,2,3,5-TeCB, which was further dehalogenated to 1,3,5-TCB. During cultivation, traces of 1,2,3,4-TeCB and 1,2,4-TCB could be detected in the PeCB and HCB treatments. 1,2,4-TCB was detected in all three cultures amended with 1,2,3,4-TeCB, and 1,2,3,5-TeCB was dehalogenated to 1,3,5-TCB (Fig. 2; Table 2).

Lindane transformation (Fig. 1d) to tetrachlorocyclohexene (TeCCH) was initially presumed to be abiotic, because TeCCH was detected in the triplicate bottles and both negative controls in low amounts. However, after 134 days of incubation, relatively high amounts of MCB, up to 30% of the added lindane (Fig. 1d), was detected in the triplicate cultures but not in the negative controls. Pentachlorophenol was dechlorinated to one of the tetrachlorophenol isomers, identified as 2,3,4,6-tetrachlorophenol. However, transformation was slow. To avoid toxic concentrations, PCP was added to the bottles at a...
concentration of 5 µM (Adrian et al., 2007 a, 2007 b) and only re-fed once after consumption of the first dose. No transformation was observed for 1,1,2-TCA, 1,1,2,2-TeCA, 1,2-D, mono-, di- and trichlorobenzenes, 1,2,4,5-TeCB, 2,3-DCP and 2,4-DCT after 4 months of incubation.

Growth of Dehalococcoides

Cell numbers of Dehalococcoides were determined in all cultures that showed dehalogenation activity by quantitative real-time PCR (qPCR) using Dehalococcoides-specific primers targeting the 16S rRNA gene. The active cultures were compared to control sets (mean controls: 2.1 ± 0.6 × 10⁷ 16S rRNA gene copies) that were inoculated with the bacteria but not amended with any electron acceptor. The cell numbers obtained are assumed to correspond to the 16S rRNA gene copy number as, so far investigated, Dehalococcoides possess only one copy of the 16S rRNA gene in their genome (McMurdie et al., 2009; Tas et al., 2009). Growth was significant in treatments containing the substrates PCE, VC, VB, 1,2-DCA, HCB and PeCB (Table 2). Although degradation products were found for sets amended with 1,2,3,4-TeCB and 1,2,3,5-TeCB, no significant increase in the gene copy number was observed, except in one bottle. However, growth was not significant (1,2,3,5-TeCB_2: 0.1 ± 0.2 × 10⁷ 16S rRNA gene copies). No significant copy number increase was observed for the triplicate cultures degrading lindane, although MCB was present as the main product. Finally, the number of produced 16S rRNA gene copies and the growth yields based on produced 16S rRNA gene copies per released chlorine or bromide were determined for PCE, VC, VB, 1,2-DCA, PeCB and lindane cultures (Table 2). Halogen (Cl⁻ or Br⁻) release was calculated based on the amount of daughter product formed or on the complete consumption of nominally added substrate.

The obtained growth yields for cultures grown on VC, VB and PCE, with 19.1 ± 12.5, 16.2 ± 11.8 and 16.5 ± 5.4 × 10⁷ 16S rRNA gene copies µmol⁻¹ halogen released, respectively, were in the same range (Table 2). Similarly, the growth yield obtained with 1,2-DCA was 11.7 ± 7.2 × 10⁷ 16S rRNA gene copies µmol⁻¹ Cl⁻, and incubation with PeCB and HCB as electron acceptor led to a growth yield of 11.4 ± 9.6 and 11.6 ± 5.0 × 10⁷ 16S rRNA gene copies µmol⁻¹ Cl⁻ released, respectively.

Microbial diversity of the enrichment culture

A clone library was prepared to investigate the diversity of the microbial community in the original enrichment culture BTF08. One hundred and three clones containing the correct size insert were obtained. One sequence proved to be a chimera and was excluded from further analysis. The analysis revealed that 83% of the 16S rRNA gene clones were derived from the genus Dehalococcoides. Seven percent of the sequences were classified to the order Bacteroidales forming the second largest group after Dehalococcoides. Five sequences were classified as representing the genus Sulfurospirillum, while four sequences were related to the family Spirohaetaceae (Table S5). Many of the analysed sequences proved to be identical or showed high similarity to sequences obtained from the previous transfers of the BTF culture (Cichocka et al., 2010).
Table 2. Dehalogenation products, incubation time, growth and growth yields of Dehalococcoides in active treatments provided with halogenated substrates. (± SD). Transient products are shown in brackets.

<table>
<thead>
<tr>
<th>Dehalogenating culture</th>
<th>Dehalogenation products</th>
<th>Incubation time (days)</th>
<th>Calculated Cl⁻ (Br⁻) released (mM)</th>
<th>16S rRNA gene copies produced (×10⁷ mL⁻¹)</th>
<th>Growth yield [16S rRNA gene copies x 10⁷ mol⁻¹ Cl⁻ (Br⁻) released]</th>
<th>Active cultures (significance of growth)*</th>
<th>Mean of growth yield [16S rRNA gene copies x 10⁷ mol⁻¹ Cl⁻ (Br⁻) released]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCE_1</td>
<td>Ethene</td>
<td>137</td>
<td>2.04</td>
<td>47.0 ± 10.6</td>
<td>23.1 ± 5.2</td>
<td>+</td>
<td>16.5 ± 5.4</td>
</tr>
<tr>
<td>PCE_2</td>
<td>Ethene</td>
<td>137</td>
<td>1.6</td>
<td>22.8 ± 5.1</td>
<td>14.2 ± 3.2</td>
<td>+</td>
<td>19.1 ± 12.5</td>
</tr>
<tr>
<td>PCE_3</td>
<td>Ethene</td>
<td>129</td>
<td>4.8</td>
<td>65.3 ± 10.8</td>
<td>13.6 ± 2.2</td>
<td>+</td>
<td>19.1 ± 12.5</td>
</tr>
<tr>
<td>VC_1</td>
<td>Ethene</td>
<td>129</td>
<td>0.99</td>
<td>7.8 ± 2.4</td>
<td>7.9 ± 2.4</td>
<td>+</td>
<td>19.1 ± 12.5</td>
</tr>
<tr>
<td>VC_2</td>
<td>Ethene</td>
<td>129</td>
<td>1.07</td>
<td>11.5 ± 12.9</td>
<td>10.7 ± 2.9</td>
<td>+</td>
<td>19.1 ± 12.5</td>
</tr>
<tr>
<td>VC_3</td>
<td>Ethene</td>
<td>104</td>
<td>0.99</td>
<td>34.5 ± 5.3</td>
<td>34.8 ± 5.4</td>
<td>+</td>
<td>19.1 ± 12.5</td>
</tr>
<tr>
<td>VB_1</td>
<td>Ethene</td>
<td>129</td>
<td>0.12</td>
<td>2.6 ± 1.4</td>
<td>21.3 ± 11.9</td>
<td>+</td>
<td>19.1 ± 12.5</td>
</tr>
<tr>
<td>VB_2</td>
<td>Ethene</td>
<td>129</td>
<td>0.20</td>
<td>0.6 ± 1.5</td>
<td>2.9 ± 0.7</td>
<td>+</td>
<td>19.1 ± 12.5</td>
</tr>
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<td>VB_3</td>
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<td>5.7 ± 0.6</td>
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<td>1,2-DCA_1</td>
<td>Ethene</td>
<td>132</td>
<td>2.06</td>
<td>14.0 ± 7.4</td>
<td>6.8 ± 3.6</td>
<td>+</td>
<td>19.1 ± 12.5</td>
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<tr>
<td>1,2-DCA_2</td>
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<td>1.76</td>
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<td>18.3 ± 4.4</td>
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<td>1,2-DCA_3</td>
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<td>10.9 ± 8.1</td>
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<td>1,2,4-TCB</td>
<td>315</td>
<td>–</td>
<td>n.q.†</td>
<td>n.c.‡</td>
<td>–</td>
<td>n.c.</td>
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<tr>
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<td>0.12</td>
<td>n.q.</td>
<td>n.c.</td>
<td>–</td>
<td>n.c.</td>
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<td>315</td>
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<td>0.1 ± 0.2</td>
<td>13.6 ± 3.5</td>
<td>+</td>
<td>19.1 ± 12.5</td>
</tr>
<tr>
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<td>1,3,5-TCB</td>
<td>315</td>
<td>–</td>
<td>n.q.</td>
<td>0.4 ± 0.4</td>
<td>–</td>
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<td>315</td>
<td>0.571</td>
<td>5.4 ± 1.1</td>
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<td>[1,2,3,4-TeCB, 1,2,4-TCB]</td>
<td>5.1</td>
<td>5 ± 1.5</td>
<td>8.7 ± 2.7</td>
<td>9.3 ± 14.1</td>
<td>+</td>
<td>19.1 ± 12.5</td>
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<td>Lindane_1</td>
<td>[TeCCH], MCB</td>
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<td>0.204</td>
<td>0.09 ± 0.4</td>
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<tr>
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</tbody>
</table>

*Active cultures: with P < 0.05 significant (+) and P > 0.05 not significant (−) growth. Growth yield was calculated per bottle if treatment showed significant growth.
†n.q. = not possible to quantify (≤ 16S rRNA gene copy number compared to controls).
‡n.c. = not possible to calculate.
§Mean of PeCB1 and 2.
Molecular screening of the rdh genes

To analyse the diversity of rdh genes present in the enrichment culture, the reductive dehalogenase homologous genes were PCR amplified using the degenerate primer set described by Krajmalnik-Brown (Krajmalnik-Brown et al., 2004) and cloned. A total of 90 clones were screened by restriction fragment length polymorphism (RFLP), and 13 different patterns among the 90 rdh clones could be distinguished using two restriction enzymes. Where available, at least two samples from each group were entirely sequenced. All sequences contained a nearly complete open reading frame for rdhA (length of 1350–1450 bp), missing only the first 2–40 nucleotides at the 5’ end of rdhA. The sequenced parts of rdhB were between 30 and 90 nucleotides long. Sequencing revealed that five of the thirteen pattern groups shared about 99.9% sequence similarity. The putative reductive dehalogenases were named RdhA1-9, combining the five almost identical sequences as RdhA1.

A phylogenetic tree was generated to show the evolutionary relationship of all newly identified reductive dehalogenase homologous genes and their closest relatives determined with BLAST (Fig. 3). Additionally, the characterised reductive dehalogenases cbrA (chlorobenzene reductive dehalogenase, named RdhA84 in GenBank and identified in strain CBDB1) (Adrian et al., 2007a), tceA (trichloroethene reductive dehalogenase, identified in strain 195) (Magnuson et al., 2000), bvCA (vinyl chloride reductive dehalogenase, identified in strain BAV1) (Krajmalnik-Brown et al., 2004) and vcrA (vinyl chloride reductive dehalogenase, identified in strain VS) (Müller et al., 2004) were included in the tree to demonstrate

![Fig. 3. Evolutionary relationships of the identified reductive dehalogenases from the enrichment culture BTF08 (highlighted with black diamond), their closest relatives determined with BLAST and characterised reductive dehalogenases (highlighted with grey square) cbrA, tceA, bvCA and vcrA were also included. Accession numbers (GenBank) are given in brackets. For their alignment, the amino acid sequence was used. The evolutionary history was inferred using the neighbour-joining method (Saitou & Nei, 1987). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl & Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the data set (Complete deletion option). There were a total of 338 positions in the final data set. Phylogenetic analyses were conducted in MEGA 4. The scale bar represents 10% sequence divergence.](https://academic.oup.com/femsec/article-abstract/83/1/176/466462/311766684642)
their position within the evolutionary relationship. For the alignment, the amino acid sequences from open reading frame A (RdhA) were used. RdhA1, 2 and 4–9 are closely related to published sequences. However, none of the newly identified reductive dehalogenase genes were closely related to cbrA, tceA, bvcA or vcrA. With RdhA3 and the partial RdhB3, a new putative reductive dehalogenase and its anchoring protein have been discovered; their sequence is not closely related to any sequences deposited in the EMBL public sequence databases. The closest match is a putative reductive dehalogenase from *D. maccartyi* strain 195. Only about a third of the *rdh3* sequence (513 bases) shows similarities to this gene with 67% identities and 4% gaps within this section.

**Discussion**

The results from the 16S rRNA gene clone library showed that the original culture used in this study was more diverse than previous transfers described in Cichocka *et al.* (2010). Clone libraries established from former transfers indicated a purity of more than 98% (Cichocka *et al.*, 2010). However, 16S rRNA gene copy numbers cannot directly be correlated to cell numbers, as the number of 16S rRNA gene copies is not equal in all bacteria. Ribosomal genes in prokaryotes average three or four per genome with the highest known copy number of 15 (Klappenbach *et al.*, 2001; Tourova, 2003). So far investigated, *Dehalococcoides* possesses only one 16S rRNA gene copy in their genome (Kube *et al.*, 2005; McMurdie *et al.*, 2009; Taş *et al.*, 2009), and if other bacteria present in the enrichment culture had several copies in their genome, still more than 83% of the community might belong to the *Dehalococcoides*. Furthermore, preferential amplification of sequences from different phylogenetic groups might occur due to minor differences in the annealing sites of the primer (Klappenbach *et al.*, 2001; Baker *et al.*, 2003), so that the frequency of detected 16S rRNA gene sequences might deviate from the true population composition. Moreover, preferential ligation during the TA-cloning is reported to contribute to the bias in microbial community structure analysis (Palatinzsky *et al.*, 2011). Nevertheless, owing to fact that the same approach (same PCR parameters, cloning approach) was used as in the previous study, we can assume certain community dynamics of the enrichment cultures between transfers. Similar to the study of Cichocka (Cichocka *et al.*, 2010), sequences representing minority populations were classified to the order Bacteroidales, the family Spirochaeta and genus *Sulfurospirillum*. *Sulfurospirillum halorespirans* and *S. multivorans* are known organohalide-respiring microorganisms, and their 16S rRNA gene sequences showed 98% similarity to the ones obtained from our enrichment culture. Clone sequences affiliated to the family *Spirohaetaceae* (99% similarity to our sequence deposited under accession number HE652867) have been recently found in PCE-degrading cultures (Ziv-El *et al.*, 2011). Sequences affiliated with the order Bacteroidales (HE652874) were mainly related to sequences obtained from biogas plants (Liu *et al.*, 2009; Riviere *et al.*, 2009).

Members of the group Bacteroidales and *Spirrochaeta* probably provide fermentation products *H*₂ and acetate, which can be utilised by *Dehalococcoides* as electron and carbon source, respectively (Ziv-El *et al.*, 2011). *Sulfurospirillum* species in our culture may contribute to some extent to the dehalogenation activity; however, thus far, *Sulfurospirillum* have not been shown to use halogenated substrates other than PCE and TCE, with the end product cis–DCE, for growth (Luijten *et al.*, 2003).

The encoded amino acid sequence of all identified *rdh* genes contained, typical for other dehalogenases, two iron-sulphur cluster-binding (ISB) motifs in the C-terminal region (Hölscher *et al.*, 2004). ISB motifs are characteristic of bacterial ferredoxins, ubiquitous electron transport proteins (Bruschi & Guerlesquin, 1988). The first ISB motif of all sequences corresponded to the conserved consensus sequence CXXCXXCXXCP. Variations of this pattern were observed in the second ISB motif. Hölscher *et al.* (2004) divided the *rdh* genes, identified in their study, into two clusters. In cluster I, the second ISB motif shared either the consensus CXXCXXCXXCP (this is the case for RdhA6 and RdhA8 obtained in this study) or the first two cysteine residues of the second ISB motif were separated by three, four or six residues instead of two. In *rdh* genes of cluster I with four or six residues between the first two cysteines of the second ISB, a fifth cysteine residue was present upstream of the second ISB motif. This was observed for RdhA4 and RdhA5 (Fig. 4). As Hölscher *et al.* (2004) stated, the second ISB motif was characterised by a long stretch of residues (8–21 amino acids) separating the first two cysteine residues in all *Dehalococcoides* *rdh* genes outside of cluster I. In RdhA1, 3, 7 and 9 obtained from the enrichment culture BTF08, ten residues separated the first two cysteine residues. In RdhA2, the first two cysteine residues of the second ISB motif were separated by 21 residues. The high similarity to earlier described *rdh* genes and the retrieval of the conserved regions characteristic for reductive dehalogenases verifies that all determined sequences are indeed candidates for reductive dehalogenases. Our study only used one broad-range specific primer set for amplification of our target gene, and the diversity of reductive dehalogenase genes may be higher in the enrichment culture. A separate study indicated the presence of *vcrA* (but not *tceA* or *bvcA*) genes in our enrichment culture (Mészáros E, Imfeld G, Nikolausz M & Nijenhuis I, 2012 Federation of European Microbiological Societies Published by Blackwell Publishing Ltd. All rights reserved
Characterisation of the BTF08 enrichment culture

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made with care and may depend on the equipment and qPCR is difficult to achieve, these comparisons are to be yields. However, an absolute quantification by means of Dehalococcoides different reductive dehalogenases might be responsible for the observed VC dehalogenation. In summary, we identified several substrates for the Dehalococcoides-dominated enrichment culture and found varying growth yields ranging from 0.32 ± 0.04 to 3.35 ± 0.32 × 10^7 16S rRNA gene copies μmol⁻¹ Cl⁻ released, for cultures amended with chlorinated ethenes. As the enrichment culture investigated in our study is not pure, other organisms present in the culture might account for some dechlorination activity (e.g. Sulfurospirillum sp.). If part of the Cl⁻ released was not produced by the Dehalococcoides present in the culture, the growth yield for Dehalococcoides might be underestimated. Furthermore, different reductive dehalogenases might be responsible for the dechlorination of the same substrate in the diverse Dehalococcoides strains, which may explain variable growth yields. However, an absolute quantification by means of qPCR is difficult to achieve, these comparisons are to be made with care and may depend on the equipment and samples used (Becker et al., 2000). The growth yield obtained with 1,2-DCA (11.7 ± 7.2 × 10^7 16S rRNA gene copies μmol⁻¹ Cl⁻) corresponded well to the value obtained by Duhamel et al. (2002) from the Dehalococcoides population within a mixed culture (16 ± 8 × 10^7 16S rRNA gene copies μmol⁻¹ Cl⁻).

In addition to the chlorinated ethenes, the Dehalococcoides in the enrichment culture were capable of growing with chlorinated benzenes, VB and 1,2-DCA. Similar to D. mccartyi strain 195, the culture dechlorinated 1,2-DCA to ethene and produced TCB as end product of the dechlorination of chlorobenzenes (Maymo-Gatell et al., 1999; Fennell et al., 2004) unlike D. mccartyi. Strains CBDB1 and DCMB5 that produce DCB as end product of PCP did not dehalogenate PCP, and strain CBDB1 produced lower chlorinated phenols (Adrian et al., 2000; Bunge et al., 2008). PCP was dehalogenated, however, to TeCP, while D. mccartyi strain 195 did not dehalogenate PCP, and strain CBDB1 produced lower chlorinated phenols (Adrian et al., 2007b). Novel substrates for Dehalococcoides identified in this study were lindane and VB. Dehalogenation of lindane to chlorobenzene was described previously for anaerobic bacteria from marine sediments and for sulphate-reducing bacteria (Boyle et al., 1999). Furthermore, we confirmed that also D. mccartyi strain 195 could dehalogenate lindane to MCB (unpublished results), suggesting that lindane may be an additional substrate for other Dehalococcoides spp. as well.

In future, identification of further substrates for dehalogenation by Dehalococcoides should aid to identify the substrate specificity for the different dehalogenase enzymes present. In combination with the sequencing of multiple Dehalococcoides spp. genomes, a correlation of activity and genes present may be possible. This investigation did not identify a possible TCE dehalogenase (TceA); however, the vcrA gene identified by Mészáros et al. (unpublished data) may be responsible for the observed VC dehalogenation.

In summary, we identified several substrates for the Dehalococcoides ribotype BTF08 in our enrichment culture derived from Bitterfeld. This includes novel identified substrates such as VB and lindane, suggesting that Dehalococcoides spp. may be highly flexible in their metabolism in the environment and may be able to use a variety of alternative electron acceptors in parallel. Therefore, Dehalococcoides spp. may play a major role in the in situ conversion of the pesticides such as lindane as well as brominated substances such as the brominated flame retardants in the environment. First
investigations indicated the presence of *Dehalococcoides* ribotype BTF08 in Bitterfeld (Nijenhuis *et al.* 2007, Mészáros *et al.*, unpublished data), which suggests that these organisms play an important role in dehalogenation of the organohalide contamination present in this field site and should be taken into account for future remediation approaches as the stimulation of these organisms may already improve the removal of contaminants.

### Acknowledgements

We kindly acknowledge Paula Martinez, Petra Bombach, Lorenz Adrian, Jan Pieter Haest, Kerstin Ethen and Ines Mäusezahl for helpful discussion and assistance in the laboratory. We gratefully thank Ute Lechner and Anke Wagner for kindly providing the clone containing the 16S rRNA gene of *Dehalococcoides* sp. strain DCMB5 as qPCR standards. M.S. was funded from the EU FP7 project Genesis (contract number: 226536). This project was financially supported by the Helmholtz Centre for Environmental Research – UFZ.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Reductive dehalogenation of VC in triplicate bottles. Average and standard deviation of concentration analysis is shown.

**Table S1.** Concentrations of electron acceptors and acetone in the different treatments.

**Table S2.** Composition of PCR mix for the amplification of the reductive dehalogenase genes.

**Table S3.** Thermocycler program: reductive dehalogenase genes.

**Table S4.** Thermocycler program for the qPCR analysis of the *Dehalococcoides* specific BTF08 16S rRNA gene.

**Table S5.** Sequencing results of representative 16rRNA gene clones.

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