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**Assessment of microbial transformation of chlorinated  
ethenes by compound specific isotope analysis and  
cultivation techniques**

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# **Assessment of microbial transformation of chlorinated ethenes by compound specific isotope analysis and cultivation techniques**

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## **THESIS**

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Dr. rer. nat.

submitted

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## ABSTRACT

Two issues related to the assessment of chlorinated ethenes biodegradation at field sites by means of Compound Specific Isotope Analysis (CSIA) are addressed: 1) stable isotope fractionation upon microbial reductive dechlorination and 2) investigation and enrichment of environmentally relevant microorganisms. The stable carbon isotope fractionation upon reductive dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE) by several microbial strains was shown to be highly variable. TCE fractionation by crude extracts of *S. multivorans*, *S. halorespirans* and *Desulfitobacterium* sp. strain PCE-S, purified enzyme and the abiotic reaction with cyanocobalamin was examined. In contrast to previous results with PCE, no rate limitations due to uptake or transport of the substrate to the reactive site were observed for TCE. Furthermore, the enrichment procedure of a *Dehalococcoides*-containing culture dechlorinating PCE to ethene from contaminated mega-site in Bitterfeld is described.

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# 1

## General introduction



### 1.1 Chlorinated ethenes

Chlorinated ethenes constitute an important group of groundwater contaminants. In the last decades they have been widely used in many industrial branches. Careless handling during production, transportation and application, improper waste disposal, spills and leakages resulted in large entry of these chemicals into the environment (Häggbloom and Bossert, 2003).

Chlorinated ethenes can be divided into two categories, higher chlorinated ethenes comprising tetrachloroethene (PCE) and trichloroethene (TCE), and lower chlorinated ethenes including vinyl chloride (VC), *cis*-dichloroethene (*cis*-DCE), *trans*-dichloroethene (*trans*-DCE) and 1,1-dichloroethene (1,1-DCE). Tetrachloroethene and trichloroethene have high boiling points, are lipophilic, volatile, and nonflammable, what makes them excellent solvents. Due to all these properties, they are commonly used in extraction processes, metal degreasing and dry cleaning industry. PCE has been also used in the production of other chemicals, such as various fluorocarbons (Euro Chlor, 1997).

**Table 1.1** Industrial production of chlorinated ethenes in the US, Europe and the World.  
Adapted in part from Field and Sierra-Alvarez (2004).

Compound	Global production		US production		European production	
	kt y <sup>-1</sup>	year of estimate	kt y <sup>-1</sup>	year of estimate	kt y <sup>-1</sup>	year of estimate
1,1-DCE	150-200	1986	104.5	1989	12	1998
VC	13 600	1985	6000	1992	5 533	1998
TCE	390	1984	110	1984	123	1998
PCE	650	1993	123	1993	122	1998

Vinyl chloride, belonging to lower chlorinated ethenes, has different characteristics. It is a flammable gas and its major industrial application is synthesis of polyvinylchloride (PVC) (Euro Chlor, 1999). All chlorinated ethenes are toxic to humans, VC being considered the most dangerous. According to Appendix 1 of Regulation (EC) No 1907/2006 referred to as "REACH regulation" VC falls into the carcinogenicity category 1,

encompassing the substances for which there is scientific evidence for association between human exposure and the development of cancer (European Parliament 2006).

**Table 1.2** Industrial emission of chlorinated ethenes in the US, Europe and the World.

Adapted in part from Field and Sierra-Alvarez (2004).

Compound	Global industrial emissions		US emissions		European emissions at 80 production plants in 1997 <sup>a</sup>		
	(t y <sup>-1</sup> )	year of estimate	Air (t y <sup>-1</sup> )	Water (t y <sup>-1</sup> )	year of estimate	Air (t y <sup>-1</sup> )	Water (t y <sup>-1</sup> )
1,1-DCE			130	0.4	1991	na	na
VC	181 800	1982	460	0.1	1993	2 470	4.7
TCE	241 400	1990	13 730	2.5	1993	na	na
PCE	366 200	1990	4 870	4.6	1993	230	2.9

na – data not available; <sup>a</sup> (Lecloux, 2003)

The production and emission of the chlorinated ethenes in the US, Europe and the World are listed in tables 1.1 and 1.2, respectively (Field and Sierra-Alvarez, 2004). According to this data the global production of vinyl chloride amounts around 13 600 kt y<sup>-1</sup> and comprises more than 90% of the global industrial production of all chlorinated ethenes. The scale of production is similar in Western Europe and the U.S., however the emission in Europe seems to be on decline in last two decades (Lecloux, 2003). Euro Chlor, the European Federation of chlor-alkali producers, observed 80 chlorinated solvents production plants in Europe over the period 1985-1997 and recorded around 85% reduction in emissions to water and around 70% reduction in emissions to air for most chlorinated organic substances (Lecloux, 2003).

With respect to biodegradability, lower and higher chlorinated ethenes also show different behavior. Lower chlorinated ethenes are generally more susceptible to aerobic, while higher chlorinated ethenes to anaerobic degradation (Vogel et al., 1987; Field and Sierra-Alvarez, 2004). Nonetheless, there is evidence for bacterial aerobic degradation of TCE (Little et al., 1988; Arp et al., 2001) as well as anaerobic transformation of VC and *cis*-DCE (Bradley and Chapelle, 1998). Recently, aerobic



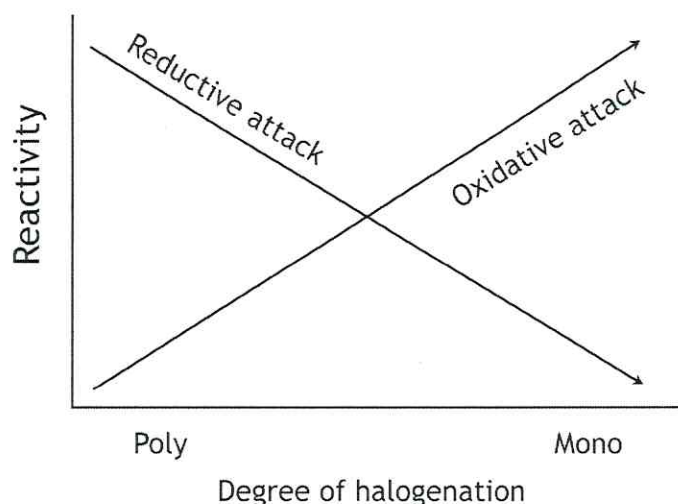
degradation of PCE and TCE by white-rot fungus was reported (Marco-Urrea et al., 2006; Marco-Urrea et al., 2008).

Chlorinated ethenes have been believed for decades to be solely of anthropogenic origin. Abrahamsson et al. (1995) revealed that tri- and tetrachloroethene can be produced by marine algae and Keppler et al. (2002) found evidence of natural production of vinyl chloride in soil. Natural occurrence of chlorinated compounds may have driven the evolution of microorganisms capable of using them as their primary growth substrates.

## **1.2 Microbial transformation of chlorinated ethenes**

Microbial transformation of chlorinated ethenes can be classified in two categories: 1) oxidative dechlorination, where chlorinated ethenes act as electron donors and/or provide carbon for growth and 2) reductive dechlorination, where chlorinated compounds serve as electron acceptors. The tendency to undergo oxidative degradation or reductive dechlorination can be displayed as a function of degree of chlorination (Figure 1.1). Upon an increase in the number of chlorine substituents the tendency to undergo reductive dechlorination increases while the tendency to undergo oxidative dechlorination decreases (Vogel et al., 1987; Vogel, 1994).

Chlorinated compounds can be transformed by microorganisms in energy yielding reduction reaction known as “halorespiration” or “dehalorespiration” (Holliger et al., 1998b; Smidt and de Vos, 2004), which is described in detail in section 1.2.2, or while growing on other primary substrates. The latter process is known as “cometabolism” and it occurs when enzymes expressed to degrade other substrates accidentally display activity also with chlorinated compounds (Field and Sierra-Alvarez, 2004). The oxidation of chlorinated compounds by the methane monooxygenase of methylotrophic bacteria can serve as an example (Alvarez-Cohen and Speitel, 2001). Other oxidative transformations of chlorinated ethenes are described in section 1.2.1.



**Figure 1.1** Relative trends of oxidative versus reductive dehalogenation as a function degree of chlorination (Bossert et al., 2003).

### 1.2.1 Oxidative dechlorination

Chlorinated ethenes can be oxidized under aerobic or anaerobic conditions. A number of studies with mixed and pure cultures demonstrated mineralization of lower chlorinated ethenes (DCEs and VC) under aerobic conditions (Verge et al., 2002; Singh et al., 2004). Aerobic oxidation of *cis*-DCE (Bradley and Chapelle, 2000b) and VC (Verge et al., 2002; Singh et al., 2004) can be coupled to energy conservation. Strain JS666 belonging to the *Comamonadaceae* family in  $\beta$ -Proteobacteria was the first aerobic isolate, which gains energy from *cis*-DCE oxidation (Coleman et al., 2002). VC is even more susceptible to undergo oxidation as it is the most reduced of the chlorinated ethenes and several strains capable of using VC as a primary substrate have been isolated. For example *Mycobacterium aurum* strain L1 (Hartmans and De Bont, 1992), *Pseudomonas aeruginosa* strain MF1 (Verge et al., 2000) and *Ralstonia* sp. strain TRW-1 can grow on VC (Elango et al., 2006).

In contrast to lower chlorinated ethenes, PCE and TCE have been observed thus far to be oxidized under aerobic conditions only in cometabolic reactions, which do not supply energy and require other

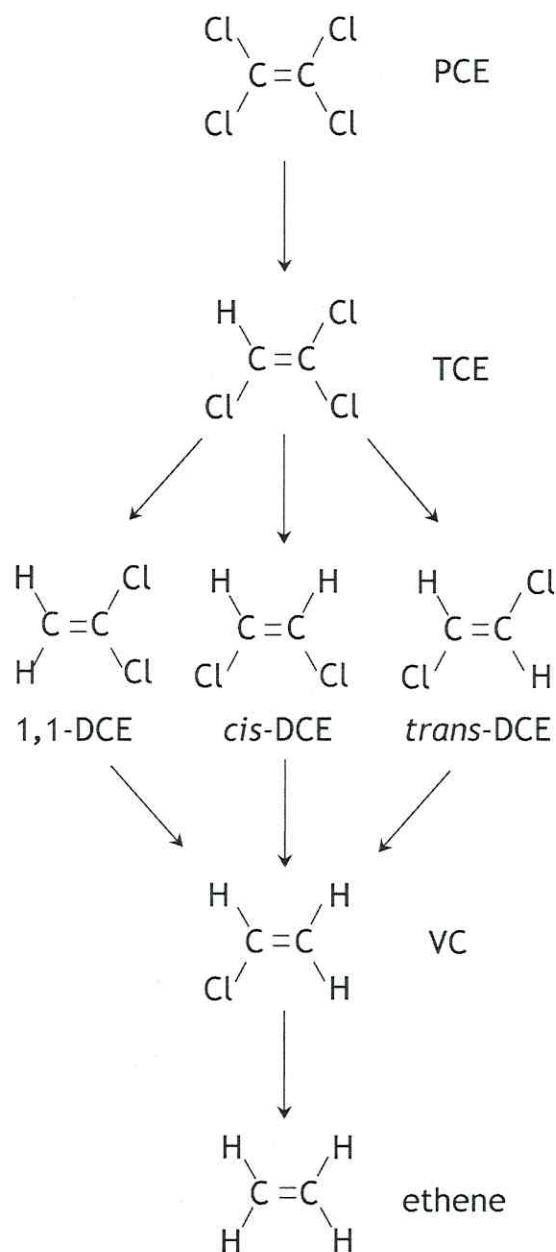
growth supporting substrates (Little et al., 1988; Ryoo et al., 2000; Ryoo et al., 2001).

In anaerobic environment, VC and *cis*-DCE can be oxidized under different electron accepting conditions, e.g. manganese, iron, sulfate, humic acid, acetogenic and methanogenic conditions (Bradley and Chapelle, 1996, 1998; Bradley et al., 1998a; Bradley et al., 1998b; Bradley and Chapelle, 2000a). To my knowledge, no pure culture capable of oxidizing VC or *cis*-DCE under anaerobic conditions has been obtained yet. Higher chlorinated ethenes are resistant to oxidative dechlorination under anaerobic conditions but can be reductively dechlorinated.

### 1.2.2 Reductive dechlorination

Reductive dechlorination is a reaction where a chlorine substituent is replaced by hydrogen and hydrochloric acid is formed as a product. Reductive dechlorination occurs most often in strict anaerobic environments (Löffler et al., 2003), where PCE can be dechlorinated in sequential reactions via TCE and DCE isomers up to VC and ethene. However, depending on the bacteria present in a given environment and on the environmental conditions, the reaction might stop at any intermediate product. Incomplete dechlorination and accumulation of *cis*-DCE and VC is a major problem at contaminated field sites, as lower chlorinated ethenes are more toxic than PCE and TCE, and VC is categorized as human carcinogen (European Parliament 2006). The scheme of reductive dechlorination of chlorinated ethenes is shown in Figure 1.2.

Reductive dechlorination can be carried out by bacteria which cometabolize the chlorinated ethenes and their growth is supported by other compounds. Examples of these type of bacteria are sulfate reducers (Cole et al., 1995), methanogens (Fathepure and Boyd, 1988) and acetogens (Terzenbach and Blaut, 1994). Other groups of reductively dechlorinating microorganisms are bacteria capable of conserving energy from this reaction, known as “halorespirers” or “dehalorespirers”.



**Figure 1.2** The anaerobic biotransformation of tetrachloroethene (PCE) to trichloroethene (TCE), *cis*-dichloroethene (*cis*-DCE), *trans*-dichloroethene (*trans*-DCE), 1,1-dichloroethene (1,1-DCE), vinyl chloride (VC) and ethene (Middeldorp et al., 1999).



The amount of energy available from reductive dechlorination is large enough to permit metabolism and growth of microorganisms and the energy available from sequential chloroethene reduction steps decreases only slightly with decreasing number of chlorine constituents (Vogel et al., 1987; Vogel, 1994; Bradley, 2003). The biochemical standard reduction potentials for the sequential steps of PCE to ethene dechlorination range from +0.58 V, for PCE to TCE, to +0.36 V for *cis*-DCE to VC, and +0.49 V for VC to ethene (Vogel et al., 1987; Bouwer, 1994; Bradley, 2003). Similarly, the standard Gibbs free energy for the sequential dechlorination steps (assuming H<sub>2</sub> as electron donor) changes only from -164 kJ/mol H<sub>2</sub> for PCE to TCE, down to -141 kJ/mol H<sub>2</sub> for *cis*-DCE to VC, and -155 kJ/mol H<sub>2</sub> for VC to ethene (Dolfing and Janssen, 1994; Löffler et al., 1999; He et al., 2002; Bradley, 2003). This makes chlorinated compounds excellent electron acceptors. The reduction potentials for all dechlorination steps of PCE to ethene are significantly higher than the values for redox couples: SO<sub>4</sub><sup>2-</sup>/H<sub>2</sub>S (-0.22 V) or CO<sub>2</sub>/CH<sub>4</sub> (-0.25 V), which implies that in competition for reducing equivalents dehalogenating organisms would out-compete sulfate reducers and methanogens (Dolfing, 2003; Field and Sierra-Alvarez, 2004).

Reductive dehalogenation was first reported in 1982 by Suflita et al. (1982), who enriched stable methanogenic consortium dehalogenating a variety of halobenzoates to methane and carbon dioxide. The first dehalorespiring organism obtained in pure culture was *Desulfomonile tiedjei* strain DCB-1, deriving energy from the reductive dechlorination of 3-chlorobenzoate to benzoate (Deweerd and Suflita, 1990; Deweerd et al., 1991). Since then, many other reductively dechlorinating microorganisms have been isolated and characterized. All of them belong to the domain bacteria and are distributed among different phylogenetic groups. They are able to degrade a wide variety of chlorinated compounds, ranging from chlorinated alkanes and alkenes, chlorinated benzenes and phenols to polychlorinated biphenyls and dioxins (Smidt and de Vos, 2004). In Table 1.3 a summary of the organisms capable of reductive dechlorination of chlorinated ethenes is presented.

### 1.2.3 Diversity of bacteria reductively dechlorinating chlorinated ethenes

The bacterial strains capable of reductive dechlorination of chlorinated ethenes that have been isolated thus far are summarized in Table 1.3. They are distributed among five phyla: Firmicutes,  $\delta$ -,  $\epsilon$ -,  $\gamma$ -Proteobacteria and Chloroflexi.

#### Firmicutes

The Firmicutes constitute the richest phylum with respect to the number of dehalogenating bacteria as well as their physiological capabilities. The isolated strains represent different genera, e.g. *Desulfitobacterium* (Gerritse et al., 1996; Miller et al., 1997; Gerritse et al., 1999; Suyama et al., 2001; Finneran et al., 2002), *Dehalobacter* (Wild et al., 1996; Holliger et al., 1998a) and *Clostridium* (Chang et al., 2000).

*Desulfitobacterium* spp. is a metabolically versatile group and can use a very wide spectrum of electron donors and acceptors for energy conservation. For example, *Desulfitobacterium* sp. strain PCE1 grows with PCE, 2-chlorophenol, 2,4,6-trichlorophenol, 3-chloro-4-hydroxyphenyl acetate, thiosulfate, sulfite and fumarate as electron acceptors and formate, butyrate, lactate, pyruvate, succinate and ethanol as electron donors (Gerritse et al., 1996). In the last decade, *Desulfitobacterium* strains capable of using humic substances and metals as terminal electron acceptors have been isolated (Finneran et al., 2002; He and Sanford, 2003). This finding confirmed the premise that the genus *Desulfitobacterium* is extremely diversified group in terms of metabolism and is capable of living also in absence of chlorinated compounds.

However, the Firmicutes phylum comprises also highly specialized organisms. The growth of the very close *Desulfitobacterium* relative *Dehalobacter restrictus* is restricted to dehalorespiration, solely with PCE and TCE as electron acceptors and hydrogen as electron donor (Holliger et al., 1998a). *Dehalobacter restrictus* was also the first isolated organism that was able to metabolically reduce PCE (Holliger et al., 1998a).

All currently known dechlorinating representatives of the Firmicutes are not able to reduce PCE or TCE further than *cis*-DCE.

### Proteobacteria

Organisms capable of reductive dechlorination of chlorinated ethenes were also found in the  $\delta$ -,  $\epsilon$ - and  $\gamma$ - subphyla of the Proteobacteria.  $\delta$ -Proteobacteria are represented by *Desulfomonile tiedjei*, the first dehalogenating isolate, which dechlorinates PCE and TCE to *cis*-DCE, but only in cometabolic reaction (Deweerd et al., 1991; Cole et al., 1995). Other isolates from this group are: *Desulfuromonas chloroethenica* (Krumholz, 1997), *Desulfuromonas michiganensis* (Sung et al., 2003) and recently isolated *Geobacter lovleyi* strain SZ (Sung et al., 2006a).

*Desulfuromonas* and *Geobacter* spp. grow with various substrates. The range of their electron acceptors is especially wide and it includes metals like iron and in case of *G. lovleyi* also manganese and uranium. Interestingly, the presence of alternate electron acceptors did not inhibit dechlorination and chlorinated ethenes and metals can be simultaneously reduced by these cultures (Sung et al., 2003; Sung et al., 2006a). This feature makes both strains very attractive for bioremediation purposes as bioavailable ferric iron is present at many contaminated sites and iron reduction may cease reductive dechlorination by other species (Sung et al., 2003; Sung et al., 2006a).

**Table 1.3** Bacterial isolates capable of reductive dechlorination of chlorinated ethenes. Adapted in part from Nijenhuis, (2002).

Phylogenetic group Species	Electron donor	Dechlorination steps performed with chl. ethenes as electron acceptors	Other electron acceptors	Reference
<b>Firmicutes</b>				
<i>Desulfitobacterium</i> sp. strain PCE1	formate, butyrate lactate, pyruvate	PCE to TCE	mono-, di-, tri chlorophenols, thiosulfate, sulfite fumarate, ClOHPA	(Gerritse et al., 1996)
<i>Desulfitobacterium</i> <i>frapperi</i> TCE1	succinate, ethanol, butyrate, crotonate, ethanol, formate, hydrogen, lactate	PCE to <i>cis</i> -DCE TCE to <i>cis</i> -DCE	sulfite, thiosulfate, fumarate, nitrate	(Gerritse et al., 1999)
<i>Desulfitobacterium</i> <i>metallireducens</i> strain 858-15A	lactate, formate, ethanol, butanol, butyrate, malate, pyruvate	PCE to <i>cis</i> -DCE TCE to <i>cis</i> -DCE	Fe(III) citrate, Mn(IV) oxide, humic substances, sulfur, ClOHPA, AQDS	(Finneran et al., 2002)
<i>Desulfitobacterium</i> sp. strain Viet1	hydrogen	PCE to TCE		
<i>Desulfitobacterium</i> sp. PCE-S	formate, hydrogen, pyruvate, yeast	PCE to <i>cis</i> -DCE TCE to <i>cis</i> -DCE	trichlorophenol, pentachlorophenol, fumarate, sulfite	(Miller et al., 1997)
<i>Desulfitobacterium</i> sp. strain Y51		PCE to <i>cis</i> -DCE	hexa-, penta-, tetra- chloroethanes	(Suyama et al., 2001)
<i>Dehalobacter restrictus</i>	hydrogen	PCE to <i>cis</i> -DCE TCE to <i>cis</i> -DCE		(Holliger et al., 1998a)
<i>Dehalobacter</i> sp. TEA	hydrogen	PCE to <i>cis</i> -DCE TCE to <i>cis</i> -DCE		(Wild et al., 1996)
<i>Clostridium bifermentans</i> strain DPH1	hydrogen	PCE to <i>cis</i> -DCE TCE to <i>cis</i> -DCE		(Chang et al., 2000)
<b><math>\delta</math>-proteobacteria</b>				
<i>Desulfomonile tiedjei</i>	formate, hydrogen, pyruvate	comet. PCE to <i>cis</i> -DCE TCE to <i>cis</i> -DCE	3-chlorobenzoate, pentachlorophenol, sulfate, sulfite, thiosulfate	(Deweerd et al., 1991) (Cole et al., 1995)
<i>Desulfuromonas</i> <i>chloroethenica</i>	acetate, pyruvate, polysulfide	PCE to <i>cis</i> -DCE TCE to <i>cis</i> -DCE	fumarate, Fe(III)	(Krumholz, 1997)



<i>Desulfuromonas michiganensis</i>	acetate, lactate, pyruvate, succinate, malate, fumarate	PCE to <i>cis</i> -DCE TCE to <i>cis</i> -DCE	malate, fumarate, Fe(III), sulfur	(Sung et al., 2003)
<i>Geobacter lovleyi</i> strain SZ	acetate, hydrogen, pyruvate	PCE to <i>cis</i> -DCE TCE to <i>cis</i> -DCE	nitrate, fumarate, Fe(III), malate, Mn(IV), U(VI), sulfur	(Sung et al., 2006a)
<b><i>ε</i>-proteobacteria</b>				
<i>Sulfurospirillum multivorans</i>	ethanol, formate, hydrogen, lactate, pyruvate	PCE to <i>cis</i> -DCE TCE to <i>cis</i> -DCE	arsenate, fumarate, nitrate, selenate	(Scholz-Muramatsu et al., 1995)
<i>Sulfurospirillum halorespirans</i>	lactate, hydrogen, formate, pyruvate	PCE to <i>cis</i> -DCE TCE to <i>cis</i> -DCE	sulfur, arsenate, selenate, nitrate, nitrite, fumarate	(Luijten et al., 2003)
<b><i>γ</i>-proteobacteria</b>				
<i>Enterobacter</i> MS-1	acetate, amino acids, formate, glucose, lactate, pyruvate, yeast extract	PCE to <i>cis</i> -DCE TCE to <i>cis</i> -DCE	nitrate, oxygen	(Sharma and McCarty, 1996)
<b><i>Chloroflexi</i></b>				
<i>Dehalococcoides ethenogenes</i> strain 195	hydrogen	PCE, TCE, <i>cis</i> -DCE and 1,1-DCE to ethene, (VC to ethene comet.)	chloroethanes, chlorobenzenes, chloronaphthalenes, dioxins, polychlorobiphenyls, chlorophenols	(Maymo-Gatell et al., 1997), (Fennell et al., 2004), (Adrian et al., 2007)
<i>Dehalococcoides</i> sp. strain BAV1	hydrogen	DCEs to ethene, VC to ethene (PCE and TCE comet.)		(He et al., 2003a), (He et al., 2003b)
<i>Dehalococcoides</i> sp. GT	hydrogen	TCE, <i>cis</i> -DCE, 1,1-DCE, VC to ethene		(Sung et al., 2006b)
<i>Dehalococcoides</i> sp. strain FL2	hydrogen	TCE, <i>cis</i> -DCE and trans-DCE and trans-DCE (PCE and VC comet.)		(He et al., 2005)
<i>Dehalococcoides</i> sp. strain CBDB1	hydrogen	PCE to trans-DCE comet	chlorobenzenes, chlorinated dioxins, chlorophenols	(Adrian et al., 2000), (Bunge et al., 2003), (Adrian et al., 2007), (Adrian, 2001)

AQDS – anthraquinone-2,6-disulfonate, ClOHPA - 3-chloro-4- hydroxyphenyl acetate

Other dechlorinating proteobacteria isolated in pure cultures are, belonging to the  $\epsilon$ -subphylum, *Sulfurospirillum multivorans* (Scholz-Muramatsu et al., 1995) and *Sulfurospirillum halorespirans* (Luijten et al., 2003) and belonging to the  $\gamma$ -subphylum *Enterobacter* MS-1 (Sharma and McCarty, 1996). The latter is the only known facultative anaerobic bacterium capable of reductive dechlorination.

Similar to the Firmicutes, thus far known Proteobacteria dechlorinating representatives dechlorinate tetrachloroethene and trichloroethene only to *cis*-DCE.

### Chloroflexi

*Dehalococcoides* spp., which are most closely affiliated with the Chloroflexi, are the only so far described group of organisms capable of reductive dechlorination of lower chlorinated ethenes (DCEs and VC). The first isolate, *Dahalococcoides ethenogenes* strain 195, was obtained by Maymo-Gatell in 1997 at Cornell University and it was shown to completely dechlorinate PCE to ethene (Maymo-Gatell et al., 1997). PCE, TCE, *cis*-DCE and 1,1-DCE supported the growth, while VC was converted cometabolically (Maymo-Gatell et al., 1999; Maymo-Gatell et al., 2001). Later studies proved the growth of this strain with other chlorinated compounds, e.g. chlorinated benzenes, naphthalenes, biphenyls and dioxins (Fennell et al., 2004).

*Dehalococcoides* have limited substrate range, they can only use halogenated compounds as electron acceptors and solely hydrogen as electron donor. They are strict anaerobes and are very sensitive even to traces of oxygen. They have complex nutrient requirements and grow slowly. Due to all these features, *Dehalococcoides* are relatively difficult in cultivation. Despite these constraints, a few *Dehalococcoides* strains have been isolated to date and several other have been described in mixed cultures.

Strain FL2 can conserve energy from the dechlorination of TCE, *cis*-DCE and *trans*-DCE (He et al., 2005). PCE and VC are dechlorinated only cometabolically in the presence of growth supporting chloroethenes

(He et al., 2005). *Dehalococcoides* sp. strain BAV1 was the first isolate capable of using VC as growth supporting electron acceptor (He et al., 2003a; He et al., 2003b). This strain can also capture energy from the dechlorination of all DCE isomers and it cometabolically dechlorinates PCE and TCE in the presence of VC or DCEs (He et al., 2003a). Later, other strains growing on VC were described, including strains GT (Sung et al., 2006b), VS (Cupples et al., 2003; Müller et al., 2004) and KB-1/VC (Duhamel et al., 2004). However, the latter two have never been purified and are available only in enrichment cultures. The growth of strain GT, in addition to the vinyl chloride, can be coupled to dehalogenation of TCE, *cis*-DCE and 1,1-DCE (Sung et al., 2006b). Strain VS can use *cis*-DCE besides VC (Cupples et al., 2003; Müller et al., 2004) and KB-1/VC TCE and *cis*-DCE (Duhamel et al., 2004) as terminal electron acceptor.

Finally, *Dehalococcoides* strain CBDB1 is an isolate, which cannot gain energy from dehalogenation of chlorinated ethenes. However, it is worth mentioning here due to its interesting characteristics (Adrian et al., 2000). Strain CBDB1 grows on chlorinated benzenes (Adrian et al., 2000; Jayachandran et al., 2003), chlorinated dioxins (Bunge et al., 2003) and it was recently found to dechlorinate chlorinated phenols (Adrian et al., 2007). Kube et al. (2005) sequenced the genome of strain CBDB1 and found 32 reductive dehalogenase genes, which suggests that the dehalogenating potential of these bacteria may be even higher and further halogenated compounds can be included in its electron acceptor spectrum.

The discovery of *Dehalococcoides* sp. was a breakthrough in terms of remedial treatment contaminated sites with chlorinated ethenes. Finding that chlorinated ethenes can be microbiologically transformed to non-toxic ethene opened the door for bioremediation in place of expensive physical and chemical remediation approaches.



### 1.3 Natural attenuation of chlorinated ethenes

Until 1980s chlorinated ethenes were considered to be not significantly biodegradable in groundwater systems (Bradley, 2000). Parsons et al. (1984) made one of the first observations of tetrachloroethene degradation in the natural environment. Following this finding, Kleopfer et al. (1985) obtained an evidence of trichloroethene biotransformation in soil. Since then, a body of evidence for biodegradation of chlorinated ethenes has emerged and natural attenuation became a recognized strategy to remediate the sites contaminated with chlorinated ethenes.

Dechlorinating microorganisms are ubiquitous in the environment and the genera of *Dehalococcoides*, *Desulfitobacterium*, *Desulfuromonas* and *Dehalobacter* have been found at many chloroethene-contaminated sites (Löffler et al., 2000; Hendrickson et al., 2002; Yang et al., 2005; Nijenhuis et al., 2007; Imfeld et al., 2008). Hendrickson et al. (2002) investigated 24 sites scattered throughout North America and Europe and they detected *Dehalococcoides*-like sequences at 21 of them. This implies that *Dehalococcoides* can survive in a wide range of geographical locations, in a wide range of geological matrices, and in a variety of climatic zones (Hendrickson et al., 2002).

Despite the wide distribution of reductively dechlorinating microorganisms in chloroethene-contaminated aquifers, the extent of dechlorination varies at different sites (Bradley, 2000). Moreover, the reductive dechlorination is very often incomplete and results in the accumulation of *cis*-DCE or VC, which is a major concern due to VC carcinogenicity.

The crucial factors, determining the presence or absence as well as type of microbial transformation chlorinated ethenes undergo in the aquifer, are redox potential of the environment and availability of the substrates (electron donors and acceptors). Additionally, other environmental factors, like pH, salinity, temperature, presence of toxic or inhibitory compounds etc. may affect the microbial community structure and activity (Bossert et al., 2003). A summary of the factors influencing the

biodegradation of chlorinated compounds is presented in Table 1.4. Some of these factors can be manipulated, e.g. electron donors or other nutrients can be added, in order to stimulate the biodegradation (Bossert et al., 2003). This approach is referred to as “enhanced natural attenuation”.

In the context of risk assessment and management of the contaminated sites, the monitoring of microbial contaminant transformation in polluted aquifers is of high importance. A few methods enabling assessment of biodegradation in groundwater have been demonstrated until now. Wiedemeier et al. (1999) proved the biodegradation of organic contaminants by the measurement of contaminant concentrations and monitoring depletion of electron acceptors. Another method is detection of metabolites specific for certain degradation pathway, for example less chlorinated compounds for reductive dechlorination (Witt et al., 2002). A relatively new approach to detect biodegradation is the determination of isotope composition of individual contaminants, known as Stable Isotope Fractionation Analysis (SIFA) or Compound Specific Isotope Analysis (CSIA) (Meckenstock et al., 2004). The principles of this method are described in section 1.4.

**Table 1.4** Summary of factors affecting contaminant biodegradation rates in soil (Bossert et al., 2003)

Factor	Impact	Target Range	Reference
Temperature	Phase distribution of contaminants; Metabolic rates and respiratory quotient ( $Q_{10}$ ); changes in dehalogenation patterns	10° to 35 °C optimum; <5° to > 50 °C reported	(Valo et al., 1985; Kohring et al., 1989; Holliger et al., 1992; Suffita and Townsend, 1995; Wu et al., 1996; Mohn et al., 1997; Wu et al., 1997a, b; Männisto et al., 1999; Männisto et al., 2001; Männisto and Puhakka, 2002) (Bossert and Compeau, 1995)
pH/Buffering	Contaminant and mineral speciation and mobilization; physiological reactions	Optimum 6 to 8.5	
Moisture	Availability measured by water activity ( $a_w$ ); contaminant concentration and partitioning; aeration/redox conditions	50 to 70% water holding capacities in soils, corresponding to $a_w$ of 0.9 $a_w$ > 0.9 (bacteria), or > 0.6 (fungi)	(Ehrlich, 1996; Bartha and Atlas, 1998; Arands et al., 1999)
O <sub>2</sub> and alternate electron acceptors; H <sub>2</sub> Donors	Aerobic vs. anaerobic pathways; changes in metabolite profiles, i.e., complete vs. incomplete metabolism	Non-limiting amounts, based on stoichiometric need of electron donor (contaminant)	(Genthner et al., 1989a, b; Allard et al., 1992; DiStefano et al., 1992; Häggblom et al., 1993b, a; Häggblom and Young, 1995; Kazumi et al., 1995; Fennell et al., 1997; Monserrate and Häggblom, 1997; Sanford and Tiedje, 1997; Bradley and Chapelle, 1998; Yang and McCarty, 1998; Boyle et al., 1999; Bradley and Chapelle, 2000b; Milligan and Häggblom, 2000) (Arands et al., 1999)
Mineral nutrients (N, P)	Microbial activity and growth; mobility; enzyme induction (e.g. laccases and other ligninolytic enzymes)	Use of bioavailable forms; determination of needs (i.e. limiting or non-limiting) C/N, 10 to 200 C/P, 50 to 800	

Inhibitory elements			
High salinity	Lowered water potential; changes in Osmotic pressure and ionic strength	Optimum salinity 0.9 to 3.5 %, or water potential <-10bar; Higher for halophiles in extreme Environments	(Ehrlich, 1996; Bartha and Atlas, 1998)
Heavy metals	Enzyme inactivation; protein and metabolic inhibitors	Low heavy metal concentrations; low mobilization with pH control; redox effect on non-toxic oxidation states	(Wall and Stratton, 1994a, b; Kuo and Genthner, 1996; Pardue et al., 1996)
Contaminant type	Effect of degree of halogenation on intrinsic biodegradability and bioavailability (e.g. phase distribution; uptake)	Varies with substrate and environment	(Bossert and Compeau, 1995)
Concentration	Toxicity toward microbiota; bioavailability; Interaction with contaminant matrix	ppb range higher (very compound -specific and requires site-specific assessment)	(Alexander, 1981, 1985; Abramowicz et al., 1993; Rhee et al., 1993; Suflita and Townsend, 1995)



## 1.4 Stable isotope fractionation

During microbial degradation of organic compounds kinetic isotope effects (KIE) may occur. Kinetic isotope effects result from the fact that biogenic elements, e.g. carbon, hydrogen and many other exist in at least two stable isotopes (see Table 1.5), differing in the activation energies needed to break or form chemical bonds in the molecule. Thus, chemical bonds formed by light isotopes are weaker and light isotopes react faster compared to heavy isotopes (Hoefs, 1997). Different reaction rates of the compounds containing heavy and light isotopes lead to isotope fractionation and enrichment of heavy isotopes in the not yet degraded residual fraction.

**Table 1.5** Selected elements and the relative natural abundance of isotopes, mass differences and international standards (Hoefs, 1997)

Element	Relative isotope abundance	Relative mass difference	Standard
H/D	99.9844/0.0156	2.00	V-SMOW
<sup>12</sup> C/ <sup>13</sup> C	98.89/1.11	1.08	V-PDB
<sup>14</sup> N/ <sup>15</sup> N	99.64/0.36	1.07	Air
<sup>16</sup> O/ <sup>18</sup> O	99.76/0.02	1.13	V-SMOW
<sup>32</sup> S/ <sup>34</sup> S	94.02/4.21	1.06	V-CTD
<sup>35</sup> Cl/ <sup>37</sup> Cl	75.53/24.47	1.06	24.47

### 1.4.1 Mathematical description of isotope fractionation

Isotope ratios of a given compound can be measured mass-spectrometrically and they are reported as difference in per mil in comparison to an international standard and expressed in  $\delta$  units (Hoefs, 1997). Carbon isotope compositions are reported relative to the Vienna Pee Dee Belemnite standard (V-PDB, IAEA-Vienna) and can be calculated from eq. 1.1, (Coplen et al., 2006).

$$\delta^{13}\text{C}[‰] = \left( \frac{(^{13}\text{C}/^{12}\text{C})_{\text{Sample}}}{(^{13}\text{C}/^{12}\text{C})_{\text{Standard}}} - 1 \right) \times 1000 \quad (\text{eq. 1.1})$$



The commonly used mathematical description of isotope fractionation processes is the Rayleigh equation, relating the changes in contaminant concentrations to the changes in isotope compositions (eq. 1.2), (Hoefs, 1997):

$$\ln\left(\frac{R_t}{R_0}\right) = (1/\alpha - 1) \times \ln\left(\frac{C_t}{C_0}\right). \quad (\text{eq. 1.2})$$

$R_t$  is the  $^{13}\text{C}/^{12}\text{C}$  isotope ratio at time  $t$ ,  $R_0$  is the initial isotope composition of the substrate,  $C_t/C_0$  is the fraction of substrate remaining at time  $t$ , and  $\alpha$  is the isotope fractionation factor, which is a constant for the reaction at given experimental conditions (Hoefs, 1997).

#### 1.4.2 Primary vs. secondary isotope effects

The molecules of organic contaminants usually consist of several atoms of given element (e.g. H, C, Cl etc.) present at various locations. Significant isotope fractionation of a certain element occurs only if this element is involved in the rate limiting reaction step of cleavage or formation of the chemical bond. Isotope effects are referred to as "primary", if the heavy atom is directly present in the reacting bond, and as "secondary", if it is located adjacent to the reacting position (Meckenstock et al., 2004; Elsner et al., 2005). Secondary isotope effects are typically one to two orders of magnitude lower than primary effects (Meckenstock et al., 2004).

Processes acting on the whole compound, such as advective and dispersive transport, volatilization, sorption and desorption cause much lower changes in isotope composition than chemical and biochemical reactions, and they are often under detection limits of commonly used analytical methods (Elsner et al., 2005).

### 1.4.3 Assessment of *in situ* biodegradation

As the isotope fractionation during microbial transformations of most organic contaminants is significantly higher than fractionation caused by physical processes (advective and dispersive transport, volatilization, sorption, phase partitioning etc.), change in isotope composition may serve as an indicator of biodegradation of organic pollutants at contaminated field sites. The Compound Specific Isotope Analysis (CSIA) is a tool frequently used to determine the isotope compositions of many organic contaminants *in situ* as well as in laboratory experiments (Meckenstock et al., 2004). In order to describe isotope fractionation during microbial degradation, the concentrations and isotope ratios of the residual, not yet degraded substrate fraction, are analyzed and isotope fractionation factor ( $\alpha$ ) can be calculated from the Rayleigh equation (eq. 1.2).

The methods to measure isotope signatures of many elements have been developed. Concerning chlorinated ethenes, the on-line gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) methods to measure isotope compositions of hydrogen ( $^2\text{H}/^1\text{H}$ ), carbon ( $^{13}\text{C}/^{12}\text{C}$ ) and recently also chlorine ( $^{37}\text{Cl}/^{35}\text{Cl}$ ) (Shouakar-Stash et al., 2006) are available.

Furthermore, the extent of biodegradation *in situ* can be quantified applying a modified Rayleigh equation (eq. 1.3 and 1.4), provided that an appropriate fractionation factor ( $\alpha$ ) is known (Hoefs, 1997).

$$\frac{R_t}{R_0} = f_t^{(1/\alpha-1)}, \quad \text{eq. 1.3}$$

where  $f_t$  is the remaining contaminant fraction at time  $t$  ( $f_t = C_t/C_0$ ). The percentage of biodegradation ( $B$  [%]) can then be expressed as:

$$B[\%] = (1 - f_t) \times 100 = (1 - (R_t/R_0)^{[1/(1/\alpha-1)]}) \times 100. \quad \text{eq. 1.4}$$

However, it should be taken into account that abiotic chemical reactions, which are also associated with large isotope effects, can also contribute to the degradation of organic contaminants (Dayan et al., 1999). For example, reductive dechlorination with metallic iron ( $\text{Fe}^0$ ), which is used for the cleanup of groundwater contaminated with chlorinated ethenes, induces large carbon isotope effects (Bill et al., 2001). The uncertainty associated with the quantification of pollutant biodegradation by the Rayleigh-equation approach increases when abiotic processes cause a significant decrease in contaminant concentrations (Fischer et al., 2007).

Recently, microbial degradation of chlorinated ethenes at different contaminated sites has been monitored using stable carbon isotope analysis (Hunkeler et al., 1999; Sherwood Lollar et al., 2001; Song et al., 2002; Vieth et al., 2003; Chartrand et al., 2005; Morrill et al., 2005). Isotope shifts in the non-degraded residual substrate fraction of contaminant and/or in its degradation products demonstrated that biodegradation was occurring. Also the attempts to apply the Rayleigh model to quantify the *in situ* biodegradation have been carried out (Sherwood Lollar et al., 2001; Vieth et al., 2003; Chartrand et al., 2005; Morrill et al., 2005; Fischer et al., 2007). For example, Sherwood Lollar et al. (2001) used the isotope fractionation factors for reductive dechlorination of PCE and TCE available in the literature and calculated the extent of biodegradation in groundwater at area 6, Dover Air Force Base. Chartrand et al. (2005) used the isotope signatures of the TCE degradation products, *cis*-DCE and VC, to quantify the extent of biodegradation at the TCE contaminated site, where the bioaugmentation was implemented. Morrill et al. (2005) investigated a PCE-contaminated site, where *cis*-DCE accumulated due to incomplete reductive dechlorination. Likewise, in the case of the previous site, the culture dechlorinating chlorinated ethenes to non-toxic ethene was added and fractionation factors from previous studies together with field isotopic values were used to estimate the *cis*-DCE degradation rates.

The Rayleigh model has been also successfully applied to estimate the biodegradation of other contaminants. For instance, Fischer et al. (2007) used stable isotope fractionation analysis to characterize and quantitatively assess the *in situ* biodegradation of benzene in a BTEX-contaminated aquifer located in the area of a former hydrogenation plant.

#### 1.4.4 Factors influencing isotope fractionation

The extent of the kinetic isotope effect depends on rate limitation in the transition state of the bond cleavage and the nature of the chemical reaction (Northrop, 1981). Consequently, similar degradation pathways should cause similar fractionation. The isotope fractionation during anaerobic toluene degradation by different bacterial cultures using the benzylsuccinate pathway may serve as an example (Meckenstock et al., 1999).

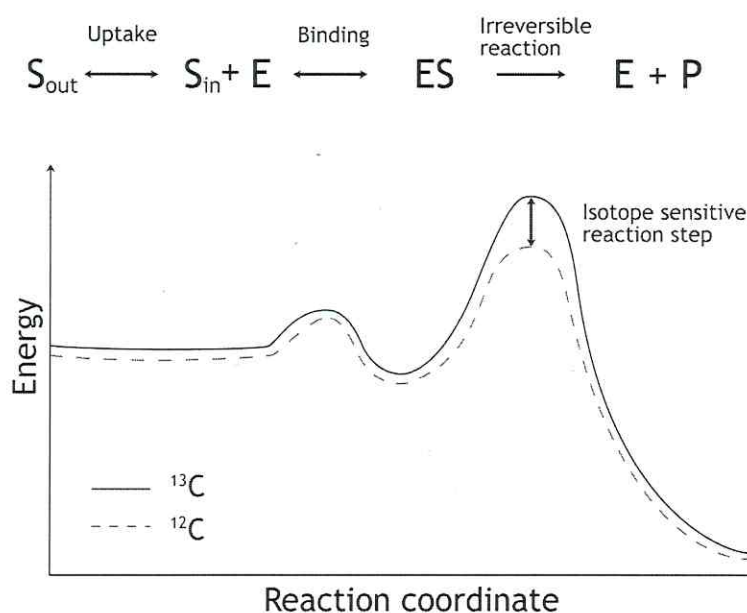
However, rate-limiting reaction steps, lowering the “apparent” isotope effect observed in the experiment, may precede the isotope sensitive bond cleavage (Northrop, 1981). In biodegradation reactions, rate limitations associated with the substrate mass transfer towards the cell, uptake of substrate by the cell, transport of substrate within the cell or binding to the enzyme complex can affect the kinetics of the reaction. Figure 1.3 shows the scheme of an enzymatic reaction comprising the steps controlling the overall kinetics of the reaction, which can cause rate limitations, as well as a diagram of reaction coordinate illustrating the irreversible reaction step associated with the isotope discrimination (Northrop, 1981; Nijenhuis et al., 2005).

Nijenhuis et al. (2005) demonstrated that physiological factors inside the bacterial cell can lower the overall observed isotope fractionation. They investigated fractionation of PCE by enzyme, crude extract and growing cultures of *Sulfurospirillum multivorans* and *Desulfitobacterium* sp. strain PCE-S and observed that with decreasing system complexity fractionation increased. This suggests that membranes can hinder the substrate flow into the cell and may lead to isotope non-equilibrium conditions between substrate inside and outside the cell.



Moreover, formation of the enzyme-substrate complex can be a rate-limiting step in the reaction (Nijenhuis et al., 2005).

Besides rate limitations, other factors like type of microorganism and type of enzyme involved in the degradation reaction can affect the extent of the isotope fractionation. Nikolausz et al. (2006) observed that isotope fractionation during aerobic dichloromethane degradation can vary to some extent due to structural variability of the enzymes of the microorganisms involved in the reaction. Nijenhuis et al. (2005) showed differences in fractionation of tetrachloroethene by two reductively dechlorinating strains: *Sulfurospirillum multivorans* and *Desulfitobacterium* sp. strain PCE-S.



**Figure 1.3** A scheme of enzymatic reactions showing the steps controlling the overall kinetics of the reaction ( $S_{out}$  – substrate in the medium,  $S_{in}$  – substrate in the cell, E – enzyme, ES – enzyme-substrate complex, P – product) and a reaction coordinate diagram illustrating isotope sensitive reaction step. Adapted from: (Nijenhuis et al., 2005) and (Northrop, 1981)

The variability of fractionation during microbial degradation of organic contaminants is of major concern as it poses a limitation for the application of CSIA for quantitative assessment of *in situ* biodegradation. The selection of a representative fractionation factor becomes complicated and the uncertainty associated with the estimated biodegradation can be very high. Hence, efforts should be made to gain knowledge on variability of fractionation during degradation of different compounds as well as to better understand the factors potentially influencing isotope fractionation.

### 1.5 Outline of the thesis

The aim of this thesis was to get better understanding of microbial transformations of chlorinated ethenes under anaerobic conditions in order to facilitate application of Compound Specific Isotope Analysis (CSIA) to assess *in situ* biodegradation of these widely distributed groundwater contaminants. The research presented in this dissertation addresses two issues related to qualitative and quantitative evaluation of chlorinated ethenes biodegradation at field sites: 1) isotope fractionation upon microbial reductive dechlorination and 2) investigation and enrichment of environmentally relevant microorganisms.

**Chapter 1** gives a general overview on microbial transformations of chlorinated ethenes, including the characteristics and sources of chlorinated ethenes in the environment, natural degradation processes, diversity of dechlorinating organisms, as well as natural attenuation and means by which it can be assessed with special attention on CSIA.

**Chapter 2** provides insights in the processes controlling the carbon isotope fractionation of tetrachloroethene (PCE) and trichloroethene (TCE) by *Sulfurospirillum* spp. and *Desulfitobacterium* sp. strain PCE-S. Fractionation of TCE by growing cells, crude extracts, enzymes as well as during abiotic reaction with cyanocobalamin was determined and compared with previously reported data for PCE.

**Chapter 3** reports on the variability in carbon isotope fractionation during reductive dechlorination by different microbial strains. Isotope fractionation by a set of bacterial strains, comprising *Dehalococcoides*

*ethenogenes* strain 195, *Desulfitobacterium* sp. strain Viet1, *Desulfuromonas michiganensis* and *Geobacter lovleyi*, was investigated and juxtaposed with other fractionation factors, available thus far in the literature, for pure cultures. The consequences of variability in fractionation for application of CSIA to monitor biodegradation at contaminated sites are discussed.

**Chapter 4** describes the carbon isotope fractionation of chlorinated ethenes and polychlorinated dibenzo-*p*-dioxins (PCDDs) by a mixed culture containing *Dehalococcoides ethenogenes* strain 195 and evaluates the potential of CSIA for assessment of microbial transformations of PCDDs in the environment.

**Chapter 5** focuses on our attempt to enrich new dechlorinating organisms from contaminated aquifer in Bitterfeld (East Germany). Our efforts resulted in enrichment of a *Dehalococcoides*-containing culture dechlorinating tetrachloroethene to ethene. The enrichment procedure and molecular methods used to assess the purity and phylogenetic affiliation of the culture are explained.

In **Chapter 6** the main findings presented in this thesis are summarized and evaluated with respect to the application of CSIA for qualitative and quantitative assessment of the biodegradation. In the end, the future perspectives and directions of this research are proposed.

**Factors controlling the carbon isotope  
fractionation of tetra- and trichloroethene  
during reductive dechlorination by  
*Sulfurospirillum* spp. and *Desulfitobacterium*  
sp. strain PCE-S**

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Kirsten Beck, Gabriele Diekert, Hans-Hermann Richnow and Ivonne  
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### **Abstract**

Carbon stable isotope fractionation of tetrachloroethene (PCE) and trichloroethene (TCE) was investigated during reductive dechlorination. Growing cells of *Sulfurospirillum multivorans*, *Sulfurospirillum halorespirans*, or *Desulfotobacterium* sp. strain PCE-S, the respective crude extracts and the abiotic reaction with cyanocobalamin (vitamin B<sub>12</sub>) were used. Fractionation of TCE ( $\alpha_C=1.0132$ - $1.0187$ ) by *S. multivorans* was more than one order of magnitude higher compared to values previously observed for tetrachloroethene (PCE) ( $\alpha_C=1.00042$ - $1.0017$ ). Similar differences in fractionation were observed during reductive dehalogenation by the close relative *S. halorespirans* with  $\alpha_C=1.0046$ - $1.032$  and  $\alpha_C=1.0187$ - $1.0229$  for PCE and TCE respectively. TCE carbon isotope fractionation ( $\alpha_C=1.0150$ ) by the purified PCE-reductive dehalogenase from *S. multivorans* was more than one order of magnitude higher than fractionation of PCE ( $\alpha_C=1.0017$ ). Carbon isotope fractionation of TCE by *Desulfotobacterium* sp. strain PCE-S ( $\alpha_C=1.0109$ - $1.0122$ ) as well as during the abiotic reaction with cyanocobalamin ( $\alpha_C=1.0154$ ) was in a similar range compared to previously reported values for fractionation by mixed microbial cultures. In contrast to previous results with PCE, no effects due to rate limitations, uptake or transport of the substrate to the reactive site, could be observed during TCE dechlorination. Our results show that prior to a mechanistic interpretation of stable isotope fractionation factors it has to be carefully verified how other factors such as uptake or transport affect the isotope fractionation during degradation experiments with microbial cultures.

## 2.1 Introduction

In recent years, compound specific stable isotope analysis (CSIA) has been developed as a tool to monitor the fate of organic contaminant in the environment (Meckenstock et al., 2004). CSIA takes advantage of the observation that during biodegradation molecules with lighter isotopes ( $^1\text{H}$ ,  $^{12}\text{C}$ ) are preferentially reacted over the heavier isotope ( $^2\text{D}$ ,  $^{13}\text{C}$ ) resulting in an enrichment of the heavier stable isotope in the residual, non-degraded, fraction. The extent of stable isotope fractionation can be expressed as the stable isotope fractionation factor ( $\alpha$ ) and may be used to quantify *in situ* biodegradation of a pollutant (Meckenstock et al., 2004).

The chlorinated ethenes, tetrachloroethene (PCE) and trichloroethene (TCE) are common ground water contaminants. In various studies it has been observed that during reductive dechlorination of these compounds carbon stable isotope fractionation occurs (Bloom et al., 2000; Slater et al., 2003; Nijenhuis et al., 2005). Fractionation factors determined in the laboratory may enable qualitative and quantitative assessment of *in situ* biodegradation (Meckenstock et al., 2004).

Kinetic stable isotope fractionation of substrates in microbial degradation processes is mainly a result of the mass dependent isotope effect upon cleavage of a chemical bond in the first irreversible reaction step and is largely affected by the biochemical reaction mechanism. The biochemical reaction mechanism and kinetic control depends on properties and structure of the dehalogenating enzyme involved in reductive dechlorination.

Cobalamins are important cofactors of reductive dehalogenases in organisms capable of dehalorespiration (Neumann et al., 1996; Miller et al., 1998; Krasotkina et al., 2001; Holliger et al., 2002; Neumann et al., 2002; Kräutler et al., 2003). The microbial dehalogenation of chlorinated ethenes has been proposed to proceed by alkylating a super reduced corrinoid containing Co(I) at the reactive site with the chloroethene (Neumann et al., 1996). The chemical mechanism of reductive dehalogenation of chlorinated ethenes catalyzed by cobalamin has been subject of previous studies and has been suggested to occur via a single electron transfer from a reduced cob(I)alamin leading to the formation of

chloride and vinyl radicals as intermediates (Glod et al., 1997; Wohlfarth and Diekert, 1999; Shey and van der Donk, 2000; Holliger et al., 2002). The cleavage of the carbon-chlorine bond during dehalogenation by cobalamin containing dehalogenases may produce the primary carbon isotope effect. The protonation reaction of the substrate only to some extent controls the overall kinetic of the reaction and may occur after the transfer of the electron and the cleavage of the carbon-chlorine bond, as was indicated by the moderate solvent kinetic effect of deuterated water (Krasotkina et al., 2001). This reaction scheme would explain the carbon isotope and chlorine isotope fractionation observed during the dehalogenation of PCE and TCE and halogenated benzenes (Sherwood Lollar et al., 2001; Numata et al., 2002; Griebler et al., 2004a; Nijenhuis et al., 2005). However, the extent of kinetic carbon stable isotope fractionation during reductive dechlorination by living microorganisms could also be affected by factors such as substrate binding to enzymes, transport processes across membranes and cell wall, which can modify the kinetic of the overall reaction. For example, O'Leary showed that transport of the substrate across the membrane can affect carbon isotope fractionation (O'Leary, 1981; Roeske and O'Leary, 1984).

Enzyme catalyzed reactions involve a sequence of steps which may be represented by binding of the substrate to the enzyme in the first and dissociation of the product from the enzyme in the last step. Although the number of intermediate steps in unidirectional biochemical reactions may be unknown, the kinetic isotope fractionation should be caused by the first irreversible reaction step in the reaction sequence associated with the conformational change of a chemical bond. The resistance of membranes can modify the substrate flow into the cell and may lead to isotope non-equilibrium conditions between substrate in the cell and the medium. Furthermore, the formation of the enzyme substrate complex can be a rate limiting step in the reaction before the biochemical reaction takes place. Thus, kinetic limitation due to physiological processes can influence isotope fractionation and depress the extent of fractionation by the pure biochemical reaction (O'Leary, 1981; Roeske and O'Leary, 1984).



Our previous investigations showed that microorganisms belonging to different genera, *Sulfurospirillum multivorans* and *Desulfitobacterium* sp. strain PCE-S, had a fractionation factor for PCE differing by one order of magnitude (Nijenhuis et al., 2005). Fractionation of PCE was the highest with cyanocobalamin, a model compound for the corrinoid cofactor of the reductive dehalogenases. Additionally, a decrease in fractionation was observed with an increasing integrity of the cells, comparing fractionation by the model reactive site, cyanocobalamin, the purified enzyme, the crude extracts after cell wall destruction, and metabolically active cells. Apparently, the increasing complexity of the reactive system caused a rate limitation lowering the extent of isotope fractionation.

Interestingly, although PCE fractionation during dechlorination by *S. multivorans* was very low, TCE was obviously fractionated. Therefore, in this study, we investigated carbon isotope fractionation of TCE compared to PCE during reductive dechlorination by *S. multivorans*, and its close relative *S. halorespirans*, belonging to the  $\epsilon$ -proteobacteria. Studies with *Desulfitobacterium* sp. strain PCE-S as a model strain for gram positive microorganisms were performed. These three strains have been studied in detail before and therefore were used as model systems representing two bacterial genera (Scholz-Muramatsu et al., 1995; Miller et al., 1996, 1997, 1998; Luijten et al., 2003). Kinetic rate limitation of transport processes, due to uptake and membrane barriers, and substrate binding was investigated comparing TCE fractionation by the metabolically active cells, crude extracts of the three strains and the purified PCE-reductive dehalogenase of *S. multivorans*. Furthermore, effects of growth substrate and resulting physiological changes on fractionation were investigated using crude extracts of *S. multivorans* prepared with cells grown with TCE or PCE. Cyanocobalamin was included in this study as the model for the biochemical reaction at the centre of reductive dehalogenases.

## 2.2 Materials and methods

### 2.2.1 Chemicals

All chemicals were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich Chemie, Munich, Germany) at the highest purity available unless stated elsewhere. Gases were purchased from Airproducts (Hattingen, Germany).

### 2.2.2 Cultivation of bacteria and preparation of crude extracts

*S. multivorans* (Scholz-Muramatsu et al., 1995; Miller et al., 1996), *S. haloferax* (Luijten et al., 2003) and *Desulfitobacterium* sp. strain PCE-S (Miller et al., 1997) were cultured as described previously with PCE as the electron acceptor except as indicated. Pyruvate and fumarate (*S. multivorans*), lactate (*S. haloferax*) and pyruvate (*Desulfitobacterium* sp. strain PCE-S) were added as the electron donor and carbon source. Crude extracts and the purified PCE-reductive dehalogenase were prepared as described previously and assays were performed using reduced methyl viologen as the electron donor (Nijenhuis et al., 2005). All preparations were performed anoxically within an anaerobic glovebox (Coy Laboratory Products Inc., USA) with N<sub>2</sub>/H<sub>2</sub> (96%/4%) atmosphere or under a stream of nitrogen.

### 2.2.2 Analytical methods

Gas chromatography system (Varian Chrompack CP-3800, Middelburg, The Netherlands) with flame ionisation detection (GC-FID) equipped with a 30m x 0.53mm GS-Q column (J&W Scientific, Waldbronn, Germany) was applied to analyze ethene and the chlorinated ethenes and gas chromatography combustion isotope ratio mass spectroscopy (GC-C-IRMS) was applied to determine the stable carbon isotope composition of the chlorinated ethenes as described before (Nijenhuis et al., 2005). All samples were measured in at least three replicates.



### 2.2.3 Determination of stable isotope fractionation factors

Dehalogenation experiments of TCE with cyanocobalamin were performed under the reaction conditions described elsewhere (Lesage et al., 1998; Nijenhuis et al., 2005). TCE was added at an initial concentration of 1 mM. Ti(III)citrate was used to reduce cyanocobalamin. The samples were stored at 4°C until headspace analysis.

For the determination of stable isotope fractionation by growing cultures of *S. multivorans* and *Desulfitobacterium* sp. strain PCE-S, several parallel cultures were prepared which were sacrificed after appropriate growth periods for analysis of the concentration and isotope composition of the chlorinated ethenes as described before (Nijenhuis et al., 2005). All cultures received PCE (500  $\mu\text{mol l}^{-1}$ ) or TCE (600  $\mu\text{mol l}^{-1}$ ) and pyruvate (*Desulfitobacterium* sp. strain PCE-S and *S. multivorans*) or lactate (*S. halorespirans*) as the electron donor. At various incubation times, 500  $\mu\text{l}$  gas phase was removed from one culture and analyzed for chlorinated ethenes by GC-FID and the respective culture was then killed by addition of 5 ml aerobic, saturated  $\text{Na}_2\text{SO}_4$ , pH 1. For extraction of the PCE, TCE and dichloroethene (DCE), 2 ml pentane was added to each culture which was incubated on a rotary shaker overnight (25°C). The pentane phase was then removed, transferred to autosampler vials and analyzed by GC-C-IRMS for isotopic composition of the chlorinated ethenes. Due to the relatively high losses for DCE during the extraction, mass balances for the chlorinated ethenes could not be completely closed. Isotope fractionation by crude extracts and partially purified enzyme was determined as described before (Nijenhuis et al., 2005). PCE or TCE were added dissolved in ethanol in a final concentration of 1 mM and methyl viologen (1.6 mM), reduced with Ti(III)citrate, was used as the electron donor. Ti(III)citrate was added in various concentrations to reach approx. 0, 20, 40, 60, 80, or 100% dechlorination of the substrate. After complete reaction time, 0.5 ml headspace was removed and analyzed by GC-FID for determination of the concentrations of the chlorinated ethenes, then 1 ml pentane was added to each vial to extract the chlorinated ethenes for analysis by GC-C-IRMS.

### 2.2.4 Calculations and definitions

The carbon isotope composition (R) is reported in  $\delta$ -notation [‰] relative to the Vienna Pee Dee Belemnite standard (V-PDB, IAEA-Vienna) (eq. 2.1) (Coplen et al., 2006).

$$\delta^{13}\text{C}[\text{‰}] = \left( \frac{\left( \frac{^{13}\text{C}}{^{12}\text{C}} \right)_{\text{Sample}}}{\left( \frac{^{13}\text{C}}{^{12}\text{C}} \right)_{\text{Standard}}} - 1 \right) \times 1000 \quad \text{eq. 2.1}$$

The isotope fractionation during the dehalogenation reaction was calculated applying the Rayleigh-equation (eq. 2.2) (Hoefs, 1997).

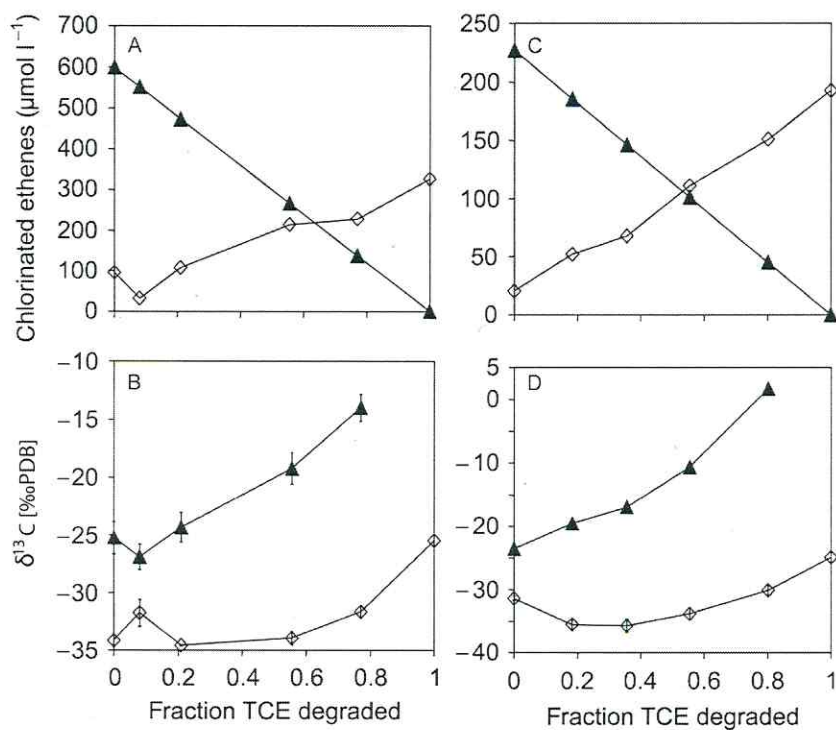
$$\ln \left( \frac{R_t}{R_0} \right) = (1/\alpha_C - 1) \times \ln \left( \frac{C_t}{C_0} \right) \quad \text{eq. 2.2}$$

The kinetic carbon isotope fractionation factor  $\alpha_C$  is a constant for the reaction at given experimental conditions.  $R_t$  and  $R_0$  are the isotope compositions and  $C_t$  and  $C_0$  are the concentrations of the compound at times  $t$  and zero, respectively. The isotope fractionation factor  $\alpha_C$  relates the changes in the isotope composition to changes in concentration of the residual fraction during the transformation. The 95% confidence interval [CI (95%)] was determined on the slope of the linear regression of the  $\ln R_t/R_0$  vs.  $\ln C_t/C_0$  as defined in eq. 2.2 and reported as  $\alpha_C \pm \text{CI (95\%)}$ . The enrichment factor ( $\varepsilon$ ) is defined as  $\varepsilon = (\alpha_C - 1) \times 1000$ .

## 2.3 Results

### 2.3.1 Carbon isotope fractionation during growth

Previously it was shown that isotope fractionation of PCE by *S. multivorans* was one order of magnitude lower compared to fractionation by *Desulfotobacterium* sp. strain PCE-S (Nijenhuis et al., 2005).



**Figure 2.1** Change in concentrations (A, C) and carbon isotope composition (B, D) of TCE (▲) and the product *cis*-DCE (◊), during reductive dechlorination by growing cultures of *Desulfotobacterium* sp. strain PCE-S (A, B) and *S. multivorans* (C,D). The standard deviation of typically less than 0.5 ‰-units for the isotope measurement is usually smaller than the symbol.

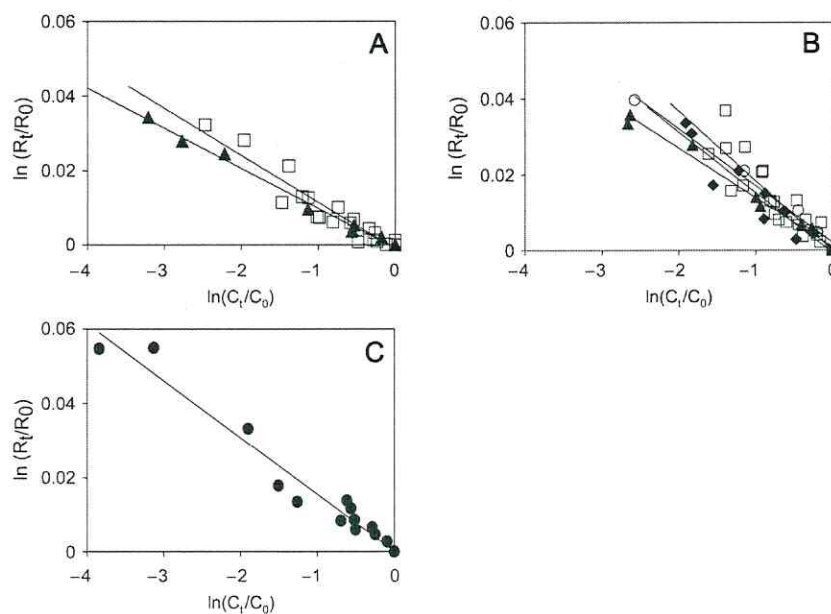
*Desulfitobacterium* sp. strain PCE-S dechlorinates PCE via TCE to *cis*-DCE (Miller et al., 1997). It is assumed the same enzyme performs the reaction of PCE to TCE and of TCE to *cis*-DCE. During the subsequent reductive dechlorination of TCE by *Desulfitobacterium* sp. strain PCE-S the concentration of TCE decreased from 600 to 140  $\mu\text{mol l}^{-1}$  with a corresponding change of the isotope signature from -25.2‰ to -14.0‰ (Fig. 2 A, B). The isotope signature of *cis*-DCE changed from -34.2‰ to -25.5‰ at the end of the experiment, similar to the initial signature of the added TCE indicating a closed isotope balance. Fractionation of TCE, with  $\alpha_C=1.0122\pm0.0023$ , was higher compared to previous reported values for PCE ( $\alpha_C=1.0054\pm0.0015$ ) (see Table 2.1) (Nijenhuis et al., 2005).

*S. multivorans* also dechlorinates TCE to the end product *cis*-DCE and it is assumed that both dechlorination steps, PCE to TCE and TCE to *cis*-DCE are performed by the same enzyme (Neumann et al., 2002). Opposite to previous experiments with PCE, significant changes were observed in the isotope composition of TCE ranging from -23.5‰ up to +0.8‰ (Fig. 2.1 C, D). The corresponding isotope fractionation factor,  $\alpha_C=1.0187\pm0.0042$  for *S. multivorans*, was more than one order of magnitude higher than isotope fractionation for PCE ( $\alpha_C=1.00042\pm0.00021$ ) (Nijenhuis et al., 2005). Similar changes in carbon isotope composition were observed for the close relative *S. halorespirans* for TCE with  $\alpha_C=1.0189\pm0.0010$  (Table 2.1). Fractionation of PCE by *S. halorespirans* ( $\alpha_C=1.00046\pm0.00019$ ) was similar to values found previously for *S. multivorans* (Nijenhuis et al., 2005).

### 2.3.2 Carbon isotope fractionation by crude extracts; effect of uptake processes

Our previous investigation showed that the observed PCE fractionation was apparently affected by rate limitation. To investigate if rate limitation plays a role during TCE fractionation, crude extracts and the purified enzyme were investigated. Crude extracts of *Desulfitobacterium* sp. strain PCE-S had a similar fractionation factor compared to the growing cells ( $\alpha_C=1.0109\pm0.0011$ ) and no significant difference could be

observed (Fig. 2.2 A, Table 2.1). Similarly, for *S. multivorans* carbon stable isotope fractionation by crude extracts prepared from cells grown with TCE as the substrate was in the same order of magnitude ( $\alpha_C=1.0162\pm0.0037$ ) compared to growing cells ( $\alpha_C=1.0187\pm0.0042$ ) (Fig. 2.2 B; Table 2.1).



**Figure 2.2** Stable isotope fractionation of the residual fraction of TCE during reductive dechlorination by A) growing cells ( $\square$ ;  $R^2=0.876$ ); and crude extracts ( $\blacktriangle$ ;  $R^2=0.991$ ) of *Desulfitobacterium* sp. strain PCE-S, and B) growing cells ( $\square$ ;  $R^2=0.838$ ), crude extracts ( $\blacktriangle$ ;  $R^2=0.986$ ), crude extracts prepared from cells grown with TCE ( $\blacklozenge$ ;  $R^2=0.921$ ) and the purified reductive dehalogenase ( $\circ$ ;  $R^2=0.988$ ) of *S. multivorans*, and C) reduced cyanocobalamin ( $\bullet$ ;  $R^2=0.961$ ).



**Table 2.1** Compound specific carbon stable isotope fractionation factors ( $\alpha_C$ ) for reductive dechlorination of PCE by *S. multivorans*, *Desulfotobacterium* sp. strain PCE-S and abiotic reactions with cyanocobalamin (vitamin B<sub>12</sub>).

	PCE		TCE			
	$\alpha$ C	R <sup>2</sup> <sup>a</sup>	CI (95%) <sup>b</sup>	$\alpha$ C	R <sup>2</sup> <sup>a</sup>	CI (95%) <sup>b</sup>
<i>Desulfotobacterium</i> sp. strain PCE-S						
growth	1.0054 <sup>c</sup>	0.931	0.0015	1.0122	0.876	0.0023
crude extract	1.0089 <sup>c</sup>	0.933	0.0018	1.0109	0.991	0.0011
<i>Sulfurospirillum multivorans</i>						
growth	1.00042	0.668	0.00021	1.0187	0.838	0.0042
growth substrate						
PCE	1.00097 <sup>c</sup>	0.949	0.0002	1.0132	0.986	0.0018
TCE	1.0014	0.924	0.0003	1.0162	0.921	0.0037
enzyme	1.0017 <sup>c</sup>	0.910	0.0021	1.0150	0.988	0.0039
<i>Sulfurospirillum halorespirans</i>						
growth	1.00046	0.763	0.00019	1.0189	0.993	0.0010
growth substrate						
PCE	1.0024	0.926	0.0008	1.0229	0.977	0.0037
TCE	1.0032	0.997	0.0004	1.0187	0.995	0.0032
Abiotic						
Cyanocobalamin	1.0132 <sup>c</sup>	0.983	0.0015	1.0154	0.961	0.0021

Eq. 2.2 and the data from Fig. 2.2 were used to calculate  $\alpha_C$ .

<sup>a</sup> The co-efficient of determination (R<sup>2</sup>) was calculated from the complete data set.

<sup>b</sup> CI(95%)= 95% confidence interval determined on the slope of the linear regression of the  $\ln R_t/R_0$  vs.  $\ln C_t/C_0$  as defined in eq. 2.2.

<sup>c</sup> Nijenhuis et al. (2005).

Fractionation by crude extracts of *S. halorespirans* prepared from cells grown with TCE ( $\alpha_C=1.0187\pm0.0032$ ) was almost identical to the fractionation by growing cells ( $\alpha_C=1.0189\pm0.0010$ ) (Table 2.1). The highest fractionation was observed for the reductive dechlorination of TCE by crude extracts prepared from *S. halorespirans* cells grown with PCE as the electron acceptor ( $\alpha_C=1.0229\pm0.0037$ ). On the contrary, fractionation by crude extracts prepared from *S. multivorans* cells grown with PCE as the electron acceptor had much lower fractionation ( $\alpha_C=1.0132\pm0.0018$ ), the lowest observed for this organism (Fig. 2.2 B; Table 2.1).

### 2.3.3 Carbon isotope fractionation by the purified enzyme *S. multivorans*

Since PCE fractionation by *S. multivorans* was more than one order of magnitude lower compared to TCE by both growing cultures and crude extracts, we considered the possibility of the presence of another dehalogenase. Therefore, the purified reductive dehalogenase of *S. multivorans* was used to investigate carbon isotope fractionation of TCE. Interestingly, the purified enzyme also had a similar fractionation ( $\alpha_C=1.015\pm0.0039$  for TCE and  $\alpha_C=1.0017\pm0.0021$  for PCE (Nijenhuis et al., 2005) compared to the crude extracts and growing cells. This may indicate that the difference in fractionation between PCE and TCE by growing cells of *S. multivorans* was most probable not due to reaction with different enzymes.

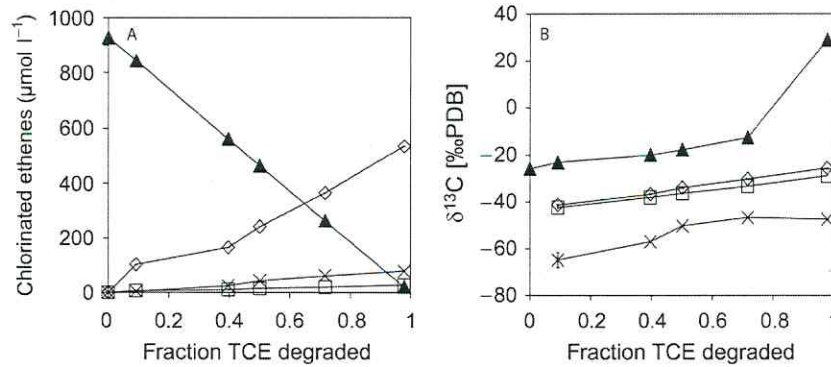
### 2.3.4 Comparison of crude extract from PCE vs. TCE grown cells

To investigate, if the growth substrate had an overall effect on the physiology and on fractionation, crude extracts were prepared from cells grown with either PCE or TCE present as the electron acceptor. Fractionation of PCE was similar when crude extracts were prepared from cells grown with PCE or TCE with  $\alpha_C=1.00097\pm0.0002$  and  $\alpha_C=1.0014\pm0.0003$  for *S. multivorans* crude extracts from PCE and TCE grown cells respectively; and  $\alpha_C=1.0024\pm0.0008$  and  $\alpha_C=1.0032\pm0.0004$  for *S. halorespirans* crude extracts from PCE and TCE grown cells

respectively (Table 2.1). Fractionation of TCE was not significantly different: crude extracts of *S. multivorans* fractionated TCE similarly when prepared from PCE grown cells ( $\alpha C=1.0132\pm0.0018$ ) or TCE grown cells ( $\alpha C=1.0162\pm0.0037$ ). Fractionation of TCE by crude extracts of *S. halorespirans* pregrown with PCE ( $\alpha C=1.0229\pm0.0037$ ) or TCE grown cells ( $\alpha C=1.0187\pm0.0032$ ) was not significantly different either (Table 2.1). Although some variation in fractionation could be observed, the differences were very small and within the 95% confidence interval.

### **2.3.5 Carbon isotope fractionation at the model reaction site (cobalamin)**

Cyanocobalamin (vitamin B<sub>12</sub>) was used as the model compound for the cofactor of the reductive dehalogenases. Thus far all identified reductive dehalogenases, except for the 3-chlorobenzoate reductive dehalogenase of *Desulfomonile tiedjei*, were found to contain a corrinoid cofactor (Holliger et al., 2002). TCE was dechlorinated to *cis*-DCE as well as minor amounts of *trans*-DCE and ethene during the abiotic reaction with reduced cyanocobalamin (Fig. 2.3). The isotope signature of TCE increased from -25.8‰ to +29.0‰; the products *cis*-DCE, *trans*-DCE and ethene changed from -41.2‰ to -25.6‰, -42.4‰ to -28.8‰ and -64.8‰ to -47.4‰ respectively. The isotope fractionation factor for TCE was, in this case,  $\alpha C=1.0154\pm0.0021$  (Table 2.1; Fig. 2.2 C) similar to the fractionation previously observed by Slater et al. (2003).



**Figure 2.3** Change in concentrations (A) and carbon isotope composition (B) of TCE (▲) and the products *cis*-DCE (◇), *trans*-DCE (□) and ethene (×) during reductive dechlorination by abiotic reactions with reduced cyanocobalamin. The standard deviation of typically less than 0.5 δ-units for the isotope measurement is usually smaller than the symbol.

## 2.4 Discussion

### 2.4.1 Isotope fractionation effects of the reaction mechanism

Kinetic stable isotope fractionation of substrates in microbial degradation processes largely depends on the biochemical reaction mechanism. Therefore, if the biochemical reaction mechanism determines the observed isotope fractionation, fractionation would be similar in case the same reaction mechanism is used. The model for the cofactor of reductive dehalogenases, cyanocobalamin (Vitamin B<sub>12</sub>) had a fractionation factor for TCE ( $\alpha_C=1.0154$ ) within the range observed for *Sulfurospirillum* spp. ( $\alpha_C=1.0132$  to  $1.0229$  as the upper and lower limits); but was higher compared to *Desulfitobacterium* PCE-S ( $\alpha_C=1.0109$  to  $1.0122$ ). Usually, it is expected that the fractionation is highest with the pure chemical reaction, in this case with the corrinoid as the direct catalyzer. The kinetic isotope fractionation should be caused by the first irreversible reaction step in a reaction sequence, but any preceding step, such as uptake of substrate by the degrading microorganism or binding of



the substrate to the enzyme, may affect the overall kinetics of the reaction. As an example, membrane resistance to a substrate can modify the substrate flow into the cell and may lead to isotope non-equilibrium conditions between substrate in the cell and the medium reducing the observed fractionation (O'Leary and Yapp, 1978). Because the fractionation is mainly affected by the cleavage of the chemical bond in the case of reaction with the corrinoid, factors like enzyme binding or transport across membranes influencing the reaction kinetics can be excluded.

However, the cobalamin cofactor of the PCE dehalogenase of *S. multivorans* is a novel and unusual type of corrinoid characterized as a norpseudob<sub>12</sub> (Kräutler et al., 2003) and the PCE dehalogenase of *Desulfitobacterium* sp. strain PCE-S contains another different type of corrinoid cofactor (A. Schilhabel, personal communication). The structure of the cofactor could potentially affect the enzymatic dehalogenation reaction and may explain the differences in fractionation between our model corrinoid and the purified enzyme of *S. multivorans*.

The isotope fractionation of TCE was, in all cases, larger than of PCE as has been observed before (Table 2.1) (Hunkeler et al., 1999; Bloom et al., 2000). For *Desulfitobacterium* sp. strain PCE-S, fractionation of TCE was about double compared to PCE (factor of 2.4). More interestingly, TCE fractionation by *S. multivorans* and *S. haloalkaliphilus* was more than one order of magnitude higher (38 to 47.5 fold respectively) compared to PCE although a similar mechanism for dehalogenation is suggested (Neumann et al., 2002). This indicates that a kinetic bottleneck, resulting from uptake and binding of the substrate to the enzyme, is present for PCE, lowering the kinetic isotope effect, but not for TCE. Apparently, a rate limiting step is present before the reaction with PCE resulting in a isotope non-equilibrium of the substrate in the different compartments for example surrounding atmosphere, vacuoles and reactive enzyme as observed during the overall CO<sub>2</sub> fixation process in green plants by O'Leary and Yapp (1978). For TCE, there is apparently no such transport limitation and it appears that TCE concentration and isotope composition are in equilibrium between all cell compartments and medium resulting in similar fractionation in the various biological systems.



Our investigation also demonstrated that the interpretation of isotope fractionation factors for the analysis of biochemical degradation mechanisms as suggested recently (Zwank et al., 2005b) may become very difficult when substrate uptake or binding lower the extent of isotope fractionation significantly. In case of microbial growth experiments preceding rate limiting steps such as uptake of substrates by the cell, transport or binding to the enzyme can be rate limiting and thus may decrease the maximal possible isotope fractionation of the pure biochemical reaction. These factors have to be taken in account for mechanistic interpretation of isotope effects.

The relative difference in fractionation of TCE to PCE by *S. multivorans* was in the same order of magnitude comparing growing cultures, crude extracts and the purified enzyme. Apparently, the same enzyme is involved in the dechlorination of both substrates therefore, the difference in TCE and PCE fractionation must be due to the reaction at the enzyme. Possibly, the dehalogenation reactions of PCE and TCE proceed by different mechanisms but are catalyzed by the same enzyme. The previously reported values for enzyme kinetics ( $K_m$  and  $V_{max}$ ) for PCE and TCE were quite similar (Neumann et al., 2002). Thus, the overall kinetics of the complex biochemical reaction gives no clue to explain these observed differences in fractionation.

*S. halorespirans*, a close relative of *S. multivorans*, is thought to have a similar enzymatic system with only minor differences (identity of 91% of the catalytic unit of the reductive dehalogenase PceA; (AY013367) NCBI database ([www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)) compared to *S. multivorans* (RdhA; AJ539530)). On the one hand, the observed differences between *S. multivorans* and *S. halorespirans*, could be due to the diversity in enzyme structure as observed before for aerobic dichloromethane degradation (Nikolausz et al., 2006). On the other hand, the enzyme of *Desulfitobacterium* sp. strain PCE-S (AY216592; [www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)) has a much lower identity (27% compared to both dehalogenases from the *Sulfurospirillum* strains) coinciding with the different fractionation properties.

#### **2.4.2 Effects of uptake and transport over membrane barriers on isotope fractionation**

Membrane barriers and uptake of a substrate may cause rate limitation, reducing the extent of observed isotope fractionation (O'Leary, 1981; Nijenhuis et al., 2005). Our previous investigation showed that when PCE was used as the substrate the observed isotope fractionation due to uptake and binding to the enzyme was reduced comparing intact growing cells to crude extracts and crude extracts to the purified enzyme (Nijenhuis et al., 2005). For TCE, factors like uptake and binding apparently did not decrease the observed isotope fractionation since no significant differences could be observed when comparing equivalent systems (pre-grown with the same substrate) (Table 2.1). Fractionation of TCE by growing cells compared to crude extracts was very similar for the three investigated strains while fractionation of PCE by crude extracts was 1.7, 2.3 and 5.2 fold higher compared to fractionation by growing cells of *Desulfitobacterium* sp. strain PCE-S, *S. multivorans* and *S. halorespirans*, respectively.

#### **2.4.3 Effects of growth substrate on fractionation by crude extracts**

In addition to rate limitation and enzyme mechanism other factors, such as the growth substrate used to prepare crude extracts, may influence the observed isotope fractionation. The growth substrate (PCE or TCE) may affect the membrane composition and therefore affect substrate transport due to the membrane toxicity of solvents (Ramos et al., 2002). For the application of stable isotope fractionation analysis for the assessment of *in situ* biodegradation it is important to know potential effects due to the presence of specific growth substrates, PCE or TCE, or both. In many cases pollutants occur in mixtures and at any PCE contaminated site, TCE may be present as degradation product. The growth substrate did not affect the extent of isotope fractionation to a significant extent when PCE or TCE was tested with crude extracts. PCE fractionation by crude extracts when cells were grown with TCE was similar ( $\alpha_C=1.0014\pm0.0003$  and  $\alpha_C=1.0032\pm0.0004$  for *S. multivorans* and *S. halorespirans* respectively) to crude extracts prepared from cells grown

with PCE ( $\alpha_C=1.00097\pm0.00018$  and  $\alpha_C=1.0024\pm0.0008$  for *S. multivorans* and *S. halorespirans* respectively). Correspondingly, for TCE, fractionation was within the 95% confidence intervals comparing TCE grown crude extracts to crude extracts prepared from cells pre-grown on PCE for both *Sulfurospirillum* spp. Only very minor differences, which were not significant with respect to the confidence intervals (95% CI), were observed.

Thus, the growth substrate did not have significant effects on isotope fractionation. Previously it was observed that the reductive dehalogenase of *S. multivorans* was growth-substrate dependent, the PCE-reductive dehalogenase was found facing the cytoplasm in absence of PCE or TCE, while it was facing the periplasm in presence of these chlorinated ethenes (John et al., 2006). Therefore, although *S. multivorans* adapts to the presence or absence of certain substrates, this adaptation did not influence fractionation of PCE or TCE significantly, suggesting that presence of contaminant mixtures also will not change biochemical fractionation patterns.

#### **2.4.4 Implication for the assessment of *in situ* biodegradation using isotope fractionation**

In recent years, compound specific isotope fractionation analysis (CSIA) has become a powerful tool to assess *in situ* biodegradation of pollutants in aquifers (Sherwood Lollar et al., 2001; Richnow et al., 2003; Griebler et al., 2004b; Meckenstock et al., 2004). Before CSIA can be applied quantitatively, selection of an appropriate and representative fractionation factor for the active biodegrading microbial community within the aquifer is necessary. Knowledge on the variability of isotope fractionation associated with physiological factors and mechanisms is important for evaluating the uncertainty when using isotope pattern to quantify *in situ* biodegradation. We reported that the isotope fractionation of PCE by *S. multivorans* was about one order of magnitude lower compared to *Desulfitobacterium* sp. strain PCE-S and fractionation by mixed cultures (Sherwood Lollar et al., 2001; Nijenhuis et al., 2005) which should be taken into account when interpreting field data. In contrast,



fractionation of TCE was in a similar order of magnitude for both *Sulfurospirillum* spp. and *Desulfitobacterium* strain PCE-S potentially allowing a more precise assessment of *in situ* degradation by means of CSIA. Therefore, a further systematic work with pure cultures is important to assess the range of isotope fractionation during reductive dehalogenation. Furthermore, our investigation showed that the growth substrate (PCE or TCE) did not affect fractionation to a significant extent suggesting that CSIA could also be applied for contamination with mixtures of chlorinated ethenes. A wider knowledge on the variability of fractionation factors and the composition of the microbial consortium in the aquifer may be necessary for selection of an appropriate fractionation factor for quantitative analysis as various groups of microorganisms may be present at one field site (Nijenhuis et al., 2007).

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**Variability in microbial carbon isotope  
fractionation of tetra- and trichloroethene  
upon reductive dechlorination**

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### Abstract

The variability of stable carbon isotope fractionation upon reductive dechlorination of tetra- and trichloroethene by several microbial strains was investigated to examine the uncertainties related to the *in situ* application of compound specific isotope analysis (CSIA) of chlorinated ethenes. Carbon isotope fractionation was investigated with a set of microorganisms representative for the currently known diversity of dehalorespirers: *Dehalococcoides ethenogenes* strain 195, *Desulfitobacterium* sp. strain Viet1, *Desulfuromonas michiganensis* and *Geobacter lovleyi* strain SZ and compared to the previous reports using *Sulfurospirillum* spp. and *Desulfitobacterium* sp. strain PCE-S. Carbon isotope fractionation of tetrachloroethene (PCE) and trichloroethene (TCE) was highly variable ranging from the absence of significant fractionation to carbon isotope fractionation ( $\epsilon_C$ ) of 16.7 and 3.5 to 18.9 for PCE and TCE, respectively. Fractionation of both compounds by *D. ethenogenes* strain 195 (PCE:  $\epsilon_C$ =6.0; TCE:  $\epsilon_C$ =13.7) was similar to the literature data for mixed cultures containing *Dehalococcoides* spp. *D. michiganensis* (PCE: no significant fractionation; TCE:  $\epsilon_C$ =3.5) and *G. lovleyi* sp. strain SZ (PCE no significant fractionation; TCE:  $\epsilon_C$ =8.5) generated the lowest fractionation of all studied strains. *Desulfitobacterium* sp. strain Viet1 (PCE:  $\epsilon_C$ =16.7) gave the highest enrichment factor for PCE.

**Keywords:** PCE, TCE, dehalorespiration, enrichment factors

### 3. 1 Introduction

The chlorinated ethenes, tetrachloroethene (PCE) and trichloroethene (TCE), are among the most common groundwater contaminants worldwide (Rivett et al., 2006). In the last decade, assessment and quantification of natural attenuation of these and other contaminants has become of great interest because of all processes contributing to natural attenuation such as dilution, absorption or biodegradation, only biodegradation is considered to be a sustainable sink of pollutants (www.natural-attenuation.de; Pope and Jones, 1999).

Compound specific isotope analysis (CSIA) is one of the monitoring methods which is increasingly used to assess and quantify *in situ* biodegradation (Mancini et al., 2002; Meckenstock et al., 2004; Kuder et al., 2005; Zwank et al., 2005a; Fischer et al., 2007; Holmstrand et al., 2007). CSIA takes advantage of stable isotope fractionation during degradation of organic contaminants. Isotope fractionation results from the lower activation energies needed to break chemical bonds formed by light (e.g.  $^{12}\text{C}$ ,  $^1\text{H}$ ) compared to heavy (e.g.  $^{13}\text{C}$ ,  $^2\text{D}$ ) isotopes. Light isotopes form weaker bonds and thus react faster than the heavy isotopes, leading to an enrichment of heavy isotopes in the not yet degraded residual fraction (Hoefs, 1997; Meckenstock et al., 2004). *In situ*, enrichment in  $^{13}\text{C}$  in the residual contaminant fraction may then be used as a qualitative indicator for biodegradation (Meckenstock et al., 2004). Additionally, by use of the Rayleigh equation, the extent of biodegradation at contaminated sites may be quantified (Fischer et al., 2006). This approach has been successfully applied to assess the biodegradation of aromatic hydrocarbons like benzene, toluene and *o*-xylene (Richnow et al., 2003; Griebler et al., 2004b; Mak et al., 2006; Fischer et al., 2007).

For a quantitative assessment of *in situ* biodegradation using the Rayleigh concept selection of an appropriate isotope fractionation factor ( $\alpha\text{C}$ ), which is representative for the microbial degradation pathway, is necessary (Sherwood Lollar et al., 2001; Richnow et al., 2003). The kinetic isotope fractionation factor relates changes in isotope composition to changes in concentration and is determined in reference experiments (Hoefs, 1997). Isotope fractionation also can be expressed as the

enrichment factor ( $\epsilon_C$ ). Isotope fractionation depends on the biochemical reaction mechanism, and therefore fractionation factors should be determined in experiments with cultures whose degradation pathways are known. Meckenstock et al. (1999) have shown that the variability of isotope fractionation upon anaerobic degradation of toluene was low in experiments with three different pure bacterial cultures ( $\epsilon_C=1.7$  to  $1.8$ ) which use the benzylsuccinate pathway for degradation. Thus, for toluene the isotope fractionation factor representative of the anaerobic degradation pathway could be selected and used for the quantification of *in situ* degradation.

Nevertheless, isotope fractionation of a substrate can vary to some extent due to structural variability of the enzymes of the microorganisms involved in the reaction, as found during aerobic dichloromethane degradation ( $\epsilon_C=43$  to  $71$ ) (Nikolausz et al., 2006). Recently, carbon stable isotope fractionation during reductive dechlorination of the common groundwater pollutants tetrachloroethene (PCE) and trichloroethene (TCE) was investigated (Hunkeler et al., 1999; Bloom et al., 2000; Sherwood Lollar et al., 2001; Nijenhuis et al., 2005; Cichocka et al., 2007). Biochemical analysis of chloroethene reductive dehalogenases, key catalytic enzymes in the respiratory chain of dehalorespirers, has revealed that all thus far investigated enzymes contain a corrinoid cofactor (Smidt and de Vos, 2004). Similarities between enzymes and presence of the same degradation pathway (Smidt and de Vos, 2004) would suggest that reductively dechlorinating organisms generate a similar isotope fractionation. Interestingly, recent studies showed that microorganisms belonging to two different phylogenetic groups generated a different extent of stable isotope fractionation (Nijenhuis et al., 2005; Cichocka et al., 2007). Fractionation of PCE by *Desulfitobacterium* sp. strain PCE-S ( $\epsilon_C=5.2$ ) was one order of magnitude higher than for *Sulfurospirillum* spp. ( $\epsilon_C=0.4$  to  $0.5$ ) (Nijenhuis et al., 2005). In contrast, fractionation of TCE was in the same range for both genera ( $\epsilon_C=12.2$  to  $18.9$ ) (Cichocka et al., 2007). Fractionation of PCE and TCE by *Desulfitobacterium* sp. strain PCE-S was similar to previously published values for mixed cultures while



fractionation by *Sulfurospirillum* spp. was unlike the fractionation described previously in the literature (Hunkeler et al., 1999; Bloom et al., 2000).

Since various groups of dehalogenating bacteria have been found at field sites (Löffler et al., 2000; Nijenhuis et al., 2007) and it is usually not clear which group dominates the *in situ* reductive dehalogenation process, variability in isotope fractionation by dehalogenating strains would pose a limitation for the application of CSIA. In case of reductive dechlorination of chlorinated ethenes, selection of a representative fractionation factor would become complicated and the estimated biodegradation would vary widely especially if only a small shift in isotope composition was present *in situ* (Meckenstock et al., 2004). The selection of an appropriate fractionation factor would require knowledge about the *in situ* microbial community actively involved in the transformation. Therefore, the aim of this study was to assess the variability of isotope fractionation of PCE and TCE during reductive dechlorination.

The extent of the kinetic isotope effect depends on rate limitation in the transition state of the bond cleavage and the nature of the chemical reaction (Northrop, 1981). However, in biological systems the enzyme catalyzed reactions can be complex. Rate limiting reaction steps, lowering the “apparent” isotope effect observed in the experiment, may precede the isotope sensitive bond cleavage (Northrop, 1981). Thus, the expression of isotope fractionation in biological experiments can be affected by processes like: uptake into the cell, transport across membranes or binding to the enzyme. Factors controlling reaction steps taking place before bond cleavage are likely to be dependent on physiological characteristic of the degrading microorganism. Therefore, the variability of carbon isotope fractionation during reductive dechlorination may be due to 1) differences in reaction mechanism, 2) properties of the dehalogenases, 3), location of the enzyme, and/or 4) to physiological properties of the dehalogenating microorganism (e.g. membranes). Consequently, significant differences in isotope fractionation during reductive dechlorination may be present among the known phyla of dehalogenators since these are highly diverse.



For this work we investigated the variability of isotope fractionation factors during dehalogenation which is one important factor posing uncertainty on the application of CISA to characterise *in situ* degradation of PCE and TCE. Selected strains, representatives of different genera of dehalogenating bacteria were therefore investigated: *Desulfitobacterium* sp. strain Viet1 from the Firmicutes phylum, *Desulfuromonas michiganensis* and *Geobacter lovleyi* sp. strain SZ belonging to the  $\delta$ -subclass of the Proteobacteria and *Dehalococcoides ethenogenes* strain 195 from the Chloroflexi phylum. We compared our results to fractionation factors from previous studies with pure strains (*Sulfurospirillum* spp. and *Desulfitobacterium* sp. strain PCE-S) and mixed cultures and provided an overview of the variability of isotope fractionation of PCE and TCE during reductive dehalogenation.

## 3.2 Materials and methods

### 3.2.1 Chemicals

All chemicals were purchased from Fluka (Seelze, Germany), Sigma-Aldrich Chemie (Seelze, Germany) or Merck (Darmstadt, Germany) at the highest purity available. Gases were purchased from Airproducts (Hattingen, Germany).

### 3.2.2 Cultivation of bacteria

*Desulfitobacterium* sp. strain Viet1 (Löffler et al., 1997; Löffler et al., 1999), *Desulfuromonas michiganensis* (Sung et al., 2003), *Geobacter lovleyi* sp. strain SZ (Sung et al., 2006a) were cultured as described previously with PCE or TCE as the electron acceptor. For *D. michiganensis* and *G. lovleyi* sp. strain SZ acetate and for *Desulfitobacterium* sp. strain Viet1 pyruvate was used as an electron donor and carbon source. *Dehalococcoides ethenogenes* strain 195 was grown as described by Maymo-Gatell et al. (1997) with some modifications. The sludge supernatant was not required, when the mineral salts medium described by Sanford et al. (1996) was used. Hydrogen

served as the electron donor and acetate as the carbon source. All preparations were performed anaerobically under a stream of nitrogen.

### **3.2.3 Determination of stable isotope fractionation by *Desulfitobacterium* sp. strain Viet1, *D. michiganensis* and *G. lovleyi* sp. strain SZ**

For the determination of stable isotope fractionation by growing cultures of *Desulfitobacterium* sp. strain Viet1, *D. michiganensis* and *G. lovleyi* sp. strain SZ the experimental set-up described by Nijenhuis et al. (2005) was used. Several parallel cultures were inoculated using 5% inoculum and were sacrificed for analysis, after appropriate growth periods. All cultures were prepared with a small headspace (100 ml liquid in 120 ml vials) to limit the isotope fractionation effect of volatilization of the chlorinated ethenes from the liquid phase to the gas phase, although these effects are considered to be negligible (Slater et al., 1999). Cultures received PCE or TCE (50  $\mu\text{mol}$ ) as pure solvent and were incubated at 25°C in a shaker. At various incubation times, gas phase samples were taken for analysis of chlorinated ethene concentrations by GC-FID and then cultures were killed by addition of 5 ml aerobic, saturated  $\text{Na}_2\text{SO}_4$ , pH 1. Nonane (5  $\mu\text{mol}$ ) was added to serve as internal standard for concentration analysis on GC-C-IRMS in order to check growth. Two ml of pentane was injected to each vial and cultures were incubated on a rotary shaker overnight (25°C). The pentane phase was removed, transferred to autosampler vials and analyzed by GC-C-IRMS for the concentration and isotope composition of the chlorinated ethenes as described below. Concentrations measured by GC-C-IRMS using nonane as internal standard were used for calculation of fractionation factors.

### **3.2.4 Determination of stable isotope fractionation by *D. ethenogenes* strain 195**

An alternative approach for analysis of the carbon stable isotope composition of all chlorinated ethenes was developed for the experiments with *D. ethenogenes* strain 195. Due to the fact that *D. ethenogenes* strain 195 has complex nutrient requirements and is difficult to cultivate, a non-

sacrificial set-up was used to enable the experiments with low amounts of biomass. Parallel 100 ml batch cultures in 250 ml vials were prepared and received PCE or TCE (30 or 50  $\mu\text{mol}$  respectively, as pure solvent) as electron acceptor. Cultures were incubated at 34°C in a rotary shaker at 100 rpm. At the beginning of the experiment and at various time points, 0.5 ml gas phase samples were taken, and concentrations of chlorinated ethenes were analyzed by GC-FID. Simultaneously, an aliquot of 2 ml of culture was removed and added to 10 ml vials containing 2 ml saturated  $\text{Na}_2\text{SO}_4$  (pH 1), closed with Teflon<sup>®</sup> coated stoppers and crimped. Samples were stored at 4°C. Prior to isotope composition analysis, the 10 ml vials were heated up to 60°C. Aliquots (500  $\mu\text{l}$ ) of headspace were analyzed by GC-C-IRMS. In this experiment, concentrations measured by GC-FID were used for final calculations of fractionation factors.

### 3.2.5 Analytical methods

Gas chromatography combustion isotope ratio mass spectroscopy (GC-C-IRMS) was applied to determine the stable carbon isotope composition of the chlorinated ethenes. All samples were measured in at least three replicates. Generally, the standard deviation was  $<0.5$   $\delta$  unit. The temperature of the oxidation oven was set at 980°C and samples were calibrated using reference  $\text{CO}_2$  gas (refer to section 3.2.6 for the detailed definition). In case of the experiment with *D. ethenogenes* strain 195 aliquots (500  $\mu\text{l}$ ) of the headspace were injected into a gas chromatograph (Agilent 6890; Palo Alto, USA) in split mode (split 1:3) using a split/splitless injector at 250°C. In case of all other strains, chlorinated ethenes were extracted from the medium using pentane as a solvent, as described above. 2  $\mu\text{l}$  of extracts were injected in split mode (split 1:10) using an autosampler (CTC Combipal, Chromtech, Idstein, Germany). For chromatographic separation of chlorinated ethenes from headspace samples a RTX-Q-Plot (30 m  $\times$  0.32 mm  $\times$  10  $\mu\text{m}$ , Restek, Bellefonte USA) was used. The temperature programme was as follows: initial temperature was set to 40°C for 5 min, then was increased at a rate of 20°C  $\text{min}^{-1}$  to 150°C, and next increased at a rate of 5°C  $\text{min}^{-1}$  to a final temperature of 250°C, where it was held for 3 min.



A Zebron ZB-1 capillary column (60 m × 0.32 mm, 1 µm film, Phenomex Inc., USA) was used for separation of chlorinated compounds extracted by solvent. The temperature was held at 40°C for 6°C min, then increased at a rate of 6°C min<sup>-1</sup> to 120°C, and then increased at a rate of 20°C min<sup>-1</sup> to a final temperature of 200°C. The helium flow was kept constant at 2 ml min<sup>-1</sup> for both methods.

Gas chromatography (Varian Chrompack CP-3800, Middelburg, the Netherlands) with flame ionisation detection (GC-FID) equipped with a 30 m × 0.53 mm GS-Q column (J&W Scientific, Waldbronn, Germany) was used to analyze concentrations of ethene and the chlorinated ethenes. The temperature programme used was as follows: 1 min at 100°C, 50°C min<sup>-1</sup> to 225°C, hold 2.5 min. The FID was operated at 250°C and helium was used as carrier gas (0.69 × 10<sup>5</sup> Pa; 11.5 ml min<sup>-1</sup>). This method allowed the separation of ethene, vinyl chloride, 1,1-, *trans*- and *cis*-DCE, TCE and PCE. The sampling was automated using an HP 7694 headspace autosampler (Hewlett Packard, Palo Alto, USA), adding 0.5 ml headspace samples to 10 ml autosampler vials flushed with helium, which were closed with a Teflon<sup>®</sup> coated butyl rubber septum and crimped.

### 3.2.6 Calculations and definitions

The carbon isotope composition is reported in δ-notation [‰] relative to the Vienna Pee Dee Belemnite standard (V-PDB, IAEA-Vienna) (eq. 3.1) (Coplen et al., 2006):

$$\delta^{13}\text{C}[\text{‰}] = \left( \frac{\left( \frac{^{13}\text{C}}{^{12}\text{C}} \right)_{\text{Sample}}}{\left( \frac{^{13}\text{C}}{^{12}\text{C}} \right)_{\text{Standard}}} - 1 \right) \times 1000. \quad \text{eq. 3.1}$$

The isotope fractionation during the dehalogenation reaction was calculated applying the Rayleigh equation (eq. 3.2) (Hoefs, 1997):

$$\ln\left(\frac{R_t}{R_0}\right) = (1/\alpha - 1) \times \ln\left(\frac{C_t}{C_0}\right) \quad \text{eq. 3.2}$$



The kinetic carbon isotope fractionation factor  $\alpha_C$  is a constant for the reaction at given experimental conditions.  $R_t/R_0 = (\delta_t + 1000)/(\delta_0 + 1000)$  where  $\delta_t$  and  $\delta_0$  are the isotope signatures of the substrate at time  $t$  and time 0, respectively.  $C_t$  and  $C_0$  are the concentrations of the compound at times  $t$  and zero, respectively. The data from the microcosm experiments were plotted applying the linearization of the Rayleigh distillation equation (regression  $\ln(R_t/R_0)$  on  $\ln(C_t/C_0)$ ). Data ( $n > 11$ ) from at least two, but in most cases three, separate experiments were combined into a single standard linear regression to derive the estimate of the slope via the standard least-squares solution and further permit a direct estimation of the isotope fractionation factor ( $\alpha_C$ ) for each evaluated strain. The isotope fractionation factor ( $\alpha_C$ ) relates the changes in the isotope composition to changes in concentration of the residual fraction during the transformation. The fractionation factor is also expressed as isotope enrichment factor  $\epsilon_C$  (eq. 3.3):

$$\epsilon_C [\text{‰}] = (\alpha_C - 1) \times 1000. \quad \text{eq. 3.3}$$

To calculate the fraction of biodegraded contaminant *in situ* the Rayleigh equation can be modified with the fraction remaining  $f_t = C_t/C_0$  at time  $t$ .

$$\frac{R_t}{R_0} = f_t^{(1/\alpha_C - 1)} \quad \text{eq. 3.4}$$

The percentage of biodegradation ( $B$  [%]) can then be expressed as:

$$B [\text{‰}] = (1 - f_t) \times 100 = (1 - (R_t/R_0)^{[1/(1/\alpha_C - 1)]}) \times 100. \quad \text{eq. 3.5}$$

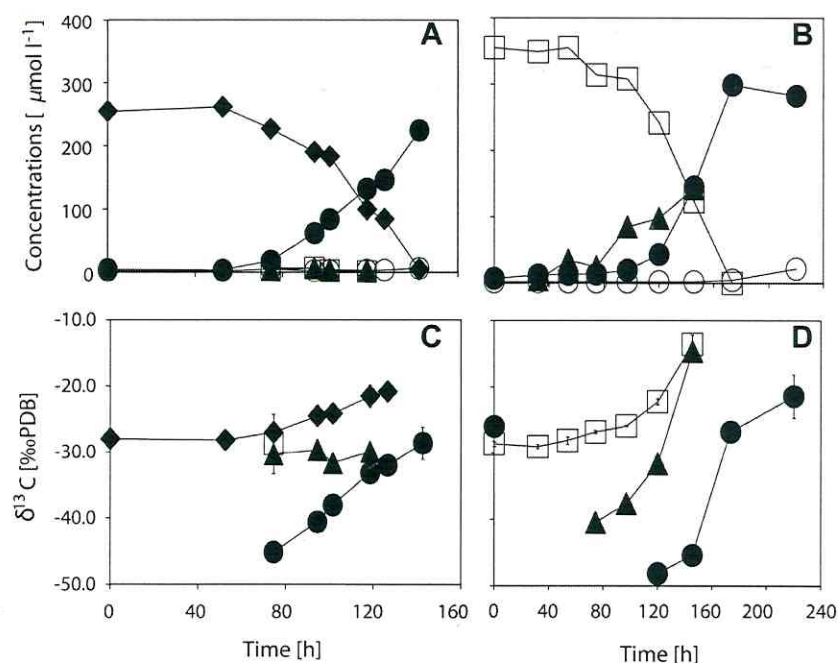
### 3.2.7 Statistical analysis

Ninety-five percent confidence intervals (95% CI) were determined on the slope of the linear regression, which was not forced through zero, and converted into ‰ units as described in the previous section. The statistical significance of the linear relationships was tested using *F*-statistics (ANOVA). The linear models were considered to significantly fit the data at  $P < 0.01$ . Except for the experiments with PCE for *G. lovleyi* and *D. michiganensis*, all data were found to fit significantly to the linear models.

## 3.3 Results

### 3.3.1 *Dehalococcoides ethenogenes* strain 195

*D. ethenogenes* strain 195 dehalogenates PCE to ethene, with the last step, dechlorination of vinyl chloride (VC) to ethene, being a slow co-metabolic reaction (Maymo-Gatell et al., 2001). In the experiment presented in Fig. 3.1 (A, C) PCE was dechlorinated from the initial concentration of  $250 \mu\text{mol l}^{-1}$  to about  $5 \mu\text{mol l}^{-1}$ . Dechlorination began after a lag phase of approximately 50 h. The isotope composition of PCE changed from -28‰ at the start of the experiment to -20.8‰ for 67% of PCE conversion after 127 h. TCE and *cis*-DCE were formed as intermediate products but their concentrations were in most cases too low to determine the isotope composition. They were further dechlorinated to VC, which was the end product during this experiment. VC at the beginning of the experiment was depleted in  $^{13}\text{C}$ , with an isotope composition of -45.2‰. In the course of degradation, VC became enriched in  $^{13}\text{C}$ , with a final composition similar to the initial composition of PCE, -28.7‰, showing a closed isotope balance. Ethene was not produced during this time period. The carbon stable isotope enrichment factor was calculated using the Rayleigh equation (Eq. 3.2, 3.3), giving  $\epsilon_{\text{C}} = 6.0$ .



**Figure 3.1** Change in concentrations (A, B) and carbon isotope composition (C, D) of PCE (◆) and TCE (□) and their products *cis*-DCE (▲), VC (●) and ethene (○), during reductive dechlorination by *D. ethenogenes* strain 195

TCE fractionation by *D. ethenogenes* strain 195 is shown in Fig. 3.1 (B, D). Dechlorination started about 50 h after the inoculation and TCE ( $350 \mu\text{mol l}^{-1}$ ) was consumed after 175 h. TCE was enriched in  $^{13}\text{C}$  from -28.6‰ to -13.6‰ for 65% conversion after 145 h. *Cis*-DCE appeared after 32 h, with initial isotope composition of -40.5‰, then it became enriched to -14.6‰ after about 145 h. The concentration of VC started to increase relatively fast after 120 h. VC, which was produced at this point, was depleted in  $^{13}\text{C}$ , the isotope composition was -48.2‰. Toward the end of the experiment, after 175 h, VC reached the isotope composition of -26.8‰, close to the initial composition of TCE. When TCE was completely consumed, the dechlorination of VC began and concentration of ethene started to increase. VC became more enriched in  $^{13}\text{C}$  with the isotope composition of -21.4‰. The enrichment factor for TCE was  $\epsilon_{\text{C}}=13.7$  as

shown in Table 3.1, and it was similar to the value found by Lee et al. (2007).

### 3.3.2 *Geobacter lovleyi* strain SZ and *Desulfuromonas michiganensis*

*G. lovleyi* strain SZ and *D. michiganensis* were examined for reductive dechlorination of PCE and TCE. Both strains reduce PCE via TCE to *cis*-DCE. Changes in the concentration and isotope composition during the growth of *G. lovleyi* and *D. michiganensis* on PCE (Fig. 3.2 A, D - *G. lovleyi*; B, E - *D. michiganensis*) and TCE (Fig. 3.3 A, C - *G. lovleyi*; B, D - *D. michiganensis*) are presented.

In the experiment with *D. michiganensis* the isotope signature of PCE changed from -29.1 to -28.4‰ for 78% conversion after 64 h. Similarly, for *G. lovleyi* strain SZ the shift in PCE isotope composition was not significant (<0.5‰ after 163 h and 71% conversion). TCE, the intermediate product of the reaction, became clearly enriched in  $^{13}\text{C}$  over time, with a change of 4.7  $\delta$  units for *D. michiganensis* (from -29.5 to -24.8‰) and 10.5  $\delta$  units for *G. lovleyi* (from -28.5 to -18.0‰). The final product *cis*-DCE was slightly depleted in heavy isotopes, compared to TCE, suggesting that the second dechlorination step, TCE to *cis*-DCE degradation, was associated with a carbon stable isotope fractionation. It has to be noted that *cis*-DCE, present at the beginning of the experiment, was transferred during the inoculation (5% v/v) from a previous experiment (Fig. 3.2 A, B, D, E) resulting in a dilution of the isotope effect. The shift in isotope signature of *cis*-DCE was likely underestimated since formed, light, *cis*-DCE was diluted with the heavier compound transferred from the inoculum.

In contrast to the experiment with PCE, both organisms significantly fractionated TCE (Fig. 3.3 C, D). In the experiment with *G. lovleyi* the isotope composition changed by about 31  $\delta$  units (from -28.1 to 2.6‰, at 24 h and for 98% conversion) whereas an enrichment of around 5  $\delta$  units (from -28.7 to -23.5‰ at 76% conversion of TCE after 91 h) was found in experiments with *D. michiganensis*. The isotope enrichment factors for TCE were  $\epsilon_{\text{C}}=8.5$  for *G. lovleyi* and  $\epsilon_{\text{C}}=3.5$  for *D. michiganensis*, in contrast to the not significant fractionation of PCE (Table 3.1).



### 3.3.3 *Desulfitobacterium* sp. strain Viet1

*Desulfitobacterium* sp. strain Viet1 dechlorinates PCE to TCE. During the experiment isotope composition of PCE was shifted from -28.9 to 2.6‰ after 90 h (97% conversion), while the concentration decreased from 380  $\mu\text{mol l}^{-1}$  to around 10  $\mu\text{mol l}^{-1}$  (Fig. 3.2 C, F). TCE was formed as the end product, being depleted in  $^{13}\text{C}$  to -31.2‰ at the beginning of experiment. In the course of degradation, it became even more depleted reaching the isotope composition of -42.4‰, and then it became enriched again with final isotope ratio of -28.3‰. The  $\epsilon_{\text{C}}$  for *Desulfitobacterium* sp. strain Viet1 was 16.7 (Table 3.1), which is the highest fractionation factor for PCE reductive dechlorination by pure culture observed so far.

**Table 3.1** Stable isotope fractionation factors ( $\alpha_C$ ), the  $R^2$  and 95% confidence interval (CI 95%) of the regression curve and enrichment factors ( $\epsilon_C$ ) for PCE and TCE dechlorination by dehalogenating bacteria.

Strain	Phylogeny	PCE			TCE				
		$\alpha C$	R <sup>2</sup>	CI (95%)	$\epsilon C$	$\alpha C$	R <sup>2</sup>	CI (95%)	$\epsilon C$
<i>S. multivorans</i>	$\epsilon$ -Proteobacteria	1.0004 <sup>a</sup>	0.67	0.0002	0.4	1.0187 <sup>b</sup>	0.84	0.0042	18.7
<i>S. halorespirans</i>	$\epsilon$ -Proteobacteria	1.0005 <sup>b</sup>	0.76	0.0002	0.5	1.0164 <sup>c</sup>	0.97	0.0015	16.4
<i>D. michiganensis</i>	$\delta$ -Proteobacteria	ns	0.33	na	-	1.0189 <sup>b</sup>	0.99	0.0010	18.9
<i>G. lovleyi</i> strain SZ	$\delta$ -Proteobacteria	ns	0.04	na	-	1.0035	0.99	0.0002	3.5
						1.0085	0.98	0.0006	8.5
<i>Desulfitobacterium</i> sp. strain PCE-S	Firmicutes	1.0052 <sup>a</sup>	0.93	0.0015	5.2	1.0122 <sup>b</sup>	0.88	0.0023	12.2
<i>Desulfitobacterium</i> sp. strain Viet1	Firmicutes	1.0167	0.84	0.0045	16.7	-	-	-	-
<i>Dehalobacter restrictus</i> PER-K23	Firmicutes	-	-	-	-	1.0033	0.98	0.0003	3.3 <sup>e</sup>
<i>D. ethenogenes</i>	Chloroflexi	1.0060	0.94	0.0007	6.0	1.0137	0.95	0.0018	13.7
						1.0096 <sup>e</sup>	0.99	0.0004	9.6
Mixed cultures		1.0055 <sup>e</sup>			5.5	1.00025 – 1.016 <sup>c,d,e</sup>			2.5 – 16.0

ns: fractionation was not significant; na: not applicable. The observed shift in isotopic signatures was within the instrumental error.

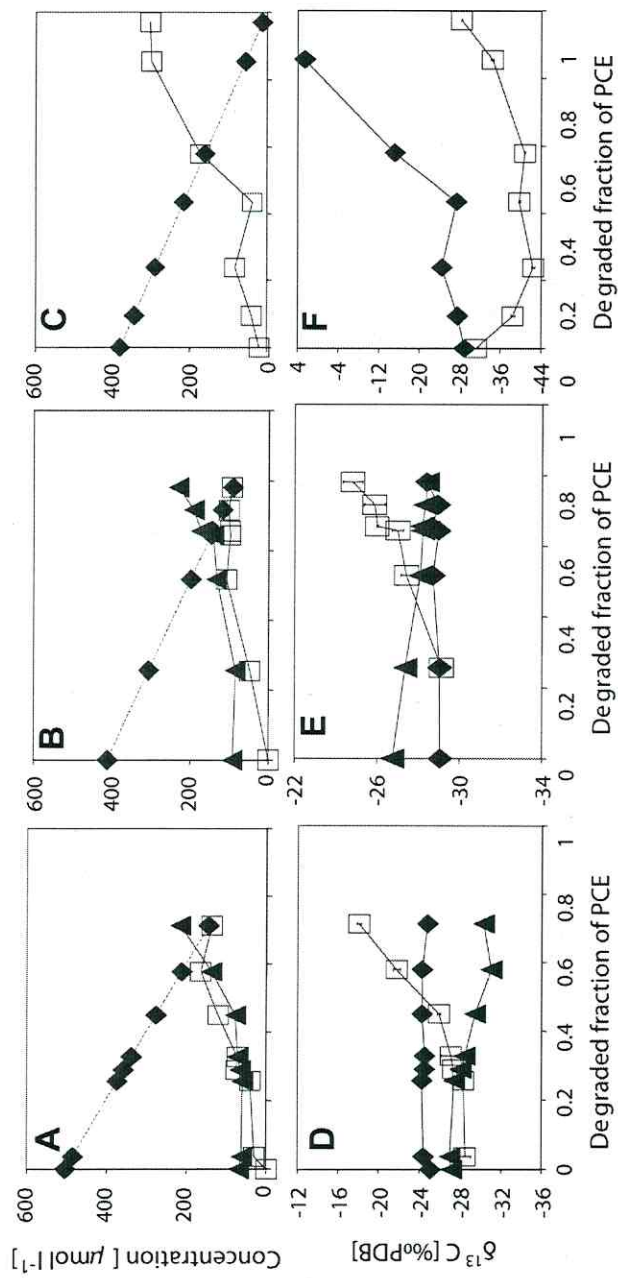
<sup>a</sup> (Nijenhuis et al., 2005)

<sup>b</sup> (Cichocka et al., 2007)

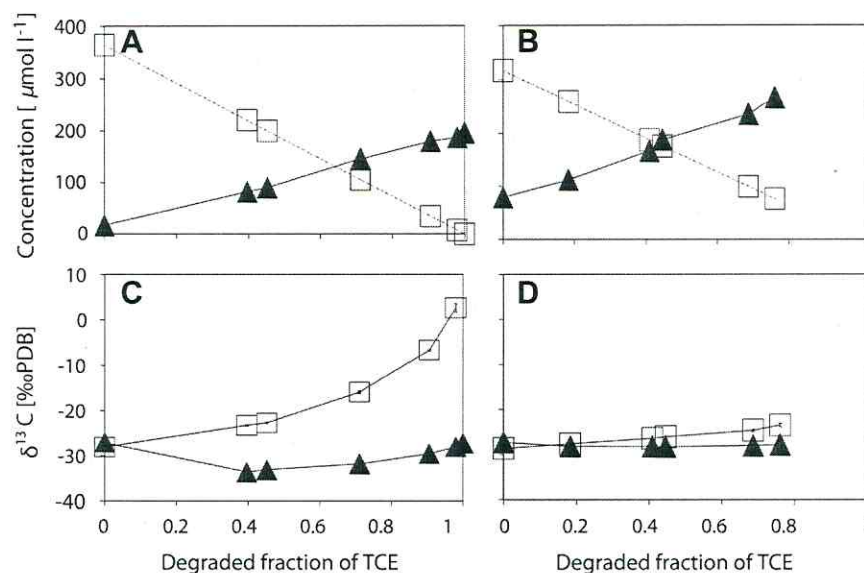
<sup>c</sup> (Slater et al., 2001)

<sup>d</sup> (Bloom et al., 2000)

<sup>e</sup> (Lee et al., 2007)



**Figure 3.2** Change in concentrations (A,B,C) and carbon isotope composition (D,E,F) of PCE (♦) and its products TCE (□) and cis-DCE (▲), during reductive dechlorination by *Geobacter lovleyi* (A,D), *Desulfuromonas michiganensis* (B,E) and *Desulfotobacterium* sp. strain Viet1 (C,F).



**Figure 3.3** Change in concentrations (A, B) and carbon isotope composition (C, D) of TCE ( $\square$ ) and its product *cis*-DCE ( $\blacktriangle$ ) during reductive dechlorination by *Geobacter lovleyi* (A, C) and *Desulfuromonas michiganensis* (B, D).

### 3.4 Discussion

Thus far, only limited information on the variability of carbon isotope fractionation of chlorinated ethenes by pure cultures is available in the literature. Previous studies revealed large differences in carbon isotope fractionation during reductive dechlorination of PCE and TCE by *Sulfurospirillum* spp. and *Desulfitobacterium* sp. strain PCE-S (Nijenhuis et al., 2005; Cichocka et al., 2007). Our results show that the variability of PCE and TCE carbon isotope fractionation by different dehalorespiring bacteria is substantial and much higher than previously reported. Thus, this variability may obviously lead to uncertainties that limit a direct application of the Rayleigh concept for estimation of biodegradation at field sites. The degradation experiments allow observing a variability ranging



from no significant fractionation to carbon isotope enrichment factors ( $\epsilon_C$ ) of 16.7 for PCE, and  $\epsilon_C$  ranging from 3.5 to 18.9 for TCE (Table 3.1). The bacteria studied here are phylogenetically and metabolically diverse and therefore our results present an interesting range of variability of biological isotope fractionation. *Desulfuromonas michiganensis* and *Geobacter lovleyi* sp. strain SZ, belonging to the  $\delta$ -subclass of the Proteobacteria, use PCE and TCE as their metabolic electron acceptors. *Desulfitobacterium* sp. strain Viet1 from the Firmicutes phylum can metabolically reduce only TCE, whereas *Dehalococcoides ethenogenes* strain 195 from the Chloroflexi phylum, can use a wide range of chlorinated ethenes, PCE, TCE, *cis*- and 1,1-DCE, as growth substrates. Interestingly, bacteria, which are similar in electron acceptor usage, did not necessarily generate similar isotope fractionation. For example, on the one hand fractionation factors for PCE and TCE for *Desulfitobacterium* sp. strain PCE-S were different from factors found for *D. michiganensis* or *G. lovleyi* which all perform the dechlorination of PCE or TCE to *cis*-DCE. On the other hand, fractionation of PCE and TCE by *Desulfitobacterium* sp. strain PCE-S and *D. ethenogenes* strain 195 were more similar even though the end products of dechlorination were *cis*-DCE and VC respectively.

Investigation of isotope fractionation applying toluene as a model compound has shown that the variability of the isotope fractionation significantly depended on the degradation pathway (Meckenstock et al., 1999; Morasch et al., 2002). The biochemical mechanism of the anaerobic degradation pathway catalysed by the benzylsuccinate synthase reaction is clearly distinct from oxidation of the methyl group as well as ring oxidation pathway catalysed by ring monooxygenase or dioxygenase reaction. However, only slight variability of carbon isotope fractionation was found within an individual (anaerobic, monooxygenase or dioxygenase) pathway. Similarly, the variability upon aerobic dichloromethane degradation by a number of methylotrophic strains using the glutathione S-transferase dependent pathway was relatively low with only a 2 fold difference ( $\epsilon_C=43$  to 71) (Nikolausz et al., 2006).

Compared to these studies, the variability in carbon isotope fractionation by the different dehalogenating strains was great, although all dechlorinating strains analyzed for isotope fractionation use similar degradation pathways (Smidt and de Vos, 2004). Also all chloroethene reductive dehalogenases investigated thus far were similar, and all investigated enzymes contained corrinoid co-factors (Smidt and de Vos, 2004), suggesting similar reaction mechanisms. A similarly large range of carbon isotope fractionation was also observed during methanogenesis (Valentine et al., 2004). During microbial methanogenesis from CO<sub>2</sub> reduction, carbon isotope enrichment factor ( $\epsilon_C$ ) varied from 2.5 to 7.9 while during acetoclastic methanogenesis,  $\epsilon_C$  ranged from 0.7 to 22 which, in this case, could be related to differences in biochemical mechanism of methanogenesis between the pathways (Valentine et al., 2004).

The variability of the observed isotope fractionation during PCE and TCE dehalogenation was high. This complicates an interpretation of the isotope effect as a result of reaction mechanism since the transition state of carbon-chlorine bond cleavage and physiological factors have to be taken into account.

As observed before, fractionation of TCE was in all cases higher compared to PCE (Bloom et al., 2000; Sherwood Lollar et al., 2001; Slater et al., 2001; Nijenhuis et al., 2005; Cichocka et al., 2007). For *D. ethenogenes* the difference was about a 2 fold increase from PCE to TCE, similar as found for *Desulfitobacterium* sp. strain PCE-S. *D. michiganensis* and *G. lovleyi* were the isolates representing the  $\delta$ -Proteobacteria, which did not generate any significant fractionation during PCE reductive dechlorination and had low enrichment factors for TCE,  $\epsilon_C=3.5$  and  $\epsilon_C=8.5$  respectively. The reductive dehalogenases of *D. michiganensis* and *G. lovleyi* have not been described yet, but are thought to be different from the currently known dehalogenases since they could not be amplified using the currently known dehalogenase specific primers (F. Löffler, personal communication). The reasons for the low fractionation may include structural variability of dehalogenases, which may affect both

binding to the enzyme and the transition state of the isotope sensitive bond cleavage reaction, but cannot be explained at this time and further studies are required. Thus far, the investigated chloroethene reductive dehalogenases were thought to be associated with the cytoplasmic membrane and located on its outer face due to the presence of signal sequences for the transport by the TAT system across the cytoplasmic membrane (Holliger et al., 2003; Smidt and de Vos, 2004). In a few cases, experimental data confirms location of the dehalogenase on the outside of the cytoplasmic membrane (Nijenhuis and Zinder, 2005; John et al., 2006). Therefore, the differences in carbon isotope fractionation cannot be explained by the location of the reductive dehalogenases alone. Potentially, low fractionation observed in *D. michiganensis*, *G. lovleyi* or *Dehalobacter restrictus* could be due to rate limiting effects of substrate transport across membranes or binding to the enzyme. Interestingly, all of the organisms belonging to the Proteobacterium phylum did not fractionate PCE or had very low enrichment factors for this compound ( $\epsilon_C \leq 0.5$ ), suggesting an effect of cell structure, e.g. the gram negative cell envelope, while the other tested bacteria either have a gram positive cell wall or are thought to have archaea-like protein S-layer envelopes (Maymo-Gatell et al., 1997; Holliger et al., 2003).

Surprisingly, fractionation of PCE and TCE by *Desulfitobacterium* sp. strain PCE-S was in the same range as by *D. ethenogenes* strain 195, although the dehalogenases of both organisms are not very closely related (identity of approximately 32% comparing TceA of *D. ethenogenes*, which reduces TCE to *cis*-DCE, to PceA of *Desulfitobacterium* sp. PCE-S, which reduces PCE to *cis*-DCE) (Holliger et al., 2003). This suggests that different enzymes could cause similar fractionation. Additionally, fractionation of both PCE ( $\epsilon_C = 6.0$ ) and TCE ( $\epsilon_C = 13.7$ ) by *D. ethenogenes* strain 195 was similar to the literature data for mixed cultures containing *Dehalococcoides* (Bloom et al., 2000; Slater et al., 2001; Lee et al., 2007).

Based on the available data, there seems to be a relation of carbon isotope fractionation during reductive dechlorination to phylogeny. Variability in fractionation of both studied compounds was generally lower within one phylum than between phyla, with only one exception for PCE



and TCE fractionation by organisms belonging to the Firmicutes. PCE fractionation by strain Viet1 was more than 3 fold larger than by strain PCE-S. These differences coincide with the differences in substrate range of these organisms: strain PCE-S dechlorinates PCE and TCE to *cis*-DCE, while strain Viet1 only dechlorinates to TCE, therefore the enzymes in these two organisms are very likely to be different. Nevertheless, only an *o*-chlorophenol dehalogenase has been described for strain Viet1 which is not likely responsible for the dechlorination of PCE (Holliger et al., 2003). Interestingly, the dehalogenases of *Dehalobacter restrictus* PER-K23 (NCBI accession number AJ439607.2, [www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)) and *Desulfitobacterium* sp. strain PCE-S (NCBI accession number AY216592) are highly similar (identity of 96%) but fractionation of TCE differs by about four fold suggesting significant effect of the specific physiology of the organism on isotope fractionation. These physiological effects may include rate limitation of uptake, transport or enzyme binding prior the dehalogenation reaction. At this time, no specific causes for these differences can be determined precisely and further research is needed.

#### **Implication for the assessment of *in situ* biodegradation**

The selection of an appropriate fractionation factor, representative for the biodegrading microbial community active in the field, is crucial for a quantitative assessment of biodegradation by means of compound specific isotope analysis. A fractionation factor could be determined by using microcosms prepared from the field material but it is extremely difficult to reproduce in the laboratory the actual environmental conditions present in the field. The risk that organisms, which are not active *in situ*, may out-compete the organisms that play much more significant role in the aquifer is very high, which may not reflect the actual field isotope fractionation factor.

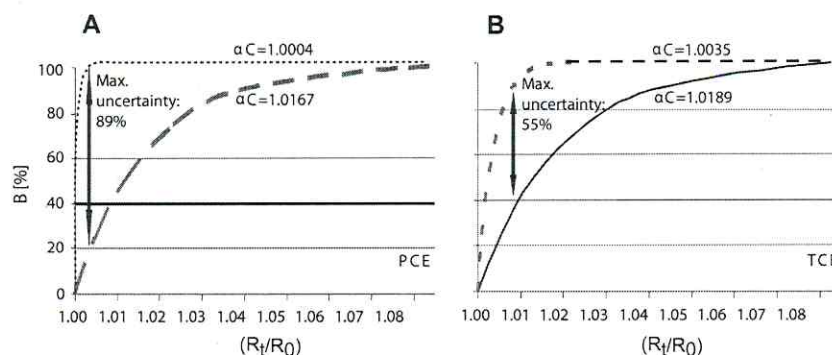
Previous investigations showed that most phylogenetic groups containing dehalorespirers are represented at contaminated field sites (Löffler et al., 2000; Nijenhuis et al., 2007) and it should be taken into account that all these microorganisms may play a role in the *in situ*



biodegradation. The collection of the strains used in this study reflects the currently known natural diversity of dehalorespiring organisms.

Our results, showing that the fractionation is highly variable between and within the different phylogenetic groups of dehalorespirers, emphasize the uncertainty of CSIA to quantify natural attenuation of PCE.

The influence of different fractionation factors retrieved in this study on the estimated proportion of biodegraded residual substrate fraction is shown in Fig. 3.4 (PCE: Fig. 3.4A and TCE: Fig. 3.4B). The percentage of biodegradation of the residual substrate fraction ( $B$  [%]) was calculated using the modified Rayleigh equation (eq. 3.5). Our maximal and minimal significant fractionation factors for PCE ( $\alpha_{C_{max}}=1.0167$ ;  $\alpha_{C_{min}}=1.0004$ ) and TCE ( $\alpha_{C_{max}}=1.0189$ ;  $\alpha_{C_{min}}=1.0035$ ) were used for biodegradation estimation, resulting in an uncertainty of more than 50% for PCE and TCE. Interestingly, the uncertainty in biodegradation estimates strongly varies as a function of the isotope shift ( $R_i/R_0$ ). It is particularly substantial when the ratio  $R_i/R_0 < 1.02$ . According to the model (Fig. 3.4) the maximal uncertainty (89%) for PCE occurs, when the ratio  $R_i/R_0=1.0018$  (corresponding to a shift of approx. 1.7‰, assuming  $\delta_0=-28$ ‰), and it decreases when the ratio value is higher. For TCE the maximal uncertainty (55%) occurs when the ratio  $R_i/R_0=1.0075$  (corresponding to a shift of approx. 7.3‰, assuming  $\delta_0=-28$ ‰). These considerations have a strong impact on the use of stable isotope fractionation approaches for quantification of PCE and TCE biodegradation at field sites where reductive dechlorination plays a major role. For example, previously reported carbon isotope signatures of TCE at Dover Air Force base ranged from -18 to -26‰, whereas only small differences in isotope signature (-32.8 to -33.9‰) were observed for PCE (Sherwood Lollar et al., 2001).



**Figure 3.4** Estimated biodegradation (B [%]) as a function of the ratio ( $R_t/R_0$ ) of the isotope composition of PCE and TCE at time  $t$  ( $R_t$ ) and zero ( $R_0$ ) during (A) PCE dechlorination by *S. multivorans* (-----) and *Desulfotobacterium* sp. strain Viet1 (—) and (B) TCE dechlorination by *D. michiganensis* (-----) and *S. haloferiens* (—).

As a consequence of the variability of stable carbon isotope fractionation associated with TCE reductive dechlorination, estimating the biodegradation levels at that site within an acceptable range of uncertainty is not feasible. Indeed, the biodegradation level estimates based on these data would range between 36% and 90% when using the fractionation factors for *S. haloferiens* and *D. michiganensis*, respectively. This demonstrates the spectrum of possible estimates and the large associated uncertainty ( $\Delta B = 54\%$  or 2.5 fold difference). For PCE, only minor differences in isotope signatures were observed at this site suggesting either no biodegradation or presence of microorganisms which dechlorinated PCE to TCE without associated carbon isotope fractionation. This example shows that uncertainty associated with the estimation of biodegradation using the Rayleigh approach is important and points out the risk of a misleading interpretation while using a fractionation factor without taking into account possible variation of the estimates. In any case, a qualitative approach remains possible and the shift in isotope composition of PCE or TCE detected in the field can serve as a proof of biodegradation. On the other hand, the lack of enrichment in heavy

isotopes in the not degraded contaminant fraction does not necessarily indicate that the biodegradation does not occur, because organisms like *D. michiganensis* and *G. lovleyi*, which do not fractionate PCE, may be present and active at a contaminated site. Therefore, for chlorinated ethenes CSIA should always be accompanied by other methods e.g. detection of intermediate products, before attesting the presence of absence of biodegradation. Additionally, molecular tools may be used to analyze the microbial dehalogenating communities in the field and help in selection of an appropriate fractionation factor for CSIA. Ideally, detection of active microbial community as well as reductive dehalogenases using RNA based techniques would provide information about the enzymes and microorganisms responsible for reductive dehalogenation. Using this information, the corresponding fractionation factor or factors, representing the microbial community in the field, could be selected.

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**Carbon stable isotope fractionation of  
chloroethenes and polychlorinated  
dibenzo-*p*-dioxins by a culture containing  
*Dehalococcoides ethenogenes* strain 195**

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Submitted to Environmental Science and Technology



**Abstract**

Carbon stable isotope fractionation occurred during dechlorination of tetrachloroethene (PCE) and 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) by a culture containing *Dehalococcoides ethenogenes* strain 195. Trichloroethene (TCE) produced during PCE dechlorination, was first depleted but later became enriched relative to PCE, suggesting fractionation during subsequent dechlorination. The *cis*-1,2-dichloroethene (DCE) could not be quantified. Vinyl chloride (VC) was depleted relative to PCE and TCE; then became enriched as ethene, the most depleted metabolite, was produced. Dechlorination of 1,2,3,4-TeCDD, loaded on sediment for realistic bioavailability conditions, in the presence or absence of PCE as an additional chlorinated substrate, consistently produced isotopically enriched 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD). The second dechlorination product, 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD), was depleted relative to both 1,2,3,4-TeCDD and 1,2,4-TrCDD implying greater fractionation during the second dechlorination step than the first. We observed average differences (significant at the 99% confidence level) of 1.6‰ between 1,2,4-TrCDD and 1,2,3,4-TeCDD and -2.8‰ between 1,3-DCDD and 1,2,4-TrCDD. The intrinsic enrichment factor,  $\epsilon_{\text{intrinsic}}$ , for PCE dechlorination was  $-14.2 \pm 1.0$  ‰. The  $\epsilon_{\text{intrinsic}}$  for 1,2,3,4-TeCDD dechlorination exhibited significant variability and uncertainty, perhaps because of bioavailability limitations. The maximum  $\epsilon_{\text{intrinsic}}$  of  $-13.0 \pm 5.3$  ‰ and  $-34.8 \pm 8.8$  ‰ were measured in replicates without PCE addition. With further study, carbon isotope fractionation could prove useful for monitoring polychlorinated dibenzo-*p*-dioxin dechlorination in the field.

## 4.1 Introduction

Biotransformation of contaminants contributes to their natural attenuation and may be enhanced for bioremediation of contaminated groundwater and sediment. Documentation and monitoring of *in situ* biotransformation by traditional means requires excessive time, money, and labor for intensive sampling and analyses. A promising tool that speeds assessment of biotransformation of environmental contaminants is Compound Specific Isotope Analysis (CSIA). Recent literature reviews of CSIA were provided by Schmidt et al. (2004) and Meckenstock et al. (2004). In CSIA the ratio of the relative abundance of the heavy and light isotopes of an element is determined in an individual compound. CSIA relies on the kinetic isotope effect (KIE) during biotransformation which is caused by different reaction rates for a compound containing the heavy isotope versus the light isotope at the location of the reaction. Since more energy is needed to break the chemical bond constituted by heavy isotopes, the reaction is normally slower for a molecule containing the heavy isotope than for the light isotope of the same element. This typically results in enrichment of heavy isotope in the residual substrate. Biologically mediated isotope fractionation has been examined under laboratory and field conditions for benzene, toluene, ethylbenzene and xylenes (BTEX) (Meckenstock et al., 1999; Hunkeler et al., 2001); methyl-tertiary-butyl ether (MTBE) (Gray et al., 2002; Somsamak et al., 2005); polynuclear aromatic hydrocarbons (PAHs) (Richnow et al., 2003; Yanik et al., 2003); phenol and benzoate (Hall et al., 1999); polychlorinated biphenyls (Drenzek et al., 2001); and chlorinated ethenes (Hall et al., 1999; Hunkeler et al., 1999; Bloom et al., 2000; Drenzek et al., 2001; Sherwood Lollar et al., 2001; Nijenhuis et al., 2005). CSIA may be used as both a qualitative and a quantitative measurement to monitor *in situ* biodegradation processes (Sherwood Lollar et al., 2001; Nijenhuis et al., 2005; Nijenhuis et al., 2007).

CSIA could be particularly useful for verifying biotransformation of complex contaminants such as the polychlorinated dibenzo-*p*-dioxins (PCDDs). The PCDDs are highly hydrophobic, ubiquitous and problematic

contaminants that accumulate in soils, sediments and biota (Czuczwa et al., 1984; Hites, 1990; Bopp et al., 1991; Cai et al., 1994; Koistinen et al., 1995; Wagrowski and Hites, 2000). PCDDs generally exist in the environment at very low concentrations, however, they are of great concern because of the toxicity of the 2,3,7,8-substituted congeners (Van den Berg et al., 2006). PCDDs undergo reductive dechlorination to less chlorinated congeners under anaerobic conditions (Adriaens et al., 1995; Beurskens et al., 1995; Ballerstedt et al., 1997; Vargas et al., 2001). However with few exceptions (Lohmann et al., 2000; Bunge et al., 2007), PCDDs with fewer than four chlorines are not routinely measured in environmental samples, and thus there is little evidence to confirm significant PCDD dechlorination or to delineate dechlorination pathways in the environment. If isotope fractionation of PCDDs occurs during dechlorination, CSIA could be applied to compare isotopic signatures between likely PCDD parent and daughter congeners or between PCDD homolog groups as a marker for biodechlorination.

Recently, Ewald et al. (2007) reported stable isotope fractionation during dechlorination of trichlorodibenzo-*p*-dioxins by a culture containing bacteria with high similarity to *Dehalococcoides* sp. strain CBDB1 (Bunge et al., 2003). To evaluate whether environmental transformation of PCDDs could be monitored by CSIA, it is important to understand whether stable isotope fractionation occurs during dechlorination of different PCDD congeners mediated by different bacteria and under different environmental conditions. Here we compared carbon stable isotope fractionation during dechlorination of 1,2,3,4-TeCDD by a mixed culture containing *Dehalococcoides ethenogenes* strain 195. *D. ethenogenes* strain 195 grows on PCE (Maymo-Gatell et al., 1997) and while it dechlorinates 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) to 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD), and subsequently to 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD) (Fennell et al., 2004), we do not yet know if strain 195 grows on PCDDs.

We first examined carbon stable isotope fractionation during PCE dechlorination to gain an understanding of isotope fractionation by this



culture under conditions of relatively high substrate availability. The PCDDs have log  $K_{ow}$  values ranging from approximately 7 to 9 (Shiu et al., 1988) and are likely sorbed to soil or sediment particles in the environment. Sorption/desorption processes may therefore limit their bioavailability and be a critical governing factor for dechlorination kinetics (Adriaens et al., 1995). A limitation on the approach of individual molecules to the enzyme could mean that isotope discrimination is less pronounced and that the expected isotope fractionation is masked (Elsner et al., 2005). We used 1,2,3,4-TeCDD loaded on a sediment carrier and thus dissolution of PCDDs from sediment particles was included inherently in the experimental design. In this way, any observed isotope fractionation during reductive dechlorination might more realistically approach that expected in PCDD-contaminated environmental systems.

## 4.2 Materials and methods

### 4.2.1 Chemicals

1,2,3,4-TeCDD, 1,2,4-TrCDD, 1,3-DCDD, and 2,2',5-trichlorobiphenyl (2,2',5-TrCB) were purchased from AccuStandard (New Haven, CT). PCE (99.9+%), trichloroethene (TCE) (99.5+%), *cis*-1,2-dichloroethene (*cis*-1,2-DCE) and butyric acid (99+%) were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). Vinyl chloride (VC) ( $\geq 99.97\%$ ) was obtained from Linde AG (Leuna, Germany). Ethene (99%) was purchased from Matheson Tri-Gas, Inc. (Montgomeryville, PA, USA).

### 4.2.2 Culture cultivation

A mixed culture containing *D. ethenogenes* strain 195 was grown at 34°C for PCE studies or at 28°C for PCDD studies, on PCE and butyric acid as described previously (Fennell et al., 1997; Fennell et al., 2004).



#### 4.2.3 Experimental setup

PCE dechlorination/fractionation studies were carried out in 250 ml serum bottles, containing 100 ml medium and inoculated with 2% volume:volume (vol:vol) culture from late exponential growth phase on 500  $\mu$ M PCE. Cultures were amended with 500  $\mu$ M PCE and 2.75 mM butyric acid as a hydrogen source (Fennell et al., 1997) and were incubated in a rotary shaker at 34°C. At set up, then periodically, 0.5 ml headspace samples were removed for determination of chloroethenes and ethene concentrations. Simultaneously, 0.5 ml of headspace was immediately analyzed for compound specific carbon isotope composition.

The 1,2,3,4-TeCDD experiments were performed in 160-ml serum bottles. One gram of dry sterile sediment from the Arthur Kill, New Jersey, USA (5.4% total carbon) was added. The sediment was completely wetted by addition of 0.5 ml of 6211  $\mu$ M 1,2,3,4-TeCDD toluene solution. Toluene was volatilized under sterile N<sub>2</sub>, leaving a coating of 1,2,3,4-TeCDD on the sediment. Culture (100 ml) was added under anoxic, sterile conditions resulting in 31  $\mu$ M 1,2,3,4-TeCDD nominal concentration assuming no phase partitioning. We routinely recovered only 6  $\mu$ M 1,2,3,4-TeCDD nominal concentration, thus, some 1,2,3,4-TeCDD was not recovered during sampling and extraction. The sediment to media ratio was 0.01 weight:volume. Assuming a maximum solubility of 1.95 pM 1,2,3,4-TeCDD (Govers and Krop, 1998), truly dissolved 1,2,3,4-TeCDD would account for only 0.0063% of the spiked 1,2,3,4-TeCDD, with greater than 99.99% of the compound sorbed to sediment particles, suspended matter, colloidal organic matter, and perhaps to the glass surface.

One set of triplicate bottles received 1,2,3,4-TeCDD as the only chlorinated substrate. Because we were unsure of the ability of the culture to dechlorinate 1,2,3,4-TeCDD in the absence of a known growth substrate, we also ran one set of triplicate bottles amended with 110  $\mu$ M PCE, periodically, as an additional chlorinated substrate, in addition to the 1,2,3,4-TeCDD. Each bottle also received 440  $\mu$ M butyric acid and 40  $\mu$ l of 50 g/l fermented yeast extract solution (Fennell et al., 1997) as a nutrient source, periodically. One set of triplicate cultures amended with 1,2,3,4-

TeCDD only, was autoclaved for one hour at 121°C to serve as a killed control. The bottles were shaken at 120 rpm at 28°C in the dark.

#### **4.2.4 Analytical methods**

Ethene and chloroethenes were quantified using a Varian Chrompack CP-3800 gas chromatograph (GC) (Middelburg, the Netherlands) with flame ionization detection using a 30 m x 0.53 mm GS-Q column (J&W Scientific, Waldbronn, Germany) (Nijenhuis et al., 2005). The temperature program was: 1 min at 100°C, increased at 50°C/min to 225°C, and held for 2.5 min. The FID was 250°C and helium was used as carrier gas ( $0.69 \times 10^5$  Pa; 11.5 ml/min). Sampling was via an HP 7694 headspace autosampler (Hewlett Packard, Palo Alto, CA, USA) using a 0.5 ml headspace sample added to a 10 ml autosampler vial flushed with helium, closed with a Teflon® coated butyl rubber septum and crimped.

Two ml of culture/sediment mixture was removed for PCDD analysis. Samples were centrifuged, the aqueous portion was removed to a separate vial, 2,2',5-TrCB was added as a surrogate standard to the sediment residue, and then the sediment phase was rinsed with 1 ml of acetone to remove water, as previously described (Vargas et al., 2001). The solid phase was extracted overnight with 3 ml of 2:1 vol:vol toluene:acetone, then for 4 h with 1 ml of 2:1 vol:vol toluene:acetone, and then rinsed with 1 ml toluene. The solvent phases were combined with the aqueous phase and the pooled solvent was back extracted by adding NaCl. The solvent phase was passed through a 2 ml glass pipette filled with Florisil (Sigma-Aldrich, St. Louis, MO, USA) and eluted with three volumes of toluene. The extract was concentrated to about 2 ml. PCDDs were analyzed using an Agilent 6890 GC equipped with a 5973N mass selective detector (GC-MS) (Agilent Technologies, Inc. Santa Clara, CA) and a HP-5MS (Agilent Technologies, Inc. Santa Clara, CA, USA) column (30 m x 0.25 mm I.D.). PCDDs were detected based on the retention times of standards and their molecular ions ( $m/z$ : 1,2,3,4-TeCDD, 322; 1,2,4-TrCDD, 286; 1,3-DCDD, 252; and 2,2',5-TrCB, 256). A qualifying ion was monitored to assure the correct identification of each congener (1,2,3,4-

TeCDD, 320; 1,2,4-TrCDD, 288; 1,3-DCDD, 254; and 2,2',5-TrCB, 186). The response factors for each PCDD congener compared to the surrogate, 2,2',5-TrCB, were calculated over five concentration levels as a linear calibration curve.

The isotope compositions of chloroethenes and ethene were analyzed by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) as described previously (Nijenhuis et al., 2007). Headspace samples (0.5 ml) were injected into a GC (Agilent 6890; Palo Alto, USA) in split mode (split 1:3) using a split/splitless injector at 250°C. Compounds were separated on a RTX-Q-Plot column (30 m x 0.32 mm x 10 µm, Restek, Bellefonte, PA, USA). The temperature program was: 40°C for 5 min, increased at 20°C/min to 150°C, increased at 5°C/min to 250°C, and held for 3 min. Samples were analyzed at least three times and averaged. The standard deviation was generally <0.5 δ unit.

CSIA of PCDDs was by GC-C-IRMS using an Agilent 6890 GC connected to a Finnigan MAT 252 mass spectrometer via a Finnigan GC-C/TC III Interface (Richnow et al., 2003). PCDDs were separated on a ZB-5 column (60 m x 0.32 mm I.D. with 0.25 µm thickness) (Phenomenex, Torrance, CA, USA). The temperature program was: 100°C for 1 min; increased at 15°C/min to 220°C; held for 5 min; increased at 6°C/min to 320°C; and held for 10 min. The injector was 280°C, operated in a pulsed splitless mode with a 0.8 min purge time, and a  $3.10 \times 10^5$  Pa purge pressure. The purge flow was 10 ml/min and the pulse time was 1 min. Helium was the carrier gas at 1.5 ml/min. At least triplicate measurements were performed for each sample. The CSIA data for each triplicate bottle were reported as averages of triplicate injections for each time point from each replicate plus or minus one standard deviation.

#### 4.2.5 Carbon stable isotope calculations

Isotope ratios are reported in standard δ notation in parts per thousand [‰] relative to an international reference standard, Vienna Pee Dee Belemnite (V-PDB, IAEA-Vienna) (Coplen et al., 2006):



$$\delta^{13}\text{C}[\text{‰}] = \left( \frac{(^{13}\text{C}/^{12}\text{C})_{\text{Sample}}}{(^{13}\text{C}/^{12}\text{C})_{\text{Standard}}} - 1 \right) \times 1000. \quad \text{eq. 4.1}$$

The Rayleigh model was used to correlate changes in concentration to changes in isotope composition (Rayleigh, 1896):

$$\ln\left(\frac{R_t}{R_0}\right) = (\alpha C - 1) \times \ln\left(\frac{C_t}{C_0}\right). \quad \text{eq. 4.2}$$

$R_t$  and  $R_0$  are the isotopic compositions (ratio of  $^{13}\text{C}/^{12}\text{C}$ ) of the substrate at time  $t$  and time 0, and  $C_t$  and  $C_0$  are the concentrations of  $^{12}\text{C}$  in the substrate at time  $t$  and time 0.  $\alpha C$  is the carbon isotope fractionation factor relating changes in concentration to changes in isotope composition. When the concentration of the heavy isotope is negligible as  $^{13}\text{C}$  is in a natural system,  $C_t$  and  $C_0$  may be approximated as the total concentration of  $^{13}\text{C}$  plus  $^{12}\text{C}$  in the substrate at time  $t$  and time 0.

The isotope fractionation was reported as an isotope enrichment factor,  $\varepsilon C$ ,

$$\varepsilon C[\text{‰}] = (\alpha C - 1) \times 1000 \quad \text{eq. 4.3}$$

The  $\varepsilon C$  reflects the overall fractionation of an element in the compound. An intrinsic isotope fractionation factor  $\varepsilon C_{\text{intrinsic}}$  was calculated to normalize the observed isotope fractionation of a molecule to the kinetic isotope effect of bond cleavage by subtracting dilution of non-reactive positions (Supporting information) and was calculated as (Morasch et al., 2004)

$$\varepsilon C_{\text{intrinsic}} = \varepsilon C \times n \quad \text{eq. 4.4}$$

where  $n$  is the number of the atoms of interest in one molecule, i.e.,  $n=12$  for PCDDs and 2 for chloroethenes. This approach can be used to compare isotope fractionation of various chemicals (Elsner et al., 2005).



#### 4.2.6 Comparison of isotope enrichment of PCDDs

Analysis of variance (ANOVA) was performed to compare the  $\delta^{13}\text{C}$  values of 1,2,3,4-TeCDD and its dechlorination products 1,2,4-TrCDD and 1,3-DCDD. Analyses were performed in Microsoft Excel using  $\delta^{13}\text{C}$  values of all time points from 0 to 153 days.

### 4.3 Results and discussion

#### 4.3.1 Dechlorination

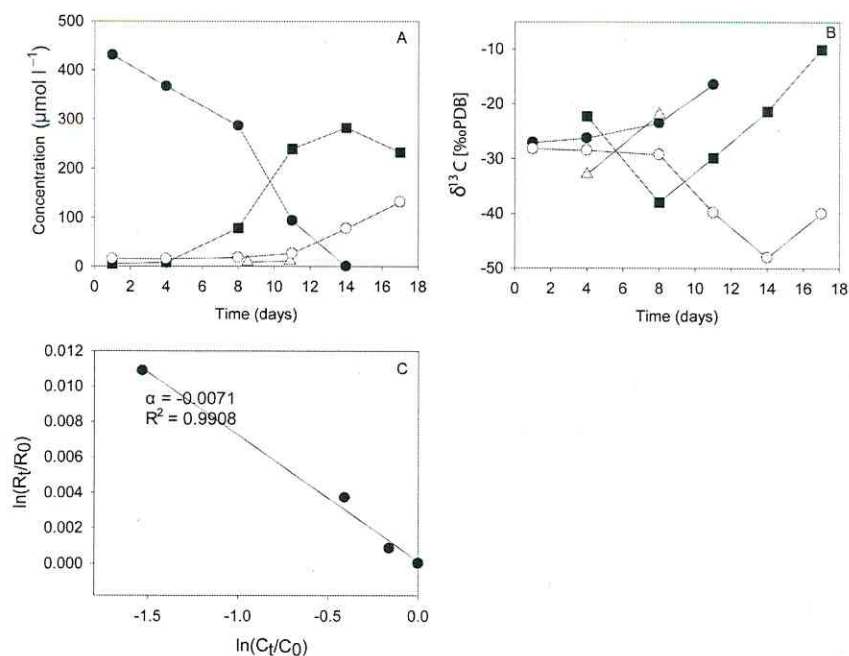
The mixed culture containing *D. ethenogenes* strain 195 dechlorinated PCE to VC and ethene via TCE and *cis*-DCE (Fig. 4.1 A). The background VC and ethene detected at day 1 were carried over from culture inoculum. TCE, appeared on day 4. On day 8 both TCE and *cis*-DCE were detected, and these intermediates underwent further, rapid dechlorination. At day 8 VC production increased rapidly and ethene was slightly increased over the background concentration. Ethene increased rapidly on day 14, when only VC was present. PCE was dechlorinated from 430  $\mu\text{M}$  to about 90  $\mu\text{M}$  within 11 days and was completely dechlorinated by day 14. By day 17, VC and ethene were 230  $\mu\text{M}$  and 130  $\mu\text{M}$ , respectively. A loss of approximately 70  $\mu\text{M}$  of chloroethenes was observed and was likely caused by removal of at least 1 ml of headspace at each sampling event. Additional leakage during incubation cannot be excluded.

The mixed culture dechlorinated 1,2,3,4-TeCDD both in the presence and absence of PCE. Dechlorination of 1,2,3,4-TeCDD to 1,3-DCDD via 1,2,4-TrCDD in two replicate cultures without PCE addition is shown in Figures 2 A and 2 C. In killed controls 1,2,4-TrCDD and 1,3-DCDD trace amounts were detected only at the final time point (Fig. S4). A slight lag time in 1,2,4-TrCDD production was observed when PCE was added as an additional chlorinated substrate (Fig. S2). By day 153 in bottles without PCE addition, total PCDDs consisted of 1,2,3,4-TeCDD, 1,2,4-TrCDD and 1,3-DCDD at  $24\pm3.8$ ,  $42\pm0.6$  and  $33\pm3.4$  mol%, respectively. In the bottles with PCE addition, total PCDDs consisted of 1,2,3,4-TeCDD, 1,2,4-TrCDD and 1,3-DCDD at  $23\pm4.5$ ,  $57\pm3.2$  and  $19\pm1.3$  mol%, respectively. Thus, the treatments exhibited different rates of product formation. In cultures

amended with both 1,2,3,4-TeCDD and PCE, PCE was dechlorinated to VC and ethene (data not shown).

#### 4.3.2 Carbon stable isotope fractionation of PCE

Carbon stable isotope fractionation was observed during dechlorination of PCE (Fig. 4.1 B). The first dechlorination step from PCE to TCE led to a depletion of  $^{13}\text{C}$  in TCE (-32.9‰) and enrichment of  $^{13}\text{C}$  in PCE (-26.3‰) (day 4). Rayleigh analysis (eq. 4.2) yielded an  $\epsilon_{\text{C}}$  of  $-7.1 \pm 0.5$  ‰ for PCE (Fig. 4.1 C). Fractionation of TCE could not be quantified because TCE is an instantaneously formed product which is further dechlorinated to *cis*-DCE. Qualitatively, TCE became enriched in  $^{13}\text{C}$  during the second dechlorination step (day 8) as indicated by an enrichment in  $^{13}\text{C}$  (-21.9‰) compared to the substrate PCE (-23.5‰). Traces of *cis*-DCE were transiently detected; however, it was not possible to determine its isotope composition. VC, the subsequent dechlorination product, was significantly depleted in  $^{13}\text{C}$  (-38‰) compared to TCE. By day 11, VC became enriched (-29.8‰) and significantly depleted ethene (-39.8‰) appeared suggesting strong isotope fractionation in the final dechlorination step. By day 14 ethene became even more depleted (-48‰) and then became slightly enriched again (-40‰). An isotope balance was not observed because the experiment was stopped before dechlorination was complete and because the VC and ethene present at day 1 were highly  $^{13}\text{C}$  enriched products transferred with the inoculum. The isotope composition of the background ethene (-28.2‰) was near that of the substrate PCE (-27 to 28‰) indicating that the isotope balance in the inoculum was closed. Nonetheless, the ethene formed during the experiment was also highly depleted in  $^{13}\text{C}$  indicating significant isotope fractionation during its formation.



**Figure 4.1** Concentrations (A) and carbon stable isotope compositions (B) of tetrachloroethene (PCE) (●), trichloroethene (TCE) (Δ), vinyl chloride (VC) (■), and ethene (○) during the reductive dechlorination of PCE by a mixed culture containing *Dehalococcoides ethenogenes* strain 195. The isotope fractionation factor for PCE (●) was determined by the Rayleigh model in (C).

#### 4.3.3 Carbon stable isotope fractionation of PCDDs

The isotope compositions of the PCDDs in replicates 1 and 2 of the treatments without PCE are shown in Figures 4.2 B and 4.2 D. The averages and ranges of isotope compositions of PCDDs in these replicates are listed in Table 4.1. Isotope compositions for replicate 3 of the treatment without PCE and for the triplicates of the treatment with added PCE are shown in supporting information (Fig. S2). The 1,2,4-TrCDD was isotopically enriched in  $^{13}\text{C}$  compared to the substrate, 1,2,3,4-TeCDD. The 1,3-DCDD was depleted in  $^{13}\text{C}$  relative to the substrate, 1,2,3,4-TeCDD and the intermediate, 1,2,4-TrCDD. This pattern was consistent in triplicate bottles of both treatments (with and without PCE), although the isotopic ranges for each compound varied slightly. The carbon isotopic compositions of total PCDDs in the system varied from

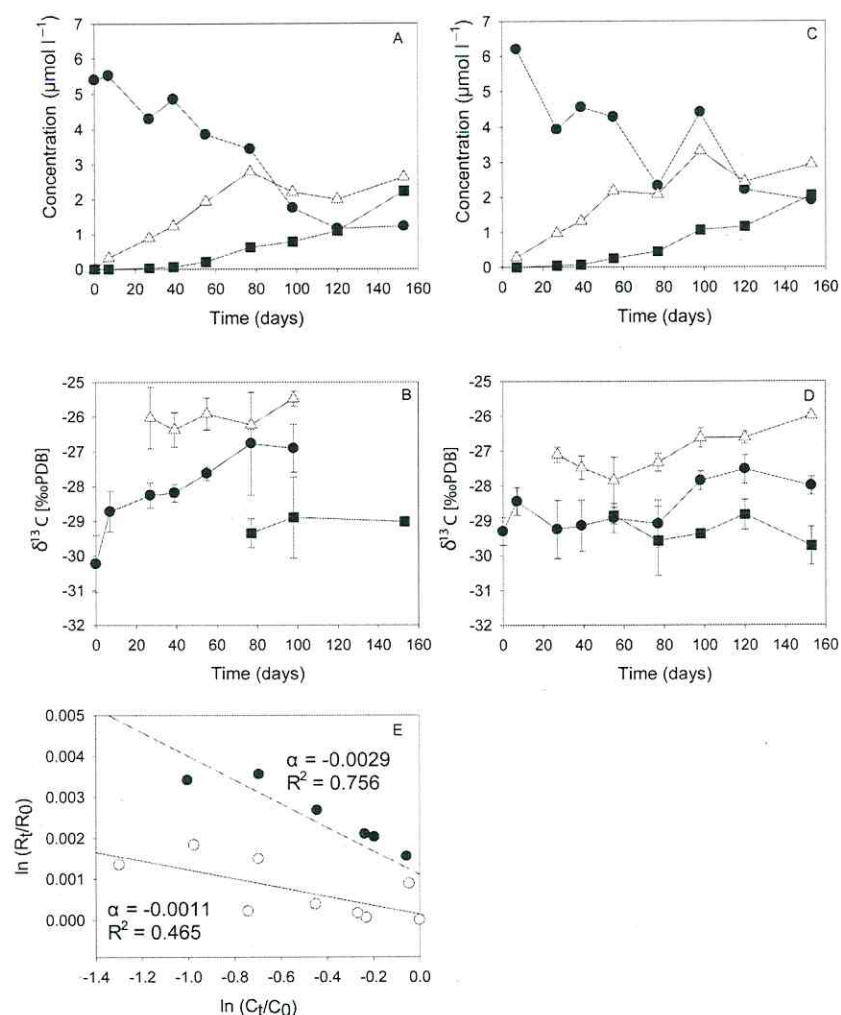
-28.6‰ to -26.6‰ (Fig. S5). ANOVA performed on the entire data set for each congener from each treatment (with and without PCE) yielded  $p$  values significantly less than 0.01 (Table 4.1) confirming that the difference between isotope compositions of the different congeners was significant at the 99% confidence level. The enrichment of  $^{13}\text{C}$  in the product 1,2,4-TrCDD is unlikely to occur as a direct result of carbon-chlorine bond cleavage in the step from 1,2,3,4-TeCDD to 1,2,4-TrCDD. Rather a greater magnitude of isotope fractionation for dechlorination of 1,2,4-TrCDD to 1,3-DCDD than for 1,2,3,4-TeCDD to 1,2,4-TrCDD may explain the observed isotope pattern. Ewald et al. (2007) reported a similar pattern for 1,2,4- and 1,2,3-TrCDD dechlorination where the first transformation step to DCDDs was thought to be associated with a lower isotope fractionation than the subsequent step to monochlorodibenzo-*p*-dioxin.

**Table 4.1** Average carbon stable isotope compositions ( $\delta^{13}\text{C}$ [‰]) of polychlorinated dibenzo-*p*-dioxins (PCDDs), isotope balance, and  $p$  value from analysis of variance (ANOVA) of the isotope compositions of the different PCDD congeners.

Treatment		1,2,3,4-TeCDD $\delta^{13}\text{C}$ [‰]	1,2,3,4-TeCD plus PCE $\delta^{13}\text{C}$ [‰]
1,2,3,4-TeCDD	Average	-28.5 ± 0.8	-29.2 ± 0.6
	Range	-30.2 ± 0.8 ~ -26.8 ± 1.5	-29.0 ± 0.2 ~ -27.2 ± 0.3
1,2,4-TrCDD	Average	-26.8 ± 1.0	-26.8 ± 0.5
	Range	-28.8 ± 0.6 ~ -25.4 ± 0.1	-27.9 ± 0.5 ~ -26.2 ± 0.4
1,3-DCDD	Average	-29.5 ± 0.6	-28.1 ± 0.5
	Range	-30.9 ± 0.8 ~ -28.8 ± 0.4	-30.1 ± 2.0 ~ -28.0 ± 0.3
Isotope balance		-27.7 ± 0.7	-27.5 ± 0.4
$p$ value		2.57E-12	1.68E-16



Rayleigh analysis (eq. 4.2) for replicates 1 and 2 amended with 1,2,3,4-TeCDD (without PCE) are shown in Figure 4.2 E. The  $\epsilon_C$  were  $-2.9 \pm 0.7\text{‰}$  ( $R^2=0.756$ ) and  $-1.1 \pm 0.4\text{‰}$  ( $R^2=0.465$ ), respectively. These results were the most significant that we obtained from our data using Rayleigh analysis. For unknown reasons, Rayleigh analysis for replicate 3 amended with 1,2,3,4-TeCDD (without PCE) yielded a net positive  $\epsilon_C$  (inverse fractionation) (Fig. S3). The  $\epsilon_C$  for cultures amended with 1,2,3,4-TeCDD plus PCE were of less magnitude ( $-0.1 \pm 0.3$  to  $-0.4 \pm 0.2\text{‰}$ ) and had poorer fits ( $R^2=0.013$  to  $0.344$ ) to the Rayleigh model (Table S1, Fig. S3) than those for cultures amended with 1,2,3,4-TeCDD without PCE.



**Figure 4.2** Concentrations (A, C) and carbon isotope compositions (B, D) of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) (●), 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD) (Δ) and 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD) (■) during reductive dechlorination by a mixed culture containing *Dehalococcoides ethenogenes* strain 195 amended with 1,2,3,4-TeCDD as the sole chlorinated substrate in replicate 1 (A, B) and replicate 2 (C, D). Isotope enrichment factors were determined for 1,2,3,4-TeCDD for replicate 1 (●, dashed line) and replicate 2 (○, solid line) using the Rayleigh model in (E).

The different resulting isotope compositions of reactant and metabolites qualitatively indicated substantial isotope fractionation during dechlorination of the PCDDs. However, quantification of the isotopic enrichment of 1,2,3,4-TeCDD using the Rayleigh approach showed high variability and uncertainty in  $\epsilon C$ . This may indicate kinetic limitations previous to the isotope sensitive step, i.e., most likely the carbon–chlorine bond cleavage. Kinetic limitations preceding the isotope sensitive step, such as sorption-desorption of PCDDs (Adriaens et al., 1995) and interaction of the substrate with enzyme, are thought to mask the magnitude of isotope fractionation (Northrop, 1981; Elsner et al., 2005) and may limit the approach. Assuming a maximum aqueous solubility of 1.95 pM 1,2,3,4 TeCDD, greater than 99.99% of the spiked 1,2,3,4-TeCDD was likely sorbed to the sediment, colloidal organic matter (including the bacteria) and the glass surface. Most of the 1,2,3,4-TeCDD, thus, had limited bioavailability. Since a portion of the culture-sediment mixture was analyzed at each time point, the isotope composition of nonreacted 1,2,3,4-TeCDD which may not be bioavailable was superimposed to some extent on the measured isotope composition. For determination of a precise Rayleigh enrichment factor, the substrate should be readily bioavailable and thus in isotopic equilibrium in all compartments of the experiment (i.e., adsorbed, dissolved, and in proximity to the cell or enzyme). Thus, the PCDD isotope fractionation we measured is only an estimate, with the highest enrichment factors observed perhaps approaching the actual biological fractionation. The fractionation may indeed be higher, if the substrate were readily bioavailable. The aqueous solubility of the first metabolite, 1,2,4 TrCDD, is ten-fold higher than that of 1,2,3,4-TeCDD (Shiu et al., 1988), implying higher bioavailability. This may have contributed to the apparently more pronounced fractionation during the subsequent dechlorination of 1,2,4-TrCDD to 1,3 DCDD, and thus may have contributed to the surprising enrichment of  $^{13}C$  in 1,2,4 TrCDD relative to 1,2,3,4-TeCDD.

The lower magnitude  $\epsilon C$  values observed in the treatment with 1,2,3,4-TeCDD plus PCE could be related to a slight lag in 1,2,3,4-TeCDD dechlorination in PCE-amended cultures (Table S1; Fig. S2 C, S2 E and

S2 G) in comparison to cultures that received no PCE (Fig. 4.2 A and 4.2 C). In separate kinetic studies, Liu (Liu, 2007) observed two-fold lower rates of 1,2,3,4-TeCDD dechlorination when PCE was present as an additional substrate. It is possible that a different dehalogenase dechlorinates 1,2,3,4-TeCDD when PCE is present, or that competition between PCE and 1,2,3,4-TeCDD for dehalogenase(s) affects the magnitude of fractionation. Our results demonstrate the complications inherent in interpreting enrichment factors with respect to biochemical mechanisms according to the transition state theory. However the results may be useful for elucidating the kinetic limitations for transformation processes in biological systems (Northrop, 1981).

#### 4.3.4 Comparison to other fractionation studies

The interpretation of  $\epsilon_C$  for characterizing the chemical mechanism of C-Cl bond cleavage is limited when nonisotope fractionating steps control the overall kinetics of the biochemical reaction (Elsner et al., 2005). We calculated  $\epsilon_{C_{intrinsic}}$  (eq. 4.4) to compare carbon isotope fractionation occurring during dechlorination of 1,2,3,4-TeCDD with dechlorination of other compounds, but these should be interpreted with caution. The  $\epsilon_{C_{intrinsic}}$  for PCE was  $-14.2 \pm 1.0\text{‰}$ , while for 1,2,3,4-TeCDD the best case examples were  $-34.8 \pm 8.8\text{‰}$  and  $-13.0 \pm 5.3\text{‰}$ , for two replicate cultures without PCE co-amendment (Table 4.2). The  $\epsilon_{C_{intrinsic}}$  were of the same magnitude for PCE and 1,2,3,4-TeCDD, which suggests the consistency of the carbon isotope fractionation during dechlorination by the mixed culture containing *D. ethenogenes* strain 195. The  $\epsilon_{C_{intrinsic}}$  of 1,2,3-TrCDD dechlorination by a *Dehalococcoides* containing mixed culture (Ewald et al., 2007) was also of the same order of magnitude (Table 4.2). Fractionation also occurred during dechlorination of 1,2,4-TrCDD, but the magnitude was significantly lower and could not be evaluated with the Rayleigh approach (Ewald et al., 2007). Our  $\epsilon_{C_{intrinsic}}$  values were similar to those reported for dechlorination of trichlorobenzenes by *Dehalococcoides* sp. strain CBDB1 (Griebler et al., 2004a) (Table 4.2). These studies all investigated carbon isotope fractionation during the reductive



dechlorination of various organohalides by *Dehalococcoides* spp. and obtained similar  $\epsilon C_{\text{intrinsic}}$ . This suggests the magnitude of carbon isotope fractionation during reductive C-Cl bond cleavage is similar across different strains and substrates and may share a similar mechanism. The maximum kinetic isotope effect according to semiclassical calculation is -54.0‰ (Huskey, 1991; Elsner et al., 2005). The  $\epsilon C_{\text{intrinsic}}$  values for 1,2,3,4-TeCDD, PCE and chlorinated benzenes are slightly lower (Table 4.2). Although these biological reactions were probably all influenced by other kinetically non-fractionating steps, e.g., sorption-desorption, the values fall in the range expected (Elsner et al., 2005).

**Table 4.2** Carbon stable isotope enrichment factors ( $\epsilon C$ ) and intrinsic carbon stable isotope enrichment factors ( $\epsilon C_{\text{intrinsic}}$ ) observed during the reductive dechlorination of 1,2,4- and 1,2,3-trichlorobenzenes (Griebler et al., 2004a), 1,2,3- and 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,3-TrCDD and 1,2,4-TrCDD) (Ewald et al., 2007), tetrachloroethene (PCE) and 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD).

Compound	Culture	$\epsilon C$ [‰]	$\epsilon C_{\text{intrinsic}}$ [‰]	Reference
1,2,4-Trichlorobenzene	<i>Dehalococcoides</i> sp. strain CBDB1	-3.2	-19.5	(Griebler et al., 2004a)
1,2,3-Trichlorobenzene	<i>Dehalococcoides</i> sp. strain CBDB1	-3.5	-22.1	(Griebler et al., 2004a)
	<i>Dehalococcoides</i> sp. strain CBDB1 with methyl viologen	-3.4	-20.8	(Griebler et al., 2004a)
1,2,3 TrCDD	<i>Dehalococcoides</i> mixed culture	-0.92	-11.0 ± 2.2	(Ewald et al., 2007)
PCE	<i>Dehalococcoides ethenogenes</i> strain 195	-7.1 ± 0.5	-14.2 ± 1.0	This study
1,2,3,4-TeCDD replicate 1	Mixed culture containing <i>Dehalococcoides ethenogenes</i> strain 195	-2.9 ± 0.7	-34.8 ± 8.8	This study
1,2,3,4-TeCDD replicate 2	Mixed culture containing <i>Dehalococcoides ethenogenes</i> strain 195	-1.1 ± 0.4	-13.0 ± 5.3	This study

Drenzek et al. (2001) did not observe significant isotope fractionation during dechlorination of 2,3,4,5-tetrachlorobiphenyl (2,3,4,5-TeCB) to 2,3,5-trichlorobiphenyl (2,3,5-TrCB) in sediment free systems. The isotope compositions of 2,3,4,5-TeCB and 2,3,5-TrCB remained nearly constant and 2,3,5-TrCB was about 0.3‰ heavier than 2,3,4,5-TeCB (significant at the 95% confidence level). The 2,3,5-TrCB was not subsequently dechlorinated, and so it was not subject to additional fractionation as our first metabolite, 1,2,4-TrCDD.

We observed an average difference of 1.6‰ between 1,2,4-TrCDD and 1,2,3,4-TeCDD and -2.8‰ between 1,3-DCDD and 1,2,4-TrCDD, in cultures without PCE addition. The differences were 1.3‰ and -2.4‰, respectively, when cultures were amended with PCE. These differences were significant at the 99% confidence level which indicated that dechlorination was associated with isotope fractionation. Our results and those of Ewald et al. (2007) and demonstrate that dechlorination can detectably alter the carbon isotope composition of compounds which have 12 carbon atoms.

#### 4.3.5 Environmental implications

The  $^{13}\text{C}$  isotope enrichment of 1,2,4-TrCDD compared with its parent compound was comparable to fractionation patterns reported previously for dechlorination of chloroethenes. For example, Bloom et al. (2000) detected enriched  $^{13}\text{C}$  in *cis*-DCE relative to TCE in the transformation of TCE to *cis*-DCE. Nijenhuis et al. (2005) described a similar pattern in the biotransformation of PCE to *cis*-DCE via TCE by *Desulfitobacterium* sp. strain PCE-S and *Sulfurospirillum multivorans*. The isotope composition of 1,2,4-TrCDD was controlled by two sequential dechlorination steps of 1,2,3,4-TeCDD. The isotope fractionation during dechlorination of 1,2,4-TrCDD to 1,3-DCDD also influences the isotope patterns and this contributes to the characteristic isotope composition of the metabolites observed during 1,2,3,4-TeCDD transformation.

As was observed by Ewald et al. (2007) with TrCDDs, our experiments confirm that  $^{13}\text{C}$  depleted lightly chlorinated PCDDs may form during

reductive dechlorination. The second dechlorination step exhibited a higher fractionation extent perhaps because the bioavailability of the metabolite was greater than that of the parent compound. The higher aqueous solubility of the metabolite and its formation near the cell or dehalogenase may reduce kinetic limitation from sorption-desorption processes and uptake or transport within the cell, all of which are known to reduce or dampen the isotope fractionation effect (Nijenhuis et al., 2005). Further research is needed for a mechanistic understanding of isotope fractionation during sequential dechlorination reactions. However, as observed for sequential PCE dechlorination the less chlorinated compounds become more strongly fractionated than their precursors.

To our knowledge, this study is the second investigation of isotope fractionation of PCDDs during biological reductive dechlorination (Ewald et al., 2007). Reductive dechlorination of highly chlorinated congeners to congeners with 1 to 3 chlorines has been well documented in sediment enrichments (Adriaens et al., 1995; Beurskens et al., 1995; Ballerstedt et al., 1997; Vargas et al., 2001). *Dehalococcoides* spp. and closely related *Chloroflexi* have been implicated in native PCDD/F dechlorination in environmental samples (Yoshida et al., 2005; Ahn et al., 2007). Confirmation of pathways of PCDD dechlorination and increases in concentrations of dechlorination daughter products is difficult to demonstrate because of multiple congeners that may be present, and because regulatory requirements have generally resulted in quantification of only the highly toxic 2,3,7,8-substituted congeners in most environmental samples. Lightly chlorinated congeners are far less toxic than highly chlorinated congeners and are rarely analyzed in environmental samples (Lohmann et al., 2000; Bunge et al., 2007). Thus, there are few data to support biodechlorination of historical PCDDs. Barabás et al. (2004a,b) used geostatistical analysis of Passaic River, New Jersey, USA, to detect a dechlorination fingerprint for formation of 2,3,7,8-TeCDD from higher chlorinated PCDDs.

Observation of isotope fractionation resulting from dechlorination of PCDDs could provide an additional method for verifying activity. The characteristic isotopic changes of PCDDs related to dechlorination could

be obtained in the laboratory and applied to the field by extrapolation. However, this procedure should be undertaken with caution since the fate of PCDDs in the environment is complex and under the influence of multiple physical, chemical and biological processes. More work is needed to decrease the variability in quantification of the fractionation and to increase the sensitivity of detection of this effect. Further research is needed to determine, if the observed fractionation patterns are related to the activity of specific dehalogenases, cometabolic versus respiratory transformation, or differences in bulk rates of reaction. This study illustrated isotope fractionation during bacterial reductive dechlorination of 1,2,3,4-TeCDD and  $\epsilon C_{\text{intrinsic}}$  were similar to those for other chlorinated compounds undergoing reductive dechlorination. The unique isotope pattern illustrated here might be used as an evidence for biotransformation processes.



## 4.4 Supporting information

### 4.4.1 Calculation of the intrinsic kinetic isotope effect

The intrinsic kinetic isotope effect (KIE) is defined as the ratio of the first order rate constants of the lighter to the heavier isotope species (Elsner et al., 2005):

$$\text{KIE} = \frac{{}^lK}{{}^hK}, \quad \text{eq. S1}$$

where  ${}^lK$  is the first rate constant of the lighter isotope species and  ${}^hK$  is that of the heavier isotope species.

The isotope fractionation factor  $\alpha$  can be defined as:

$$\alpha = \frac{{}^hK}{{}^lK}. \quad \text{eq. S2}$$

Thus,

$$\alpha = \frac{1}{\text{KIE}}. \quad \text{eq. S3}$$

Further,

$$\varepsilon = \left( \frac{1}{\text{KIE}} - 1 \right) \times 1000. \quad \text{eq. S4}$$

The  $\varepsilon$  calculated from equation S4 is the intrinsic enrichment factor which reveals the real difference between the reaction rates of molecules containing different isotope species at the reactive site. However, the enrichment factor  $\varepsilon$  calculated based on the Rayleigh equation from experimental data is the average isotope effect observed for the total molecule. Elsner et al. (2005) discussed factors that mask the intrinsic kinetic isotope effect, i.e.: the dilution effect from non-reactive position where the isotope is present in the molecule at other than the reactive site, the intramolecular competition of isotopes in indistinguishable reactive positions, and the influence of rate limiting steps prior to bond breakage/commitment to catalysis.

The dilution effect of non-reactive positions is eliminated by converting the bulk enrichment factor to a position-specific enrichment factor

$$\varepsilon_{\text{reactive position}} = n/x \times \varepsilon_{\text{bulk}} \quad \text{eq. S5}$$

where  $\varepsilon_{\text{reactive position}}$  is the position-specific enrichment factor,  $\varepsilon_{\text{bulk}}$  is the bulk enrichment factor derived from experimental data, and  $n$  is the number of atoms of the element of interest where  $x$  is the number of atoms located at the reactive site.

Further, intramolecular competition could be eliminated by calculation of  $\varepsilon_{\text{intrinsic}}$

$$\varepsilon_{\text{intrinsic}} = z \times \varepsilon_{\text{reactive position}} \quad \text{eq. S6}$$

where  $z$  is the number of indistinguishable reactive positions in one molecule that the isotope of interest might occupy. For the primary isotope effect in non-concerted reactions,  $z=x$ . Therefore, equation S6 may also be expressed as

$$\varepsilon_{\text{intrinsic}} = n \times \varepsilon_{\text{bulk}} \quad \text{eq. S7}$$

The concept of  $\varepsilon_{\text{intrinsic}}$  was defined by Morasch (2004) as the bulk enrichment factor times the number of atoms of element of interest in a molecule. Although the reasoning was done with a different approach, the final equation for calculation of the intrinsic enrichment factor is the same.

For a symmetrical molecule like tetrachloroethene (PCE),  $\varepsilon_{\text{reactive position}} = \varepsilon_{\text{bulk}}$ . Only intramolecular competition needs to be considered. So the intrinsic enrichment factor of PCE is  $2 \times \varepsilon_{\text{bulk}}$ , since the two carbon atoms are indistinguishable reactive sites for the reductive dechlorination of PCE.

For 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD),  $n=12$  and  $x=1$ . There is no intramolecular competition in the molecule of 1,2,3,4-TeCDD, thus the intrinsic enrichment factor of 1,2,3,4-TeCDD is  $12 \times \varepsilon_{\text{bulk}}$ .

The Streitwieser semiclassical limits for isotope effects at 25°C is 1.057 (Huskey, 1991; Elsner et al., 2005) for C-Cl bond breakage, which is a theoretical prediction of the maximum KIE, denoted as  $KIE_M$ . Based on eq. S4, the maximum isotope enrichment factor is thus -53.9‰. As Elsner et al. (2005) discussed, realistic values with an assumption of the transition state at 50% bond breakage would yield a predicted KIE value of 1.03. Similarly, the corresponding isotope enrichment factor is -29.1‰, comparable to the absolute values for  $\epsilon_{intrinsic}$  obtained in this study (Table 4.2).

#### 4.4.2 Linearity of GC-C-IRMS response for PCDDs

The linearity of the detector response of the gas chromatography-combustion-isotope ratio mass spectrometer (GC-C-IRMS) was assessed for 1,2,3,4-TeCDD over a detector output range of 80 to 1400 mV (Fig. S1). A linear regression of the detector response had a slope of 0.82, indicating that the non-linearity of the instrument during the analysis of 1,2,3,4-TeCDD was about 0.8‰ per 10-fold increase in the detector response between 80 and 1400 mV. This systematic error is higher than that reported for other compounds. For example, the detector response for toluene exhibited a nonlinearity of 0.1‰ per 10-fold increase in the detector response between 45 and 3500 mV (Richnow et al., 2003). This could be a result of the poor combustibility of the polychlorinated dibenzo-*p*-dioxins (PCDDs) related to their high molecular weight and stable molecular structure. These characteristics may result in higher error during GC-C-IRMS measurement of the stable carbon isotope ratios of the PCDDs. The values were not corrected for linearity and isotope values were reported for measurements where concentrations were of the same order of magnitude and thus comparable wherever possible.

#### 4.4.3 Carbon stable isotope fractionation of PCDDs

Besides the two replicates presented in the article, all other PCDD data are presented here, including replicate 3 from the treatment with 1,2,3,4-

TeCDD as the sole halogenated compound and three replicates from the treatment with PCE as an additional substrate (Fig. S2). Rayleigh model analyses for all these replicates are shown in Figure S3. The enrichment factors and intrinsic enrichment factors for all replicates are listed in Table S1. The results show that the enrichment factors for 1,2,3,4-TeCDD during the reductive dechlorination were quite low and their variability was large. This is understandable considering several factors. First, kinetic limitations preceding the isotope sensitive step, such as sorption-desorption of PCDDs and interaction of the substrate with enzyme, could mask the magnitude of isotope fractionation and limit the approach (Elsner et al., 2005). Second, the physical-chemical properties of PCDDs make them difficult candidates for isotope analysis. As discussed previously, they have a stable structure and poor combustibility, which may result in error in isotope analysis. Compound specific isotope analysis requires good separation of the components in the samples, which is sometimes difficult to achieve in practice. It also requires that the compound of interest in the samples have sufficient concentration to obtain reasonably precise measurements. Although it has been shown with variability, carbon isotope fractionation was observed in the reductive dechlorination of 1,2,3,4-TeCDD. The consistent and unique pattern of the isotope compositions of the PCDDs and the quantitative analysis with the Rayleigh equation confirmed carbon isotope fractionation during 1,2,3,4-TeCDD dechlorination.

The concentration and isotope data for the killed controls are shown in Figure S4. No significant product formation was observed in the killed controls.



#### 4.4.4 Carbon stable isotope mass balance

In a closed system, the mass balance on the isotopes of carbon in different compounds in the system is expressed as

$$R_{\text{Total},t} = \sum R_{i,t} \times f_{i,t} = \sum R_{i,0} \times f_{i,0} \quad \text{eq. S8}$$

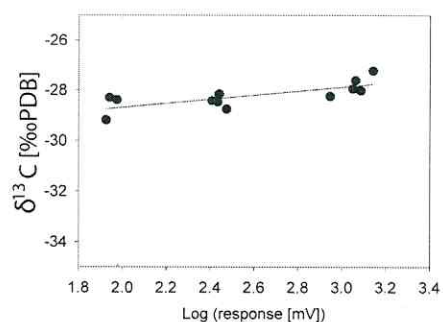
where  $R_{\text{Total},t}$  is the carbon isotope ratio of the total pool of parent compound and its metabolites in the system at time  $t$ . Theoretically,  $R_{\text{Total},t}$  should be constant over the course of the reaction.  $R_{i,0}$  is the carbon isotopic composition of a chemical  $i$  at time 0 and  $f_{i,0}$  is the fraction of chemical  $i$  at time 0. Similarly,  $R_{i,t}$  and  $f_{i,t}$  are its carbon isotopic composition and fraction at time  $t$ . The average isotope mass balance was calculated from triplicate data for each treatment (with and without PCE addition) for time points where detection and quantification of all PCDDs were possible (Fig. S5).

#### 4.4.5 Tables

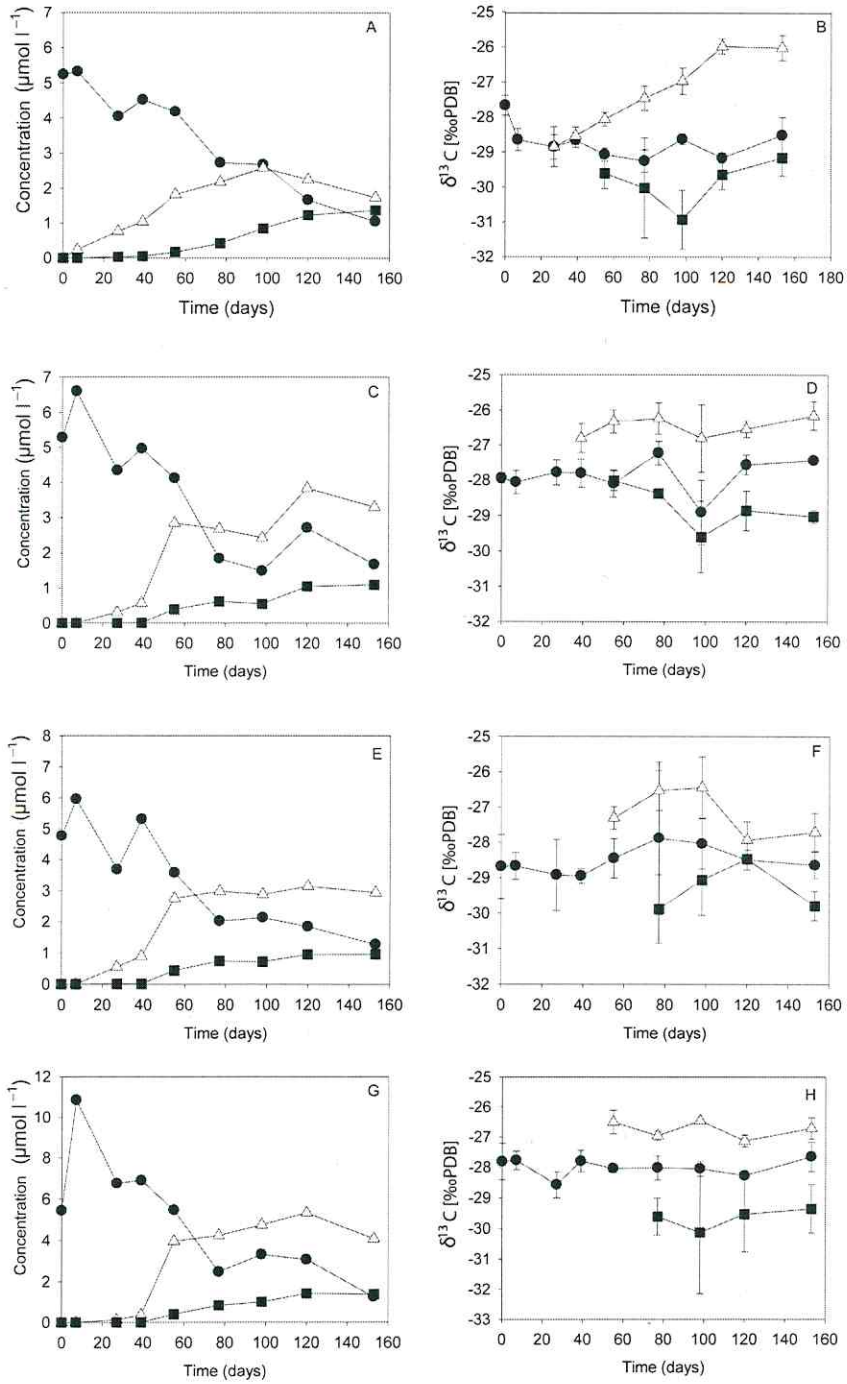
**Table S1** Carbon stable isotope enrichment factor ( $\epsilon_C$ ) and intrinsic carbon stable isotope enrichment factor ( $\epsilon_{C_{\text{intrinsic}}}$ ) observed during the reductive dechlorination of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) in a mixed culture containing *Dehalococcoides ethenogenes* strain 195 with and without addition of tetrachloroethene (PCE).

Compound	$\epsilon_C$ [‰]	$\epsilon_{C_{\text{intrinsic}}}$ [‰]	$R^2$
1,2,3,4-TeCDD replicate 1	$-2.9 \pm 0.7$	$-34.8 \pm 8.8$	0.756
1,2,3,4-TeCDD replicate 2	$-1.1 \pm 0.4$	$-13.0 \pm 5.3$	0.465
1,2,3,4-TeCDD replicate 3	$0.4 \pm 0.3$	$4.2 \pm 4.2$	0.125
1,2,3,4-TeCDD plus PCE replicate 1	$-0.1 \pm 0.3$	$-1.4 \pm 4.1$	0.016
1,2,3,4-TeCDD plus PCE replicate 2	$-0.4 \pm 0.2$	$-4.7 \pm 2.5$	0.344
1,2,3,4-TeCDD plus PCE replicate 3	$-0.1 \pm 0.2$	$-0.6 \pm 2.1$	0.013

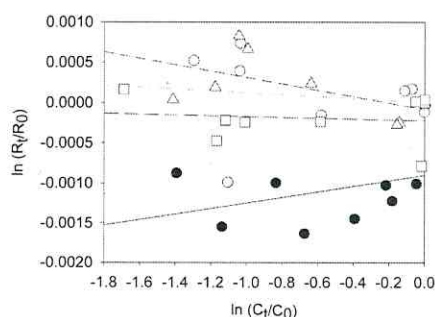
#### 4.4.6 Figures



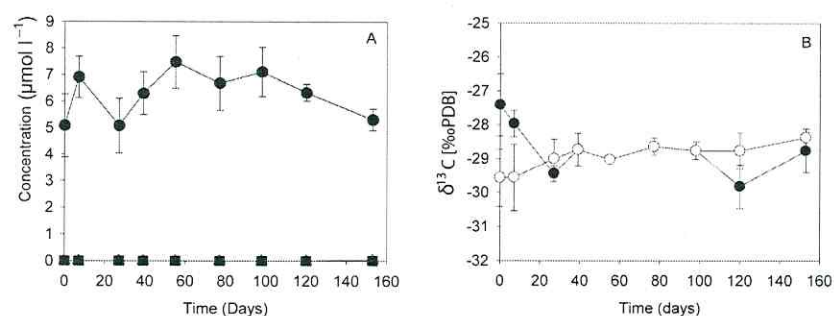
**Figure S1** Linearity of the GC-C-IRMS detector response for the 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) standard over a detector output range of 80 to 1400 mV.



**Figure S2** Concentrations (A, C, E, G) and carbon isotope compositions (B, D, F, H) of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) (●) and its products 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD) (Δ) and 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD) (■) during reductive dechlorination by a mixed culture containing *Dehalococcoides ethenogenes* strain 195. Shown are replicate 3 (A, B) from treatment with 1,2,3,4-TeCDD as the sole chlorinated substrate; and replicates 1 (C, D), 2 (E, F) and 3 (G, H) from the treatment with 1,2,3,4-TeCDD plus PCE as an additional chlorinated substrate.

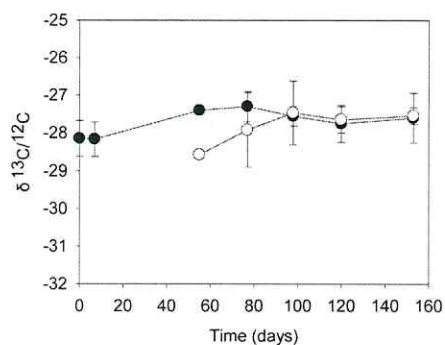


**Figure S3** Rayleigh model regression of replicate 3 (●, solid line) from culture amended with 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) as the sole chlorinated substrate; and replicates 1 (○, dashed line), 2 (Δ, dotted line) and 3 (□, dash-dot line) from culture amended with 1,2,3,4-TeCDD plus tetrachloroethene (PCE) as an additional chlorinated substrate.



**Figure S4** Average concentrations (A) of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) (●) and its products 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD) (Δ) and 1,3-dichlorodibenzo-*p*-dioxin (1,3-DCDD) (■) and carbon isotope composition (B) of 1,2,3,4-TeCDD in replicate killed (autoclaved) controls.





**Figure S5** Average isotope mass balance of polychlorinated dibenzo-*p*-dioxins in treatments amended with 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) alone (○) and 1,2,3,4-TeCDD plus tetrachloroethene (PCE) (●).

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# 5

## **Characterization of highly enriched *Dehalococcoides*-containing culture derived from contaminated mega-site in Bitterfeld dechlorinating tetrachloroethene to ethene**

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Manuscript in preparation

### **Abstract**

A culture capable of reductive dechlorination of tetrachloroethene (PCE) to ethene was enriched. Microcosms were set up using groundwater from a chlorinated ethenes contaminated anaerobic aquifer in Bitterfeld (Germany). Active, lactate amended, microcosms capable of PCE dechlorination to ethene without accumulation of intermediates were used for further enrichment. After three transfers on lactate as electron donor and PCE as electron acceptor, the enrichment was transferred to parallel cultures with one of the chlorinated ethenes as an electron acceptor and acetate and hydrogen as a carbon and energy source. After three more transfers, a highly purified culture was derived capable of dechlorinating PCE with hydrogen and acetate as the electron and carbon source respectively. PCR followed by denaturing gradient gel electrophoresis, cloning, and sequencing revealed that this culture was dominated by a *Dehalococcoides* sp. strain belonging to the Pinellas group. Investigation of substrate specificity in the parallel cultures suggested the presence of a novel *Dehalococcoides* strain which can grow with either PCE, trichloroethene (TCE), *cis*-dichloroethene (*cis*-DCE) or vinyl chloride (VC) as the electron acceptor. The strain was designated BTF08 due to its origin in Bitterfeld. Strain BTF08 has very high potential for bioremediation due to its ability to completely dechlorinate PCE to non-toxic ethene, which is particularly desirable at contaminated sites.

## 5.1 Introduction

Bitterfeld is a contaminated mega-site located in eastern Germany (Sachsen Anhalt). As a result of long history of chemical industry, soil, surface water and groundwater in this region became highly polluted with a mixture of various harmful substances, e.g. chlorinated aliphatics, BTEX, hexachlorocyclohexane, chlorobenzenes among many others. The contaminated area in Bitterfeld comprise about 25 km<sup>2</sup> corresponding to 200 million m<sup>3</sup> of contaminated groundwater (Popp et al., 2000; Wycisk et al., 2003; Heidrich et al., 2004b). Tetrachloroethene (PCE) and trichloroethene (TCE), which are among the most common groundwater contaminants in most industrialized countries and are suspected carcinogens, are present in Bitterfeld in huge amounts (Rivett et al., 2006; ASTDR, 2007). Dechlorination products of higher chlorinated ethenes, dichloroethenes (DCEs) and vinyl chloride (VC), have been also detected at this site, suggesting the microbial degradation (Heidrich et al., 2004a; Nijenhuis et al., 2007).

While higher chlorinated ethenes are persistent under aerobic conditions they can be transformed to lower chlorinated ethenes and/or harmless ethene in anaerobic environments. The main process contributing to the natural attenuation of higher chlorinated ethenes under anaerobic conditions is reductive dechlorination (Maymo-Gatell et al., 1997). Microorganisms can gain energy from this reaction and chlorinated ethenes act as terminal electron acceptors. Various electron donors can support the growth, e.g. butyrate, propionate, lactate, acetate or hydrogen, depending on the strains involved in the dechlorination as well as on the mixed microbial community present *in situ* (He et al., 2002; Freeborn et al., 2005). Diverse microbial strains, belonging to  $\delta$ -Proteobacteria,  $\gamma$ -Proteobacteria and Firmicutes can reductively dechlorinate chlorinated ethenes. However, most of them degrade PCE and TCE only to *cis*-dichloroethene (*cis*-DCE) (Holliger et al., 1998a; Löffler et al., 2003; Smidt and de Vos, 2004). The organisms capable of complete detoxification of PCE and TCE to ethene are restricted to one phylogenetic group of



bacteria, the *Dehalococcoides* genus within the Chloroflexi phylum (green non-sulphur bacteria) (Maymo-Gatell et al., 1997).

Reductive dechlorination is being taken advantage of at chloroethene-contaminated sites in bioremediation approaches such as monitored natural attenuation, biostimulation and bioaugmentation. Nevertheless, these remediation strategies often result in accumulation of intermediate degradation products, of which VC, known human carcinogen, is of particular concern (Bradley, 2000; European Parliament 2006). As *Dehalococcoides* play a crucial role in the complete detoxification of the chlorinated ethenes, they draw attention not only of the scientific community but also of the bioremediation companies. Thus far, numerous strains have been characterized. However, only few of them are able to convert VC to non-toxic ethene in relatively fast metabolic manner. *Dehalococcoides* strains isolated to date and their substrate ranges are summarized in Table 5.1.

Recently, Imfeld et al. (2008) and Nijenhuis et al. (2007) investigated a microbial community potentially involved in the reductive dechlorination in Bitterfeld and detected organisms belonging to the genera *Dehalobacter*, *Desulfuromonas*, *Desulfitobacterium* and *Dehalococcoides*. In addition, Nijenhuis et al. (2007) retrieved the *Dehalococcoides* sequences from groundwater and transfer cultures derived from Bitterfeld. Interestingly, the sequence analysis revealed that Bitterfeld *Dehalococcoides* population was dominated by only one type of microorganism, which shared 99% identity with *Dehalococcoides* strain BAV1. Strain BAV1 is one of very few isolates capable of fast, metabolic conversion of DCEs and VC to ethene (He et al., 2003a). Therefore, Nijenhuis et al. (2007) suggested that the *Dehalococcoides* strain detected in Bitterfeld could be the key organism responsible for the complete detoxification of chloroethenes at this site.

The study presented here aimed at enriching the microbial strains responsible for the conversion of PCE to ethene at the contaminated site in Bitterfeld employing standard liquid enrichment strategies with chlorinated ethenes as electron acceptors and lactate or hydrogen as

electron donors. Active cultures were transferred several times and the evolution of microbial community in subsequent enrichments was studied using DGGE and clone libraries. DGGE analysis revealed that the cultures transferred on PCE and hydrogen (4<sup>th</sup> transfer) contained highly enriched *Dehalococcoides* community. The highly enriched 6<sup>th</sup> transfer of this culture was investigated with respect to its substrate range for the chlorinated ethenes and was observed to reductively dehalogenate PCE, TCE, *cis*-DCE and VC but not *trans*- and 1,1-DCE. In this manuscript, we refer to the *Dehalococcoides* strain in our highly enriched culture as strain BTF08 due to its origin in Bitterfeld.

Our findings suggest that strain BTF08 can dechlorinate and grow with PCE, TCE, *cis*-DCE and VC, which is a novel activity pattern. Strain BTF08 is, to our knowledge, the first described *Dehalococcoides* strain originating from a European field site capable of the conversion of DCE and VC. It belongs, based on 16S rRNA gene analysis, to the Pinellas cluster. In view of bioremediation, strain BTF08 has very high potential, for example for the bioaugmentation approach, as its ability to completely dechlorinate PCE to non-toxic ethene in the fast metabolic manner, is particularly desirable at contaminated sites.

**Table 5.1** *Dehalococcoides* strains characterized to date and their substrate ranges.

<i>Dehalococcoides</i> sp. strain	Group <sup>b</sup>	Chlorinated ethenes utilization	Main end product	Other halogenated substrates	Reference
BTF	P	PCE, TCE, <i>cis</i> -DCE, VC	Ethene	ND	This study
BAV1	P	PCE <sup>a</sup> , TCE <sup>a</sup> , DCEs, VC	Ethene	ND	(He et al., 2003a) (He et al., 2003b) (Sung et al., 2006b)
GT	P	TCE, <i>cis</i> -DCE, 1,1- DCE, VC	Ethene	ND	(He et al., 2005)
FL2	P	PCE <sup>a</sup> , TCE, <i>cis</i> -DCE, <i>trans</i> -DCE, VC <sup>a</sup>	VC, ethene		(Adrian et al., 2000),(Bunge et al., 2003),(Adrian et al., 2007), (Adrian, 2001)
CBDB1	P	PCE, TCE	<i>trans</i> -DCE	chlorobenzenes, chlorinated dioxins, chlorophenols	(Duhamel et al., 2004)
KB-1/VC <sup>c</sup>	P	TCE, <i>cis</i> -DCE, VC	Ethene	ND	(Cupples et al., 2003)
VS <sup>c</sup>	V	TCE, <i>cis</i> -DCE, 1,1- DCE, VC	Ethene	ND	(Müller et al., 2004), (Maymo-Gatell et al., 1997), (Fennell et al., 2004), (Adrian et al., 2007)
195	C	PCE, TCE, <i>cis</i> -DCE, 1,1-DCE, VC <sup>a</sup>	VC, ethene	chloroethanes, chlorobenzenes, chloronaphthalenes, chlorinated dioxins, polychlorobiphenyls, chlorophenols	

ND – not determined

<sup>a</sup>Chlorinated ethenes cometabolized

<sup>b</sup>Groups according to (Hendrickson et al., 2002): C - Cornell, P - Pinellas, V - Victoria

<sup>c</sup>Strains available only in mixed cultures.

## 5.2 Materials and methods

### 5.2.1 Chemicals

All chemicals were purchased from Fluka (Seelze, Germany), Sigma-Aldrich Chemie (Seelze, Germany) or Merck (Darmstadt, Germany) at the highest purity available. Gases were purchased from Airproducts (Hattingen, Germany).

### 5.2.2 Analytical procedures

Gas chromatography (Varian Chrompack CP-3800, Middelburg, the Netherlands) with flame ionisation detection (GC-FID) equipped with a 30m x 0.53mm GS-Q column (J&W Scientific, Waldbronn, Germany) was used to analyze concentrations of the chlorinated ethenes and ethene. The temperature programme used was as follows: 1 min at 100°C, 50°C min<sup>-1</sup> to 225°C, hold 2.5 min. The FID was operated at 250°C and helium was used as carrier gas (0.69 x 10<sup>5</sup> Pa; 11.5 ml min<sup>-1</sup>). This method allowed the separation of ethene, vinyl chloride, 1,1-, *trans*- and *cis*-DCE, TCE and PCE. The sampling was automated using an HP 7694 headspace autosampler (Hewlett Packard, Palo Alto, USA), adding 0.5 ml headspace samples to 10 ml autosampler vials flushed with helium, which were closed with a Teflon<sup>®</sup> coated butyl rubber septum and crimped.

### 5.2.3 Microcosm and enrichment culture preparation

Groundwater samples were collected from the aquifer contaminated with chlorinated ethenes, located in Bitterfeld (Eastern Germany). Microcosms were prepared as described by Nijenhuis et al. (2007). In short, microcosms were prepared in 120 ml bottles filled with 100 ml of groundwater closed with Teflon coated butyl rubber septa and crimped. Lactate (3 mM) was used as electron donor and PCE (100 µmol l<sup>-1</sup>) as electron acceptor. Each microcosm was additionally amended with resazurin (1 mg l<sup>-1</sup>), yeast extract (25 mg l<sup>-1</sup>), and vitamin B<sub>12</sub> (62.5 µg l<sup>-1</sup>). Active microcosms were further transferred according to the enrichment scheme shown in Fig. 5.1.



In the first enrichment phase (Fig. 5.1, A) active microcosms were transferred three times into mineral medium described by Zinder (1998) amended with PCE as electron acceptor and lactate as electron donor and carbon source. The first transfer (0.1) was prepared by 10% dilution of original microcosm (0) in 27 ml tubes filled with 10 ml of medium. For the second transfer (0.2) 5% inoculum (0.1) was used. The third transfer (0.3) was prepared by 1% dilution of previous transfer (0.2) in 120 ml bottles filled with 50 ml of medium.

In the second enrichment phase (Fig. 5.1, B) enrichment culture (0.3) was transferred on different electron acceptors. Cultures were prepared in 50 ml glass bottles, filled with 25 ml of medium (Zinder, 1998) and were amended with  $\text{NaHCO}_3$  ( $1 \text{ g l}^{-1}$ ),  $\text{Na}_2\text{S}$  ( $25 \text{ mg l}^{-1}$ ) and Vitamins (Zinder, 1998). Cultures received PCE, TCE, *cis*-DCE, *trans*-DCE, 1,1-DCE or VC as electron acceptor (around  $10 \text{ } \mu\text{mol}$ , as pure solvent or gas) and were inoculated (4% vol/vol). Two series of enrichment cultures were prepared. In the first series, acetate (3 mM) served as carbon source and hydrogen (overpressure) as electron donor. In the second series, lactate (4 mM) was used as electron donor and carbon source. Cultures were incubated at  $20 \text{ } ^\circ\text{C}$  without shaking.

Active cultures were further transferred on the same electron acceptor, electron donor and carbon source several times (Fig. 5.1, B). Additionally, the second transfer of VC/lactate culture (culture 12.2) was retransferred on VC, acetate and hydrogen, as the initial transfer (6.1) on this electron donor and acceptor did not become active (Fig. 5.1, C).

The third transfer (1.3) enriched on PCE, acetate and hydrogen after receiving additional 3 doses of PCE (total around  $50 \text{ } \mu\text{mol}$ ) was retransferred on all chlorinated ethenes (PCE, TCE, *cis*-DCE, *trans*-DCE, 1,1-DCE or VC). Medium was prepared as described above (second enrichment phase) and hydrogen and acetate were added as electron donor and carbon source, respectively. Cultures were prepared by 4% dilution of culture 1.3 (PCE/acetate+ $\text{H}_2$ ) (Fig. 5.1, B). All treatments were set up in triplicates. Negative controls (uninoculated treatments) were prepared in triplicates for each chlorinated compound separately. At the

beginning of the experiment and at the different degradation points, headspace samples were taken and the concentrations of chlorinated ethenes were analyzed by GC-FID.

A) I. Enrichment phase	
Microcosm from well BVV3051 (24.02.03)	0 (PCE + lactate)
1 <sup>st</sup> Transfer (03.09.03)	0.1 (PCE + lactate)
2 <sup>nd</sup> Transfer (02.02.04)	0.2 (PCE + lactate)
3 <sup>rd</sup> Transfer (22.03.04)	0.3 (PCE + lactate)
B) II. Enrichment phase	

**Figure 5.1** The scheme of the enrichment procedure. <sup>a</sup> Cultures used for DGGE analysis, <sup>b</sup> cultures used for clone libraries, <sup>c</sup> complete 16S rRNA gene sequence of *Dehalococcoides* strain present in the culture was obtained, <sup>d</sup> Not active culture.

#### 5.2.4 DNA extraction and PCR amplification

DNA was extracted with the DNeasy Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions for Gram-positive cells. DNA was eluted in 60 µl of RNase-free distilled water. 16S rRNA genes were PCR amplified using HotStar *Taq* polymerase (Qiagen) in buffer supplied by manufacturer and "universal" bacterial primers 27f (Lane, 1991) and 1378r (Heuer et al., 1997). The thermocycling program was as follows: initial denaturation at 95°C for 15 min followed by 32 cycles of primer annealing at 51°C for 30 s, chain extension at 72°C for 50 s, denaturation at 95°C for 30 s and a final extension at 72°C for 30 min. The second semi-nested PCR round was performed with a second set of Bacteria primers GC968f (Nubel et al., 1996) and 1378r to obtain amplicon size appropriate for DGGE analysis. The following thermocycling program was used: initial denaturation at 95°C for 15 min followed by 30 cycles of primer annealing at 55°C for 1 min, chain extension for 1 min at 72°C, denaturation for 30 s at 95°C and a final extension at 72°C for 30 min.

#### 5.2.5 DGGE

Analysis of 16S rRNA gene PCR products by DGGE was performed as described by Imfeld et al (Imfeld et al., 2008) using the DCode Universal Mutation Detection System (Bio-Rad, Munich, Germany). The 16S PCR products were directly applied to 8% (wt/vol) acryl-bisacrylamide gel (37.5:1, Merck, Darmstadt, Germany) with 30-60% linear urea/formamide denaturing gradient (7 M urea and 40% formamide (vol/vol) as 100% denaturants). The PCR products were separated by electrophoresis for 5.5 h at constant temperature (60°C) in 1 X TAE buffer at 200 V. The gels were stained for 20 min in 0.01% Sybr Green I (Molecular Probes, Leiden, The Netherlands) in 1 X TAE solution at room temperature. DGGE patterns were visualized with UV excitation and recorded with the Chemi Doc system and Quantity One software (Bio-Rad).



#### **5.2.6 Construction of 16S rRNA gene clone libraries**

PCR products amplified from genomic DNA with primers 27f and 1378r were purified with Qiaquick spin columns (Qiagen) according to the manufacturer's protocol. Cloning was performed with the pGEM-T Promega Cloning Kit (Promega, Madison, WI). Recombinant plasmids were extracted by boiling (5 min at 98°C) an inoculation loop of bacterial cells in 50 µl water and pelleting the debris by centrifugation (2 min at 15000 g). The supernatant was transferred into fresh tubes. The inserts were further amplified using M13(-20) and M13rev primers and screened and grouped by amplified ribosomal DNA restriction analysis (ARDRA) using the restriction enzymes *HinP1* I and *BsuRI* (Fermentas, Lithuania, Vilnius) as described earlier (Nikolausz et al., 2004).

#### **5.2.7 Determination of nucleotide sequences**

The partial sequences of the 16S rRNA genes from unique ARDRA types were obtained using the PCR primer 1378r. Primers used for the almost complete 16S rRNA gene sequence determination were 27f, 1378r, 907r, 1114f, 533f and 803f (Lane, 1991), 338R (Amann and Ludwig, 2000). The sequencing reactions were performed by using the Big Dye Terminator Cycle Sequencing Kit V3.1 (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. Sequencing products were separated on a Model 3100 Genetic Analyzer (Applied Biosystems). The sequence alignment was performed using the MultAlign online software tool (Corpet, 1988). Analyses of the sequences and homology searches were performed using the BLAST algorithm with BLAST server of the National Centre for Biotechnology Information (Altschul et al., 1998). Additional phylogenetic analyses were conducted using MEGA4 software (Tamura et al., 2007). The obtained DNA sequences of this study were submitted to GenBank under the accession numbers AM981291 to AM981298.

### 5.2.8 Single nucleotide primer extension (SNUPE)

A simple SNUPE assay was developed to investigate two variable positions of the *Dehalococcoides*-type sequences (Fig. 5.4). DhcPMT1 (TTT ACTGCCCCGCGAAACGG) and DhcPMT2 (TTTTT GACAGAACAATAGGTTGCAA) primers were designed to anneal upstream from variable positions A. and B., respectively. The DhcPMT1 primer extension would result in G incorporation in case of the predominant sequence type and an A extension with the sequence variant A., while DhcPMT2 will be extended in case of predominant and the other sequence variant B. by C and T, respectively.

Cyclic primer extension reactions were performed as described before (Nikolausz et al., 2008). Briefly, in a final volume of 10 µl containing 5 µl SNaPshot multiplex kit reagent (Applied Biosystems), 4 µl of purified PCR products and 1 µl primer solution or primer mixture (10 µM of each primer). The SNUPE reactions were carried out with 35 cycles of denaturation at 96°C for 10 s, annealing at 55°C for 5 s and extension at 60°C for 30 s. In order to remove unincorporated ddNTPs, 1 unit of shrimp alkaline phosphatase (SAP) was added to each reaction and incubated at 37°C for 1 h and then at 75°C for 15 min. SNUPE reactions were run in duplicates to ensure reproducibility. Half µl of post-treated extension products was mixed with 9 µl formamide and 0.5 µl GeneScan-220 LIZ internal size standard (Applied Biosystems). The mixture was denatured at 95°C for 5 min and quickly cooled on ice. DNA fragment separation was performed on an ABI PRISM 3100 Genetic Analyzer using a 36 cm capillary filled with denaturing POP6 polymer with filter set E5 (Applied Biosystems).

## 5.3 Results

### 5.3.1 Enrichment of the BTF culture

The sampling campaign in Bitterfeld took place in February 2003. Microcosms were prepared from groundwater derived from well BVV3051 located at the fringe of a PCE and TCE plume, but with high DCE concentrations (Nijenhuis et al., 2007). In enrichment phase I all transfers were made on PCE as electron acceptor and lactate as electron donor (see Fig. 5.1, A). Duplicate microcosms became active after several months. In September 2003 first (0.1) and in February 2004 a second (0.2) transfer was made. In culture 0.2 dechlorination activity started after less than one month and PCE was degraded directly to ethene without accumulation of intermediate products. Taxon-specific 16S rRNA gene based PCR amplification indicated the presence of *Dehalococcoides*, *Dehalobacter* and *Desulfuromonas* in the culture 0.2. The amplification signal for *Dehalobacter* was very weak (Nijenhuis et al., 2007; Imfeld et al., 2008). In March 2004 the culture 0.2 was transferred to larger vials (transfer 0.3) to produce more biomass for further investigations.

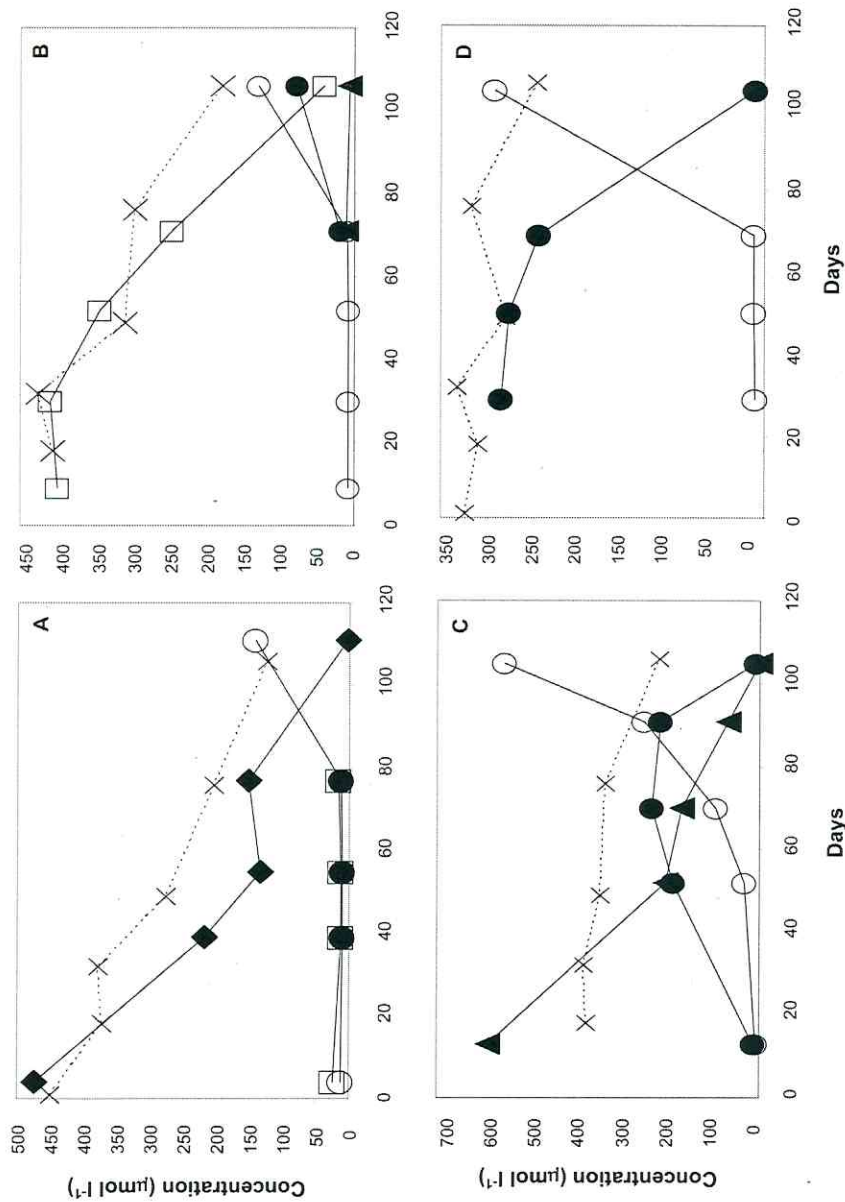
The second enrichment phase was targeted at enrichment and isolation of the *Dehalococcoides* strain detected in our PCE/lactate enrichment culture. Two enrichment series were prepared using chlorinated ethenes (PCE, TCE, *cis*-, *trans*-, 1,1-DCE or VC) as electron acceptors and two different electron donors and carbon sources (Fig. 5.1, B). The 1<sup>st</sup> series was aimed at isolation of *Dehalococcoides* strain, therefore hydrogen and acetate were used as electron donor and carbon source. The 2<sup>nd</sup> series was enriched on lactate - the electron donor used in the first enrichment phase - to maintain the growth of the culture and to have a parallel reference series for H<sub>2</sub>+acetate treatments. The third transfer culture (0.3) from the first enrichment phase was used as inoculum for all treatments.

After around 40 days first cultures showed activity: *cis*-DCE/H<sub>2</sub>+acetate (3.1), *cis*-DCE/lactate (9.1) and VC/lactate (12.1). In the course of next month degradation products were detected in other cultures: TCE/ H<sub>2</sub>+acetate (2.1), 1,1-DCE/ H<sub>2</sub>+acetate (4.1), TCE/lactate

(8.1), 1,1-DCE/lactate (10.1). Treatments having PCE as electron acceptors (1.1 and 7.1), became active the last, after around 80 days. Cultures amended with *trans*-DCE (5.1 and 11.1) as well as culture with VC/H<sub>2</sub>+acetate (6.1) did not start to grow at all. As the VC/H<sub>2</sub>+acetate treatment was not active we retransferred the VC/lactate treatment (12.1) on VC/H<sub>2</sub> +acetate (13.1). The latter culture became active and it was further transferred on VC/H<sub>2</sub> +acetate (Fig. 5.1, C). All active cultures from the 1<sup>st</sup> enrichment series - PCE/H<sub>2</sub>+acetate (1.1), TCE/ H<sub>2</sub>+acetate (2.1), *cis*-DCE/ H<sub>2</sub>+acetate (3.1) and 1,1-DCE/ H<sub>2</sub>+acetate (4.1) - were successfully transferred on the same combination of substrates at least three times (Fig. 5.1, B).

The third transfer of culture growing on PCE/ H<sub>2</sub>+acetate (1.3) was transferred back on all chlorinated ethenes (PCE, TCE, *cis*-, *trans*-, 1,1-DCE or VC) to investigate, if it maintained the ability to carry out all sequential dechlorination reactions leading to complete degradation of PCE to ethene. In this experiment, treatments amended with PCE, TCE, *cis*-DCE and VC exhibited dechlorination activity. All four chlorinated ethenes were degraded with similar efficiencies, 300 – 600  $\mu\text{mol l}^{-1}$  of each were dechlorinated to ethene in around 100 days (Fig. 5.2). The systematic losses of PCE and TCE (Fig. 5.2 A, B) were observed during the experiment. Similar losses were also detected in control treatments suggesting that they might be caused by sampling or adsorption. Only about 30% of PCE and 40% of TCE added to the flasks was left after around 100 days (Fig. 5.2 C, D). For *cis*-DCE and VC the losses were much lower and around 60% of added *cis*-DCE and 75% of VC were still present after around 100 days. No dechlorination products were detected in the cultures, for which 1,1-DCE and *trans*-DCE were used as electron acceptors.





**Figure 5.2** Dechlorination of A) PCE (♦), B) TCE (□), C) cis-DCE (▲) and D) VC (●) to ethene (○) by the highly enriched *Dehalococcoides*-culture. Negative controls for A) PCE, B) TCE, C) cis-DCE and D) VC are indicated with dotted lines (···x···).

### 5.3.2 DGGE

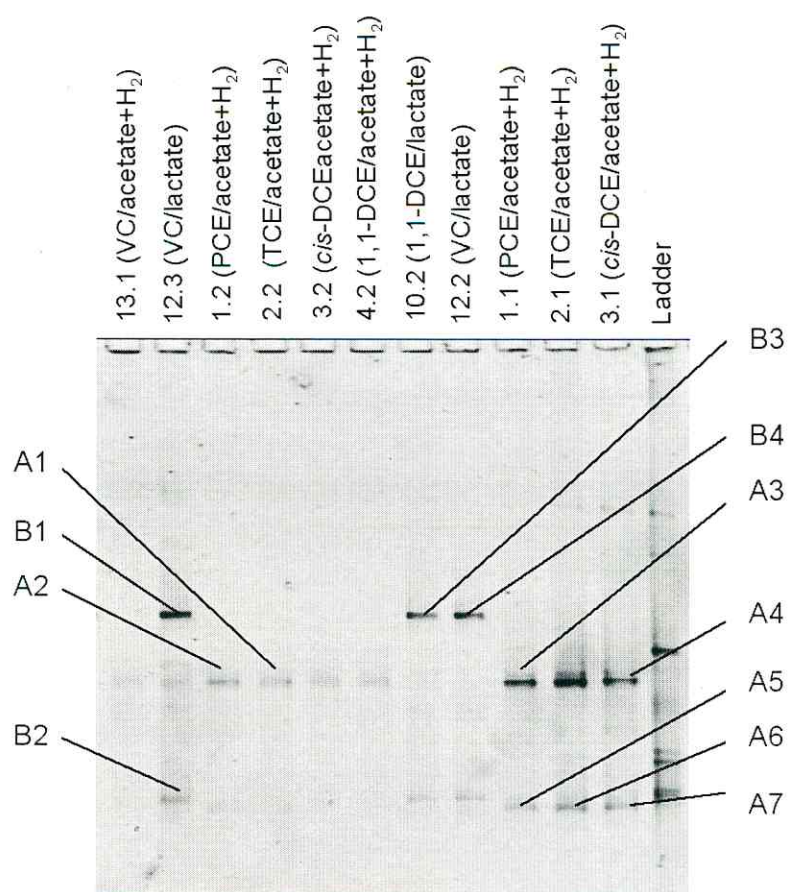
Several cultures, mainly from 1<sup>st</sup> and 2<sup>nd</sup> transfer from enrichment phase II (Fig. 5.1, B ) were subject to DGGE analysis of 16S rRNA gene to assess the changes in the bacterial community throughout the experiment. DGGE analysis was used to identify the dominant microorganisms involved in the reductive dechlorination as well as to compare the differences in microbial community structure between different treatments. The brightest bands in the denaturing gradient gel (labeled with letters in Fig. 5.3) were excised, and the reamplified DNA fragments were partially sequenced and compared to known sequences using BLASTN (Table 5.1).

We observed significant change in the community structure compared to the original groundwater. Imfeld et al. (2008) carried out the DGGE analysis of the groundwater from the well BVV3051 and detected 17 bands. All enrichment cultures investigated in this study were of very low complexity, since very few bands per lane were detected. The most important difference was observed between the treatments growing on lactate compared to the treatments growing on acetate and hydrogen. The brightest band in cultures growing on lactate corresponded to *Clostridium*-like bacterium. In the cultures growing on acetate and hydrogen the sequences obtained from the predominant band were closely related to *Dehalococcoides* strain BAV1. The *Clostridium* band was not present at all in the H<sub>2</sub>+acetate cultures, while *Dehalococcoides* band could be seen in some lactate treatments, e.g. VC/lactate (12.3), however the signal was very weak.

The separation patterns of cultures growing on various chlorinated ethenes were very similar, but the signal for *Dehalococcoides* was much stronger for the 1<sup>st</sup> transfer cultures: PCE/H<sub>2</sub>+acetate (1.1), TCE/H<sub>2</sub>+acetate (2.1), *cis*-DCE/H<sub>2</sub>+acetate (3.1), compared to the 2<sup>nd</sup> transfer: PCE/H<sub>2</sub>+acetate (1.2), TCE/H<sub>2</sub>+acetate (2.2), *cis*-DCE/H<sub>2</sub>+acetate (3.2) and 1,1-DCE/H<sub>2</sub>+acetate (4.2). The higher content of DNA in the 1<sup>st</sup> transfer corresponds with a longer cultivation time and higher dosage of the potential electron acceptor. This suggests the growth

of the *Dehalococcoides* strain BTF08 with PCE, TCE and *cis*-DCE. It was also observed that cultures from 2<sup>nd</sup> transfer lost some community members, which were still present in the 1<sup>st</sup> transfer, although gave very weak signal on the DGGE gel (see lanes 2.1 and 3.1). The cultures from the 2<sup>nd</sup> transfer (PCE/H<sub>2</sub>+acetate (1.2), TCE/H<sub>2</sub>+acetate (2.2), *cis*-DCE/H<sub>2</sub>+acetate (3.2) and 1,1-DCE/H<sub>2</sub>+acetate (4.2)) contained only the bands corresponding to the *Dehalococcoides*.

The separation of the 1<sup>st</sup> transfer cultures : PCE/H<sub>2</sub>+acetate (1.1), TCE/H<sub>2</sub>+acetate (2.1), *cis*-DCE/H<sub>2</sub>+acetate (3.1), yielded two different bands with sequence very similar to that of *Dehalococcoides* strain BAV1 (bands A3-A4 and A5-A7). The bands in the lower part of the gel (A5-A7) probably represent single-stranded DNA, a side-product due to the asymmetric nature of the PCR used for DGGE (Nikolausz et al., 2005; Zhang et al., 2005). The sequences of the bands labeled with letter A (A1-A7) were identical. Similarly, the separation of the DNA from treatments growing on lactate resulted in two *Clostridium* bands (see lane 12.3, bands B1 and B2). The sequences of all bands labeled with letter B (B1-B4) were identical.



**Figure 5.3** Image of DGGE of 16S rRNA gene fragments amplified from 11 different enrichment cultures. Letters correspond to bands that were excised and sequenced, as listed in Table 5.2. The image illustrates that the 2<sup>nd</sup> transfer of cultures growing on H<sub>2</sub> and acetate lost all but *Dehalococcoides* bands, compared to the cultures growing on lactate.



**Table 5.2** Sequence analysis of the DNA retrieved from the predominant bands from the DGGE<sup>a</sup> gel.

Band	Best matches	Accession number	Identity <sup>b</sup> (%)
A1-A7	Uncultured bacterium SHE-136	AJ290982	100
	Uncultured bacterium SHE-135	AJ290981	100
	Uncultured bacterium SHE-135	AJ290980	100
	Uncultured bacterium clone TC38	EF644514	100
	<i>Dehalococcoides</i> sp. BAV1	CP000688	100
B1-B4	Uncultured bacterium clone TANB44	AY667258	99
	Uncultured bacterium clone TANB5	AY667250	99
	Uncultured bacterium	EU287865	99
	Uncultured bacterium clone 6A	AF427938	99
	<i>Clostridium lactatifermentans</i>	AY033434	94
	<i>Clostridium propionicum</i>	X77841	94

<sup>a</sup>DGGE shown in Fig. 5.3

<sup>b</sup>Based on a BLASTn search of 360 bp fragments

### 5.3.3 Purity of the cultures (clone libraries)

The clone libraries of 16S rRNA genes were constructed from several cultures to investigate their purity and the structure of the community (Fig. 5.1). The clones were screened and grouped by ARDRA and representative members were identified by sequence analysis (Table 5.3).

All cultures growing on hydrogen as electron donor were dominated by *Dehalococcoides*. The amount of minor population varied from approximately 2% in the cultures 1.2 (PCE/H<sub>2</sub>+acetate) and 1.3 (PCE/H<sub>2</sub>+acetate) to 10% in the cultures 2.3 (TCE/H<sub>2</sub>+acetate) and 4.3 (1,1-DCE/H<sub>2</sub>+acetate). Most organisms constituting the minor population and detected in our enrichment cultures are closely related to organisms associated with other dechlorinating consortia. *Sulfurospirillum multivorans* (X82931) is a known dehalorespiring bacterium capable of dechlorination of PCE to *cis*-DCE (Scholz-Muramatsu et al., 1995). The uncultured bacterium clone IA-23 (AJ488074) was detected in a bacterial consortium dechlorinating chlorobenzenes, the uncultured spirochete clone KB-1 (AY780558) is associated with the chlorinated ethene-degrading culture KB-1 described by Duhamel and Edwards (Duhamel and Edwards, 2006). The uncultured bacterium clone TANB5 (AY667250) was found in trichloroethene-contaminated aquifer by Macbeth et al. (2004).

Unlike cultures growing on chlorinated ethenes and hydrogen, culture 12.3 growing on VC and lactate, was dominated by a bacterium closely related to the uncultured bacterium clone TANB5 (99% identity) and *Clostridium lactatifermentans* (AY033434) (95% identity). *Dehalococcoides* comprised less than 6% of the culture.

**Table 5.3** ARDRA types and partial sequence analysis results.

Culture (substrates)	Number of clones representing ARDRA type	Closest relative	Accession number	Identity % (identical/total bases)
1.1 (PCE/acetate+H <sub>2</sub> )	19	<i>Dehalococcoides</i> sp. BAV1	CP000688	99 (1316/1317)
	1	<i>Sulfurospirillum multivorans</i>	X82931	99 (619/620)
1.2 (PCE/acetate+H <sub>2</sub> )	46	<i>Dehalococcoides</i> sp. BAV1	CP000688	99 (1316/1317)
	1	Uncultured bacterium clone IA-23	AJ488074	98 (494/500)
1.3 (PCE/acetate+H <sub>2</sub> )	48	<i>Dehalococcoides</i> sp. BAV1	CP000688	99 (1316/1317)
	1	Uncultured bacterium clone BUD07	DQ447167	99 (528/530)
2.3 (TCE/acetate+H <sub>2</sub> )	17	<i>Dehalococcoides</i> sp. BAV1	CP000688	99 (1316/1317)
	1	<i>Shigella flexneri</i> 5 strain 8401	CP000266	100 (549/549)
	1	Uncultured spirochete clone KB-1	AY780558	100 (197/197)
3.3 ( <i>cis</i> -DCE/acetate+H <sub>2</sub> )	19	<i>Dehalococcoides</i> sp. BAV1	CP000688	99 (1316/1317)
4.3 (1,1-DCE/acetate+H <sub>2</sub> )	18	<i>Dehalococcoides</i> sp. BAV1	CP000688	99 (1316/1317)
	1	<i>Sulfurospirillum multivorans</i>	X82931	99 (598/600)
	1	Uncultured spirochete clone KB-1	AY780558	100 (601/601)
12.3 (VC/lactate)	1	<i>Dehalococcoides</i> sp. BAV1	CP000688	99 (1316/1317)
	16	Uncultured bacterium clone TANB5	AY667250	99 (547/550)
		<i>Clostridium propionicum</i> <sup>a</sup>	X77841	95 (527/552)
		<i>Clostridium lactatifermentans</i> <sup>a</sup>	AY033434	94 (526/554)

<sup>a</sup> closest culturable relatives

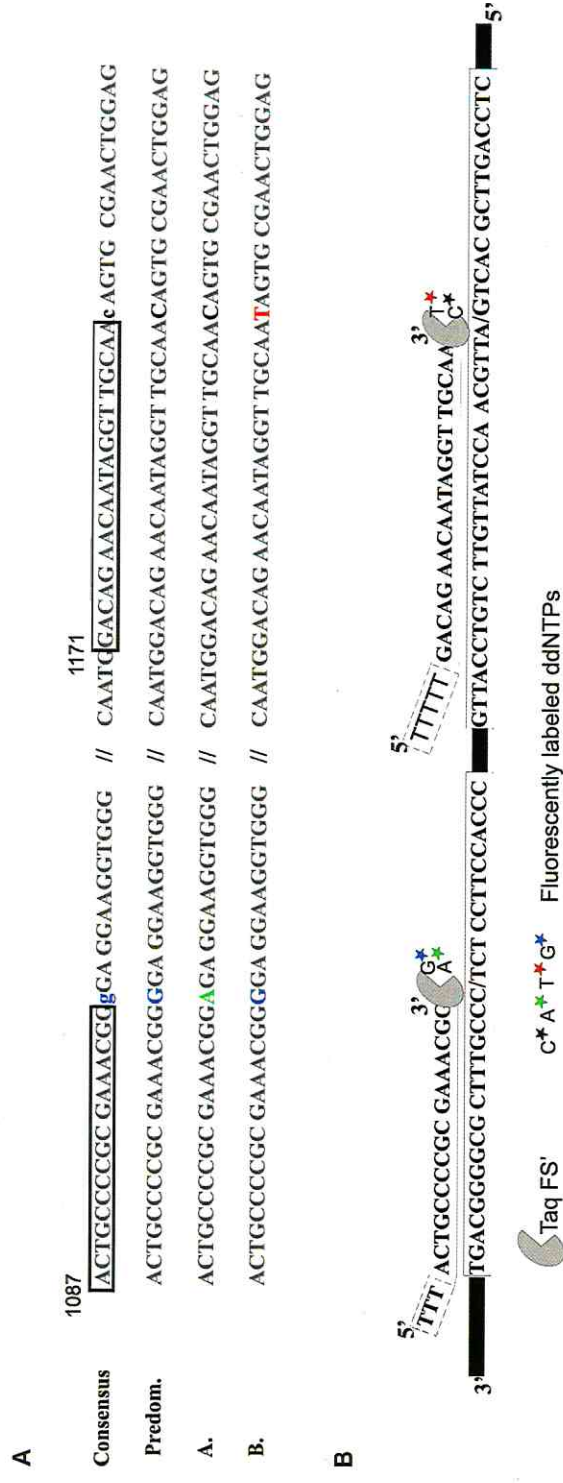
### 5.3.4 Variability of sequences

Three to four clones corresponding to the *Dehalococcoides* ARDRA pattern were selected from each clone library and almost complete 16S rRNA gene sequence (around 1260 bp) was obtained. In 58% of clones (11 out of 19) random base changes were detected (data not shown). To examine, if the sequence variability reflects the natural diversity of *Dehalococcoides* sequences in the cultures or if it is a method introduced error, one culture, PCE/H<sub>2</sub>+acetate (1.3), was further investigated. The 219 bp fragments of the 16S rRNA gene, where variations occurred, were obtained for 10 additional clones. Sequences were produced with forward and reverse primers to exclude sequencing errors. Additionally, the original PCR product used for clone library formation was sequenced directly. For 23% of screened clones (3 out of 13) the base pair changes at random positions, compared to the sequence of the original PCR product and the predominant sequence type, were detected. A single-nucleotide primer extension (SNUPE) assay was developed to investigate two variable positions of the *Dehalococcoides*-type sequences (Fig. 5.4). In this assay the fluorescently labeled ddNTP incorporation gives information about the presence of the different sequence variants in a PCR product. The first application of primer extension with four differently labeled ddNTPs was carried out by Wu and Liu (Wu and Liu, 2007) for the multiplex detection of different *Bacteroides* spp. Recently the development and application of a SNUPE assay for the detection and typing of *Dehalococcoides* spp. sequences obtained from chloroethene-contaminated groundwater samples was reported. A good dynamic range corresponding to the detection of minority template in the presence of abundant one in 1:585 ratio and a very specific detection were achieved with SNUPE (Nikolausz et al., 2008).

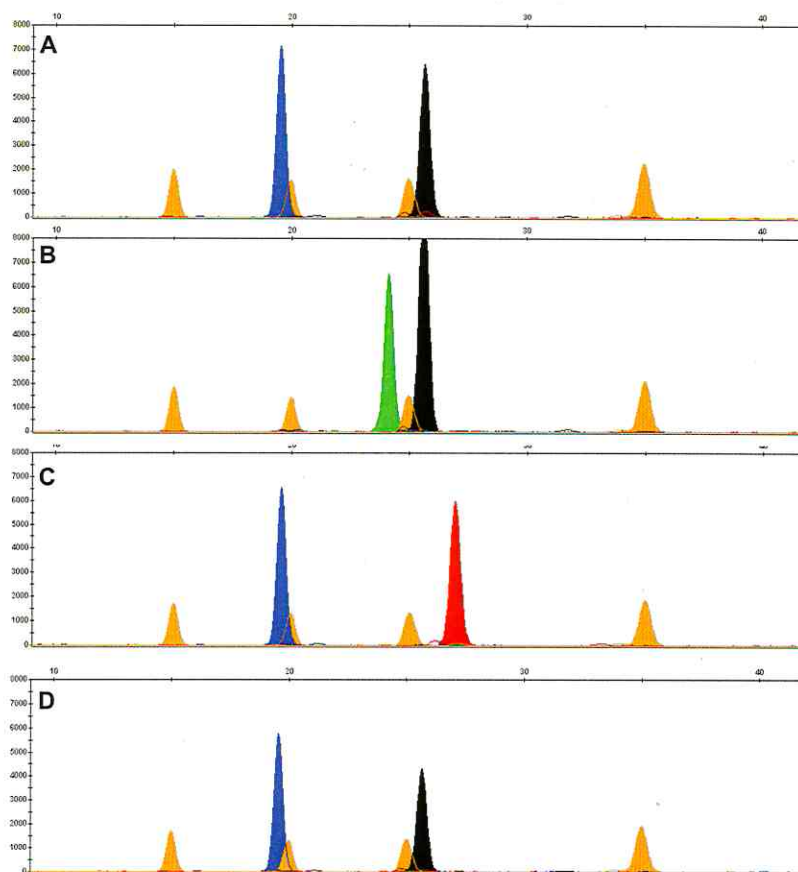
When PCR products from the selected clones were used as templates the expected incorporation of nucleotides was observed in agreement with the sequencing data. The SNUPE analysis of the predominant sequence type resulted in the expected G and C incorporation with the assigned color blue and black, respectively (Fig. 5.5 A). A (green)/C (black) and G (blue)/T (red) incorporation was observed



when sequence variants A and B were used as templates for SNuPE (Fig. 5.5, B and C.). The SNuPE analysis of the PCR product used for the establishment of the clone library revealed the presence of only the predominant sequence type (Fig. 5.5, D). We have not observed any traces of the two sequence variants represented by A and T incorporation. These sequence variants were most probably introduced by subsequent steps of cloning and PCR. This finding is in agreement with the observation of microvariation artifacts of cloning and PCR re-amplification by Speksnijder et al. (2001).



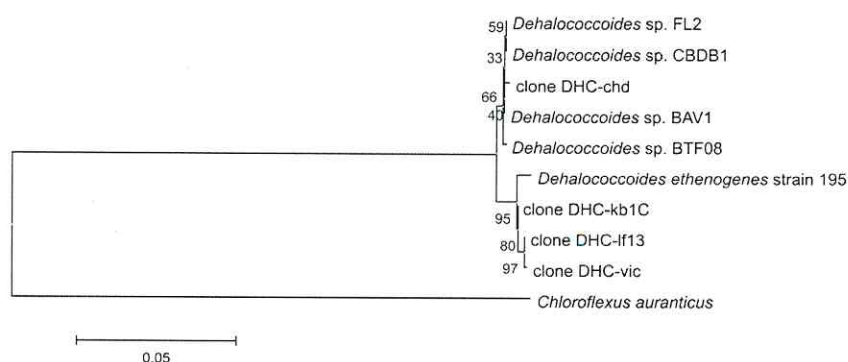
**Figure 5.4** The principle and steps of single-nucleotide primer extension (SNUPE) assay. A) Partial alignment of the target sequences used in this study. Variable loci are marked with different colors. Frames indicate the priming sites upstream from the positions of interest. B) The AmpliTaq FS polymerase incorporates a labeled dideoxynucleotide, which terminates the reaction and results in a fluorescently labeled product. The different ddNTPs are labeled with different fluorophores and the type of fluorescence provides information about the incorporated nucleotide. Multiplexing was achieved by adding different length noncomplementary tail (polyT) to the 5' end of the primers that serves as mobility modifier for the electrophoretic separation. The primers and products are subsequently separated with capillary electrophoresis but only the extended products are detected with laser induced fluorescence.



**Figure 5.5** Single-nucleotide primer extension assay for the detection of sequence variants. PCR products were obtained with M13F and M13R primers. A) SNuPE pattern obtained with the positive control DNA from a clone representing the predominant sequence type. B) and C) SNuPE patterns of the sequence variants A and B. D) SNuPE pattern obtained with the original PCR product used for the establishment of the clone library. The vertical axis represents fluorescence intensity (in relative fluorescence units); the horizontal axis represents the sizes of the extended products. Orange peaks indicate the internal size standards.

### 5.3.5 16S rRNA gene consensus sequence

In the light of the above presented results the variability of sequences was most probably introduced by the method. Therefore, the most abundant sequence was assumed to be the consensus sequence (AM981291). The phylogenetic affiliation of *Dehalococcoides* strain present in our culture is shown in Fig. 5.6. The almost complete, 16S rRNA gene sequence of strain BTF08 shows very close similarity to the 16S rRNA gene of *Dehalococcoides* strain BAV1 (CP000688) and strain CBDB1 (AJ965256). The sequence consisting of 1319 base pairs differs by 1 base pair from the sequence of *Dehalococcoides* strain BAV1 and by 2 base pairs from strain CBDB1. The sequence shares 1298 identical base pairs with the sequence of *Dehalococcoides ethenogenes* strain 195.



**Figure 5.6** Phylogenetic affiliation of the strain BTF08 based on currently available 16S rRNA gene sequences. The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987). Bootstrap values expressed as percentages of 500 replications are shown next to the branches.



#### 5.4 Discussion

We enriched a novel *Dehalococcoides* strain dechlorinating PCE to ethene from the contaminated anaerobic aquifer in Bitterfeld (East Germany). Due to its origin the strain was named BTF08. To our knowledge, this work is a first attempt to enrich the *Dehalococcoides* strain, capable of the conversion of lower chlorinated ethenes, from an European field site. Most currently known *Dehalococcoides* isolates were derived from various locations in North America (Maymo-Gatell et al., 1997; Sung et al., 2006b). Strain CBDB1 isolated from the sediment of the Saale river (Germany) grows with chlorinated benzenes and dechlorinates PCE only to *trans*-DCE (Adrian, 2001). Table 5.1 summarizes *Dehalococcoides* strains isolated thus far and their substrate ranges. For example, strain BAV1 respire DCEs and VC, strains GT and VS - TCE to ethene, and strain FL2 - TCE but only to VC (Cupples et al., 2003; He et al., 2003b; Müller et al., 2004; He et al., 2005; Sung et al., 2006b). *Dehalococcoides ethenogenes* strain 195 gains energy from PCE to VC reductive dechlorination (Maymo-Gatell et al., 1997). None of the currently known *Dehalococcoides* isolates can couple energy conservation to all steps of sequential PCE dechlorination to ethene. In this respect strain BTF08 displays novel substrate range.

Strain BTF08 was enriched using standard liquid enrichment procedure. Microcosms were prepared from groundwater derived from Bitterfeld and amended with lactate and PCE as electron donor and acceptor. After the third transfer, the enrichment culture was amended with acetate and hydrogen in place of lactate to eliminate the lactic acid fermenters and to promote the growth of *Dehalococcoides*. Three additional transfers on such combination of substrates allowed obtaining a culture which contained over 98% of *Dehalococcoides*. The BTF culture grows in purely synthetic medium containing salts, bicarbonate as pH buffer, trace elements, vitamins including B<sub>12</sub>, hydrogen as an electron donor, acetate as a carbon source, chlorinated ethenes as electron acceptors, and sodium sulfide as a reducing agent.

The *Dehalococcoides* culture enriched on PCE as electron acceptor was retransferred on different chlorinated ethenes, including PCE, TCE, *cis*-, *trans*-, 1,1-DCE and VC to investigate its dechlorination capabilities and showed activity with all except *trans*-DCE and 1,1-DCE. PCE, TCE, *cis*-DCE and VC were dechlorinated in all experiments performed for this study. Several transfers cultivated with one of these substrates as sole electron acceptor, were active. Additionally, the culture enriched on PCE as electron acceptor maintained the ability to dechlorinate TCE, *cis*-DCE and VC and dechlorinated all of them to ethane. In light of these findings, it can be assumed that PCE, TCE, *cis*-DCE and VC can be used by strain BTF08 as metabolic electron acceptors. *Trans*-DCE was not dechlorinated by any of the transfer cultures set up in this study. 1,1-DCE was dechlorinated to ethene by the transfer cultures in the second enrichment phase (Fig. 5.1, B, transfers 4.1-4.4). However, the growth on 1,1-DCE failed in the last experiment, where culture pregrown on PCE (1.3) was used as inoculum. It is possible, that over several transfers on different electron acceptors the culture lost the capability to degrade 1,1-DCE. Additionally, nutrients necessary for 1,1-DCE dechlorination could have been lacking or 1,1-DCE was added in concentrations inhibitory for growth (Maymo-Gatell et al., 1997; Nijenhuis, 2002).

The *Dehalococcoides* strain present in the enrichment culture BTF is affiliated with the Pinellas group of *Dehalococcoides*, but it differs from the other isolates with regard to electron acceptor utilization. Although our strain shares more than 99% of the 16S rRNA gene sequence with strain BAV1, it can, in contrast to the latter one, use the higher chlorinated ethenes (PCE and TCE) as electron acceptors. The 16S rRNA gene sequence of strain BTF08 is also highly similar to the sequence of strain CBDB1 (only 2 base pairs difference), which grows on chlorinated aromatic compounds. However, it was already previously reported, that the Pinellas group members cannot be distinguished solely by 16S rRNA gene sequence analysis, nor can be their dechlorination capabilities (Duhamel et al., 2002; He et al., 2003b; Ritalahti and Löffler, 2004; He et al., 2005).

While investigating 16S rRNA gene sequences of *Dehalococcoides* present in the BTF culture, we observed that some *Dehalococcoides*-type sequences differed by 1 or 2 base pairs from the predominant *Dehalococcoides* sequence. To investigate this sequence variability, a single-nucleotide primer extension (SNuPE) assay was developed and revealed that the variations in *Dehalococcoides* sequences in the Bitterfeld enrichment culture was most likely due to method introduced error. This finding is in agreement with the observation of microvariation artifacts of cloning and PCR re-amplification by Speksnijder et al. (2001). This does not exclude possibilities of the presence of further *Dehalococcoides*-like bacteria in Bitterfeld. On the other hand, it warrants the careful interpretation of microheterogeneity results obtained by cloning and sequencing approach.

### Environmental implications

The capability of strain BTF08 to degrade PCE to environmentally benign ethene and inorganic chloride is highly interesting concerning the bioremediation of chloroethene contaminated sites. At many field sites the biodegradation of PCE and TCE is incomplete and results in production of *cis*-DCE and VC. Accumulation of these intermediate dechlorination products is a major problem in bioremediation, as lower chlorinated ethenes are more toxic than PCE and TCE and VC is a known human carcinogen (ASTDR, 2007). The incomplete dechlorination is usually observed, if the organisms that reductively dechlorinate the less chlorinated ethenes are not present or active at contaminated site. When requisite microorganisms are absent, the bioremediation strategy known as "bioaugmentation" can be implemented. In this cleanup approach the reductively dechlorinating enrichment culture is added to the site (Harkness et al., 1999; Ellis et al., 2000; Major et al., 2002; Chartrand et al., 2005; Morrill et al., 2005). A few dechlorinating enrichment cultures are commercially available, for example BioDechlor INOCULUM, containing multiple *Dehalococcoides* organisms such as FL2, BAV1 and GT (Ritalahti et al., 2005) and KB-1<sup>TM</sup>, containing *Dehalococcoides* spp.

(Duhamel et al., 2004). Strain BTF08 also exhibits high potential for such application.

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# 6

## **Summary, conclusions and future directions**

## 6.1 Summary

Chlorinated ethenes have been widely used in many industrial branches in the last decades. Careless handling and inappropriate deposition resulted in an extensive contamination of soil, surface and subsurface aquifers with these chemicals. Due to their toxicity and potential carcinogenicity, chlorinated ethenes pose a serious threat for the environment (Häggblom and Bossert, 2003).

Discovery of anaerobic bacteria that can couple the reduction of chlorinated ethenes to energy conservation was an important step forward in the treatment of chloroethene contaminated sites. Among reductively dechlorinating bacteria the *Dehalococcoides* spp. are of particular interest because only this group of microorganisms is currently known to be able to dechlorinate tetrachloroethene (PCE) and trichloroethene (TCE) to non-toxic ethene. Most of the other dechlorinating isolates can only dechlorinate PCE and TCE to dichloroethenes (DCEs).

Further studies confirmed that dechlorinating microorganisms are ubiquitous at many chloroethene-contaminated sites (Löffler et al., 2000; Hendrickson et al., 2002; Yang et al., 2005; Nijenhuis et al., 2007; Imfeld et al., 2008). Reductive dechlorination started to be taken advantage of at polluted areas in innovative cleanup approaches such as monitored natural attenuation, biostimulation and bioaugmentation. Increasing interest in the bioremediation has drawn attention to the monitoring methods that could be potentially used to assess the microbial transformations of environmental pollutants *in situ*.

One approach to monitor biodegradation in the field is the determination of isotope composition of individual contaminants, known as Compound Specific Isotope Analysis (CSIA) (Meckenstock et al., 2004). Enrichment in  $^{13}\text{C}$  in the residual contaminant fraction may be used as a qualitative indicator for *in situ* biodegradation (Meckenstock et al., 2004). Additionally, by use of the Rayleigh equation, the extent of biodegradation at contaminated sites may be calculated, but the quantitative approach requires the knowledge of the fractionation factor ( $\alpha_{\text{C}}$ ), representative for the microbial degradation pathway (Sherwood Lollar et al., 2001; Richnow et al., 2003).

Recent studies on the isotope fractionation upon reductive dechlorination of PCE by two microbial strains revealed that the fractionation factors may vary, even if the identical degradation pathway is used (Nijenhuis et al., 2005). This observation implied that the other factors than reaction mechanism may cause the variability in fractionation.

The research described in this thesis aimed at better understanding of microbial transformations of chlorinated ethenes in context of CSIA application to monitor the biodegradation at contaminated field sites. The study focused on two main aspects related to the qualitative and quantitative evaluation of the chlorinated ethenes biodegradation by means of CSIA: 1) isotope fractionation upon microbial reductive dechlorination and 2) investigation and enrichment of environmentally relevant microorganisms.

In **Chapter 1** a general overview on microbial transformations of chlorinated ethenes based on the most up-to-date literature is given. The characteristics and sources of chlorinated ethenes in the environment are described, natural degradation processes are explained and the diversity of dechlorinating organisms is presented. The natural attenuation of chlorinated ethenes and the factors affecting it at contaminated sites are discussed. Finally, the methods to assess the *in situ* biodegradation with special attention on CSIA are introduced.

**Chapter 2** describes the carbon stable isotope fractionation of PCE and TCE during reductive dechlorination by growing cells of *Sulfurospirillum multivorans*, *Sulfurospirillum halorespirans* and *Desulfitobacterium* sp. strain PCE-S, the respective crude extracts and the abiotic reaction with cyanocobalamin (vitamin B<sub>12</sub>). Fractionation of TCE by *Sulfurospirillum* spp. ( $\alpha_C=1.0132-1.0229$ ) was more than one order of magnitude higher compared to values observed for PCE ( $\alpha_C= 1.00042-1.0032$ ). Interestingly, the TCE carbon isotope fractionation by the purified PCE-reductive dehalogenase from *S. multivorans* was also more than one order of magnitude higher than fractionation of PCE. Carbon isotope fractionation of TCE by *Desulfitobacterium* sp. strain PCE-S was higher than previously reported values for PCE. In contrast to previous results



with PCE, no effects due to rate limitations, uptake or transport of the substrate to the reactive site, could be observed during TCE dechlorination. Apparently, the stable isotope fractionation depends not only on the reaction mechanism and physiological factors within the organism may lower the observed fractionation. These effects should be taken into account while applying CSIA for biodegradation assessment.

**Chapter 3** reports on the carbon isotope fractionation during reductive dechlorination by different microbial strains. The tetrachloroethene and trichloroethene fractionation by *Dehalococcoides ethenogenes* strain 195, *Desulfitobacterium* sp. strain Viet1, *Desulfuromonas michiganensis* and *Geobacter lovleyi* sp. strain SZ was investigated. The carbon isotope fractionation of both compounds was highly variable ranging from the absence of significant fractionation to carbon isotope fractionation ( $\epsilon_C$ ) of 16.7 for PCE and 3.5 to 18.9 for TCE. Fractionation of both compounds by *D. ethenogenes* strain 195 was similar to the literature data for mixed cultures containing *Dehalococcoides* spp. *D. michiganensis* and *G. lovleyi* sp. strain SZ generated the lowest fractionation of all studied strains. *Desulfitobacterium* sp. strain Viet1 gave the highest enrichment factor for PCE. Our findings showed that stable isotope fractionation is highly variable among different microbial strains. As a result, the quantification of chlorinated ethenes natural attenuation by means of CSIA is associated with high uncertainty. Therefore, the quantitative approach should be made with caution and CSIA should be accompanied by other methods (e.g. molecular tools).

In **Chapter 4** the carbon isotope fractionation of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TeCDD) by mixed culture containing *Dehalococcoides ethenogenes* strain 195 was investigated and compared to the fractionation of PCE by this culture. Carbon stable isotope fractionation occurred in experiments with both substrates. The first dechlorination products, 1,2,4-trichlorodibenzo-*p*-dioxin (1,2,4-TrCDD) and TCE for 1,2,3,4-TeCDD and PCE dechlorination, respectively, became significantly enriched. For both compounds (1,2,3,4-TeCDD and PCE) the fractionation of the first dechlorination product was much stronger than fractionation of the initial substrates. The intrinsic enrichment factor,

$\epsilon_{\text{intrinsic}}$  for PCE was  $14.2 \pm 1.0$  ‰. The  $\epsilon_{\text{intrinsic}}$  for 1,2,3,4-TeCDD exhibited significant variability.  $\epsilon_{\text{intrinsic}}$  of  $13.0 \pm 5.3$  ‰ and  $34.8 \pm 8.8$  ‰ were measured in two replicate cultures. For insoluble substrates, like polychlorinated dibenzo-*p*-dioxins, the observed isotope fractionation can be significantly affected by bioavailability limitations. Before the isotope fractionation of these compounds could be applied to prove the biodegradation in the field, further studies on the factors reducing the observed fractionation, e.g. sorption-desorption processes and uptake or transport within the cell, are necessary.

Finally, **Chapter 5** reports on our attempt to characterise and enrich the organisms responsible for reductive dechlorination of chlorinated ethenes at our model contaminated site in Bitterfeld (Sachsen Anhalt, Germany). In view of the results presented in this thesis, implying that carbon isotope fractionation by dehalogenating bacteria is highly variable, the knowledge on a microbial consortium active *in situ* is essential for biodegradation assessment by means of CSIA.

To investigate the Bitterfeld dechlorinating community, microcosms were set up with anaerobic groundwater and lactate and PCE were used as electron donor and carbon source and electron acceptor, respectively. Active microcosms were transferred three times on PCE and lactate. The obtained enrichment was further transferred to different chlorinated ethenes as the electron acceptors and acetate and hydrogen as a carbon and energy source and after three transfers, a highly purified culture capable of PCE to ethene dechlorination was derived. PCR followed by denaturing gradient gel electrophoresis, cloning, and sequencing revealed that this culture was dominated by a *Dehalococcoides* sp. strain associated with the Pinellas group. Investigation of substrate specificity in the parallel cultures suggested the presence of a new *Dehalococcoides* strain, growing with either PCE, TCE, *cis*-dichloroethene (*cis*-DCE) or VC as the electron acceptor, which is a novel degradation pattern. The strain was designated BTF08 due to its origin in Bitterfeld. Strain BTF08 has a potential for application in bioremediation of chloroethene contaminated sites. For example, it might be used for bioaugmentation as its ability to

completely dechlorinate PCE to non-toxic ethene is particularly desired in this cleanup strategy.

## 6.2 Conclusions and future directions

### 6.2.1 Compound Specific Isotope Analysis

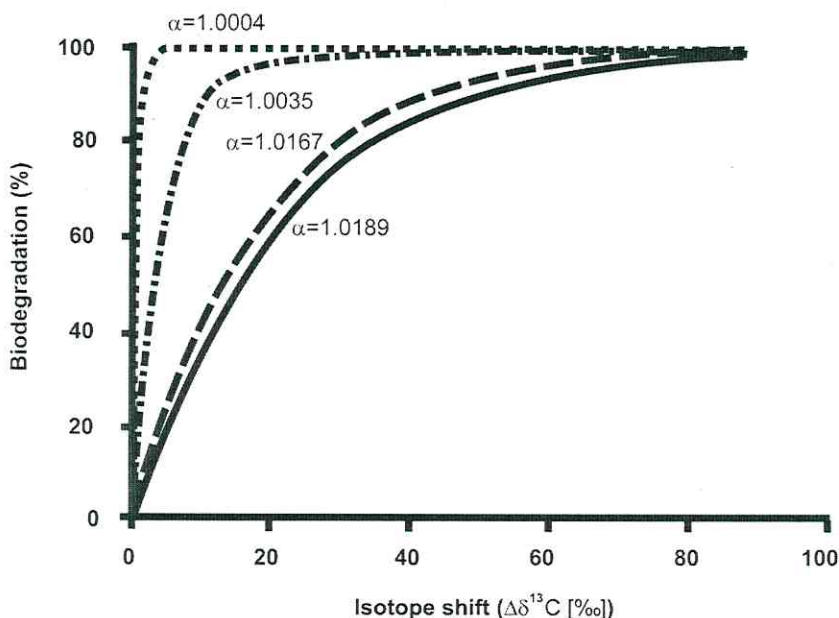
Thus far only a few methods to monitor the biodegradation of groundwater contaminants *in situ* are available. The most common approaches are monitoring of pollutants concentrations and detection of metabolites specific for certain degradation pathway (Wiedemeier et al., 1999; Witt et al., 2002). These approaches are associated with huge uncertainties as many processes, such as dilution, sorption or evaporation may influence the contaminant concentrations in groundwater aquifers. The monitoring of metabolites can be difficult especially at sites polluted with mixture of various chemicals, where intermediate or end products of certain degradation pathways also occur as primary contaminants.

Compound Specific Isotope Analysis (CSIA) is a tool that may improve the quality of the *in situ* biodegradation estimates. It proved to be successful for qualitative biodegradation assessment of chlorinated ethenes (Hunkeler et al., 1999; Sherwood Lollar et al., 2001; Song et al., 2002; Vieth et al., 2003; Chartrand et al., 2005; Morrill et al., 2005). A quantitative approach is still a challenge, although a few attempts to calculate the extent of biodegradation of these contaminants have been performed (Sherwood Lollar et al., 2001; Vieth et al., 2003; Chartrand et al., 2005; Morrill et al., 2005; Fischer et al., 2007). The work presented in this thesis showed, that one has to be cautious with such estimation as carbon stable isotope fractionation of chlorinated ethenes is highly variable and depends on the microorganisms involved in the transformation reaction. The following section describes the problems and challenges associated with CSIA as a tool to assess the biodegradation at contaminated field sites and proposes the future directions of the research presented in this thesis.



### Variability of fractionation vs. biodegradation quantification

Fig. 6.1 shows the difference in biodegradation estimates depending on the fractionation factors used for the calculation. The calculation was performed for PCE dechlorination by *S. multivorans* ( $\alpha_C=1.004$ ) and *Desulfotobacterium* sp. strain Viet1 ( $\alpha_C=1.0167$ ) and TCE dechlorination by *D. michiganensis* ( $\alpha_C=1.0035$ ) and *S. halorespirans* ( $\alpha_C=1.0189$ ).



**Figure 6.1** Theoretical calculation of the percentage of biodegradation during PCE dechlorination by *S. multivorans* (.....) and *Desulfotobacterium* sp. strain Viet1 (----) and TCE dechlorination by *D. michiganensis* (-.-.-) and *S. halorespirans* (—).  $\Delta \delta^{13}\text{C}$  indicates an isotope shift in the sample, assuming an initial isotope composition of PCE and TCE  $\delta^{13}\text{C} = -28.0$  [‰].

The influence of the fractionation factor is particularly strong for lower ranges of isotope shifts. For example, the maximal difference in biodegradation estimates (89%) for PCE occurs at the isotope shift of approx. 1.7‰ (assuming  $\delta_0 = -28$ ‰). For TCE the maximal uncertainty (55%) corresponds to the isotope shift of approx. 7.3‰ (assuming  $\delta_0 = -28$ ‰). Although, the difference in estimated biodegradation decreases with increasing isotope shift, the shifts reported at contaminated sites

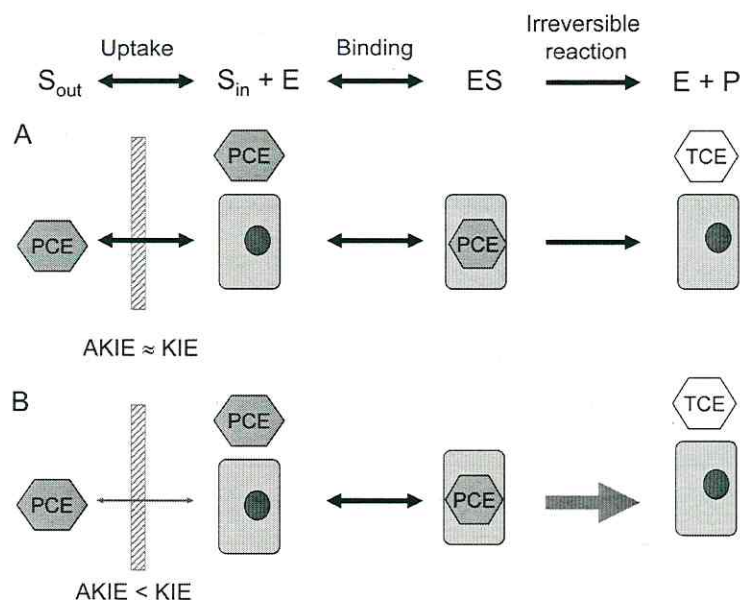


usually fall in the range associated with high uncertainty. For example, previously reported carbon isotope signatures of TCE at Dover Air Force base ranged from -18 to -26‰, whereas only small differences in isotope signature (-32.8 to -33.9‰) were observed for PCE (Sherwood Lollar et al., 2001). As a consequence of the variability of stable carbon isotope fractionation during PCE and TCE reductive dechlorination, the precise calculation of the biodegradation at the contaminated sites is not feasible.

Nevertheless, such estimation still gives a clue about the extent of biodegradation and is a significant improvement compared to the traditional methods based on concentration measurement. Additionally, to decrease the uncertainties associated with variability in fractionation of chlorinated ethenes CSIA could be accompanied by other methods. For example, molecular biology techniques could be used to identify the organisms carrying out the dechlorination reaction *in situ* and with help of mathematical modelling, an appropriate fractionation factor representative for the site could be selected.

### Rate limitations

The kinetic isotope effects (KIE) associated with the transformation of the chemical bond can only be directly observed, if this step is rate-determining for the reaction (Fig. 6.2, A). Very often, the conversion of the bond is preceded by other processes, such as transport to the cell or to the reactive site or formation of the enzyme-substrate complex. If these processes are much slower than bond cleavage, each molecule reaching the reactive site will be reacted (Fig. 6.2, B). Consequently the intrinsic KIE will be masked and measured “apparent” kinetic isotope effects (AKIE) will be much lower (Elsner et al., 2005). Northrop (1981) introduced the expression “commitment to catalysis” for this phenomenon.



**Figure 6.2** The scheme of the enzymatic reaction showing the steps associated with rate limitations ( $S_{out}$  – substrate in the medium,  $S_{in}$  – substrate in the cell, E – enzyme, ES – enzyme substrate complex, P – product). A) All reaction steps are in equilibrium. Irreversible step associated with the transformation of the chemical bond is rate-determining. B) The preceding steps (e.g. uptake or binding to enzyme) are slower than irreversible reaction, lowering apparent isotope fractionation (AKIE).

Nijenhuis et al. (2005) demonstrated that physiological factors inside bacterial cells can lower the apparent isotope fractionation. The rate limitations due to uptake and transport of the substrate through the membranes during reductive dechlorination of PCE by *Sulfurospirillum multivorans* and *Desulfitobacterium* sp. strain PCE-S occurred. Interestingly, in identical experiments with TCE no rate limitations were observed. This suggests, that rate limitations can be compound or organism specific. The masking effects, lowering the apparent isotope fractionation may cause variability in fractionation, even if the same degradation pathway and the same reaction mechanism are used.

Therefore, the laboratory-derived fractionation factor for certain degradation pathway might not be relevant for biodegradation assessment at different sites, where different organisms might be involved in the degradation reaction. Controlled laboratory studies with specific compounds and a microbial community from the investigated site are recommended to define the ranges of possible fractionation factors that may be used for biodegradation quantification.

Furthermore, in natural systems a variety of physical and/or chemical processes, such as sorption, pore size exclusion (in porous media such as aquifers and soil) and microscale transport processes around the microbial cells, take place, which may control the substrate flux towards the microbial cells, resulting in similar rate limitations as observed by Nijenhuis et al. (2005) due to membrane barriers. Theoretical considerations by Thullner et al. (in prep.) demonstrated that bioavailability restrictions due to low contaminant concentrations and mass transfer limitations may lower the apparent stable isotope fractionation. The application of laboratory derived fractionation factors for biodegradation estimates in the field, without taking into account the possible rate limitations due to the substrate low bioavailability may lead to the underestimation of the actual amount of biodegradation (Thullner et al., in prep).

To improve the biodegradation quantification by means of CSIA the studies on the factors potentially causing rate limitations should be continued. For example, threshold concentrations of different substrates resulting in detectable changes in observed isotope fractionation could be determined in the laboratory experiments (Thullner et al., in prep.).

### **Non-Rayleigh behaviour**

The biodegradation assessment by means of CSIA is based on the Rayleigh model, which relates the changes in contaminant concentration and isotope composition at given experimental conditions (Hoefs, 1997). The extent of contaminant transformation in the field can then be calculated from measured isotope compositions in the samples provided that a fractionation factor is known. This approach relies on the

assumption that isotope fractionation remains constant during degradation of the compound. However, during biotransformation reaction the “commitment to catalysis” and as a result the extent of isotope fractionation may change. This phenomenon is referred to as “non-Rayleigh behaviour” and according to Elsner et al. (2005) can be due to two main reasons: 1) changes in environmental conditions or changes in co-substrate concentrations; or 2) the degradation may include parallel pathways whose proportion changes as contaminant degradation proceeds, e.g. due to different affinity to enzyme.

Very recently, Kampara et al. (in prep.) demonstrated experimental evidence for such “non-Rayleigh behaviour”. They showed that the extent of hydrogen isotope fractionation during toluene degradation by *Pseudomonas putida* mt-2 (pWWO) depended on substrate concentration and the decrease in toluene concentration during microbial degradation was accompanied by a decrease in fractionation. The results of Kampara et al. (in prep.) are in agreement with theoretical predictions of bioavailability influence on the apparent stable isotope fractionation by Thullner et al. (in prep.). Further studies are required to investigate the impact of bioavailability limitations on fractionation under field conditions. Moreover, other factors that might have an effect on the bioavailability of the substrate in the field and thus result in “non-Rayleigh” type isotope fractionation should be investigated.

#### **Elucidation of degradation pathways**

As the extent of isotope fractionation depends on the type of transformation reaction, the isotope fractionation factors have been recently used to distinguish different biodegradation pathways (Hirschorn et al., 2004). Hirschorn et al. (2004) observed low carbon isotope fractionation ( $\epsilon=3.0$ ) for 1,2-dichloroethane by *Pseudomonas* sp. strain DCA1 (monooxygenase enzyme), while degradation via the hydrolytic dehalogenation by two pure strains (*Xanthobacter autotrophicus* GJ10, *Ancylobacter aquaticus* AD20) was characterized by much higher fractionation ( $\epsilon=32.3$ ). However, in the situation where rate limitations of



preceding reaction steps significantly affect the isotope fractionation, the characterization of the degradation pathways can lead to a huge bias.

To deal with this problem, a relatively new approach known as Two-Dimensional Isotope Fractionation Analysis (TDIFA) can be used. In this approach, the isotope compositions of two elements (e.g. carbon, hydrogen, chlorine, oxygen etc.) are measured simultaneously. The rate limiting processes would affect the isotope fractionation of both elements involved in the reaction similarly. Therefore the correlation of isotope compositions of two elements may characterize the reaction mechanism more precisely than isotope fractionation of one single element (Fischer et al., in press). The TDIFA, combining carbon and hydrogen isotope fractionation, has been recently used to characterize the biodegradation pathways of benzene and fuel oxygenates (Rosell et al., 2007; Fischer et al., in press). Nevertheless, despite growing expectation for multi-dimensional isotope analysis, its application is still largely limited by the existing analytical techniques.

Regarding chlorinated ethenes, TDIFA of carbon and chlorine could provide insights into the reaction mechanism and help to elucidate the microbial degradation pathways. However, although several methods are available for chlorine isotope analysis, most of them require large sample size and extensive sample preparation prior to isotope analysis (Tanaka and Rye, 1991; Jendrzewski et al., 1997; Numata et al., 2002; Shouakar-Stash et al., 2003). Recently, two on-line CSIA methods for chlorine isotopes in chlorinated ethenes have been developed. They simplify analysis to a great extent and could be potentially used in the future for such studies on the reaction mechanism (Shouakar-Stash et al., 2006; Sakaguchi-Soder et al., 2007).

#### **Limitations related to larger molecules**

The application of CSIA has also other limitations - the size of the molecule is one of them. Previous studies showed that the maximum size of the molecule that can be measured by stable isotope fractionation analysis is 12-14 carbon atoms. For larger molecules the dilution of the

KIE is so pronounced that the measured isotope shift is not significantly different from the analytical error (Meckenstock et al., 2004).

### **What next?**

The future work on stable isotope fractionation upon the reductive dechlorination of chlorinated ethenes may include the investigation of the fractionation by the crude extracts and enzymes of further bacteria. For example, the experiments with *Geobacter lovleyi* or *Desulfuromonas michiganensis* could be performed to see, if the observed low fractionation is related to rate limitations due to uptake and transport within a cell. The understanding of the factors altering the “commitment to catalysis” in bacterial cells (e.g. membrane barriers) as well as in the field (e.g. bioavailability) is crucial for reliable biodegradation predictions by means of Rayleigh model. Further, the characterisation of enzymes and their cofactors from other strains may help to understand what else causes the variability in fractionation in different bacteria.

For field applications, it would be also important to look at the fractionation by mixed cultures containing more than one dechlorinating strain. Such experiments may give insights into the interactions among different organisms carrying out the same reaction and can help to understand how these interactions influence overall observed fractionation by a mixed microbial community. The knowledge on fractionation by such mixed cultures can help in selection of relevant fractionation factors for biodegradation estimates in the field. Moreover, studies aiming at understanding the reaction mechanism should be continued, for which the development of analytical techniques enabling CSIA for hydrogen and chlorine is essential.

Looking ahead to the application of CSIA for biodegradation assessment, it has to be highlighted that despite its limitations it is the most promising of the methods available thus far. It does not require a long-term monitoring campaign and drilling numerous wells and the measurement of a small number of field samples can already give a clue whether the biodegradation occurs. All these aspects make CSIA a very

cost-effective tool. For some compounds CSIA enables the quantitative assessment of biodegradation, which is not possible with other methods. On the other hand, the interpretation of the results obtained by CSIA should always be done by the experts, who know the shortcomings of the method and who are able to interpret the data correctly.

### 6.2.2 Isolation of new organisms

The knowledge on the microorganisms carrying out different degradation and detoxification processes is crucial for all bioremediation strategies. Although molecular biology methods have very high potential and the fast development of metabolomics, genomics and proteomics could provide us with great deal of information, the traditional cultivation methods should not be omitted.

In this thesis, the attempt to isolate the *Dehalococcoides* strain present at the contaminated site in Bitterfeld is described. Although the purification of the strain was not possible, the highly enriched *Dehalococcoides* culture capable of reductive dechlorination of PCE, TCE, *cis*-DCE and VC was obtained. As a next step, it is necessary to prove the growth of the culture on these compounds. The further work should also include the identification of additional growth substrates as well as the overall substrate range including other chlorinated compounds, such as chlorobenzenes, chlorophenols, chloronaphthalenes, chlorobenzoates and polychlorinated dioxins.

Overall, new strains are a significant source of information allowing for better understanding of bacterial physiology, ecology and genetics. New isolates enable further studies on many aspects, the knowledge of which is essential for bioremediation, but cannot be obtained by molecular methods without cultivation. For example, the metabolic activities of new strains and factors affecting the expression of desired characteristics under different conditions can be investigated. This information could be used to better estimate the site's potential for biological treatment and can help to stimulate the biodegradation *in situ*. Novel strains, capable of degradation of various pollutants may be used as inoculum for bioaugmentation of contaminated aquifers or soils. Furthermore, the

enzymes of new strains can be taken advantage of in various biotechnological applications.



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## *Bibliography*

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## PUBLICATIONS

D. Cichocka, M. Siegert, G. Imfeld, J. Andert, K. Beck, G. Diekert, H. H. Richnow and I. Nijenhuis

**Factors controlling the carbon isotope fractionation of tetra- and trichlorethene during reductive dechlorination by *Sulfurospirillum* spp. and *Desulfitobacterium* sp. strain PCE-S**

FEMS Microbiology Ecology: 62(2007) 98-107

D. Cichocka, G. Imfeld, H.H. Richnow and I. Nijenhuis

**Variability in microbial carbon isotope fractionation of tetra- and trichlorethene upon reductive dechlorination**

Chemosphere: 71(2008) 639-648

F. Liu, D. Cichocka, I. Nijenhuis, H. H. Richnow and D. E. Fennell

**Carbon stable isotope fractionation of polychlorinated dibenzo-*p*-dioxins and chloroethenes by *Dehalococcoides ethenogenes* strain 195**

Submitted to Environmental Science and Technology

D. Cichocka, M. Nikolausz and I. Nijenhuis

**Characterization of highly enriched *Dehalococcoides*-containing culture derived from contaminated mega-site in Bitterfeld dechlorinating tetrachloroethene to ethene**

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## CONFERENCE PROCEEDINGS

### Oral presentations

D. Cichocka, M. Nikolausz\*, I. Nijenhuis (2008). **Characterisation of a highly enriched *Dehalococcoides*-containing culture dechlorinating tetrachloroethene to ethene.** VAAM-Jahrestagung, Frankfurt, Germany

D. Cichocka\*, H.H. Richnow, I. Nijenhuis (2008). **Variability of stable isotope fractionation of chlorinated ethenes during microbial reductive dechlorination.** AXIOM-VIBE-eTRAP Spring School "Electron transfer processes at biogeochemical gradients", Leipzig, Germany

D. Cichocka, H.H. Richnow, I. Nijenhuis\* (2007). **High variability of carbon stable isotope fractionation of chlorinated ethenes during microbial reductive dechlorination.** EGU General Assembly, Vienna, Austria

D. Cichocka\*, H.H. Richnow, I. Nijenhuis (2007). **Stable isotope fractionation of tetrachloroethene and trichloroethene during reductive dechlorination by different dehalogenating strains.** VAAM-Jahrestagung, Osnabrück, Germany

I. Nijenhuis\*, M. Siegert, D. Cichocka, J. Andert, K. Beck, G. Diekert, H.H. Richnow (2006). **Stable isotope fractionation of tetra- and trichloroethene by *Sulfurospirillum* spp. and *Desulfitobacterium* sp. strain PCE-S.** VAAM-Jahrestagung, Jena, Germany.

### Posters

M. Siegert\*, D. Cichocka, G. Diekert, H.H. Richnow, I. Nijenhuis (2007). **Passing Barriers- A Comparative experiment about rate limiting effects of cell barriers on carbon stable isotope fractionation.** VAAM-Jahrestagung, Osnabrück, Germany

D. Cichocka\*, H.H. Richnow, I. Nijenhuis (2006). **Carbon isotope fractionation during reductive dechlorination of chlorinated ethenes by *Sulfurospirillum halorespirans* and *Desulfuromonas michiganensis*.** VAAM-Jahrestagung, Jena, Germany.

M. Siegert\*, D. Cichocka, M. Nikolausz, H.H. Richnow, I. Nijenhuis (2006). **Investigation of microbial communities derived from the Bitterfeld aquifer capable of complete dehalogenation of chlorinated ethenes.** VAAM-Jahrestagung, Jena, Germany.

D. Cichocka\*, H.H. Richnow, I. Nijenhuis (2006). **Carbon isotope fractionation during reductive dechlorination of chlorinated ethenes by *Sulfurospirillum halorespirans* and *Desulfuromonas michiganensis*.** AXIOM-Virtual Institute Spring School "Microbial Activity at Biogeochemical Gradients". Leipzig, Germany.

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D. Cichocka\*, M. Nikolausz, A. Köth, M. Kästner, H.H. Richnow, I. Nijenhuis (2005). **Assessment of natural attenuation of chlorinated ethenes using stable isotope techniques, microcosm studies and molecular markers**. Summer School: "Biomonitoring, bioavailability and microbial transformation of pollutants in sediments and approaches to stimulate their biodegradation". Genoa, Italy.

D. Cichocka\*, M. Nikolausz, A. Köth, M. Kästner, H.H. Richnow, I. Nijenhuis (2005). **Assessment of natural attenuation of chlorinated ethenes using stable isotope techniques, microcosm studies and molecular markers**. European Marie Curie Conference "Making Europe more attractive for researchers". Pisa, Italy.

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**Summer School:** Hopkins Microbiology Course, Stanford University, Hopkins Marine Station  
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From 02.04.2006 to 06.04.2006

**Spring School:** Microbial Activity at Biogeochemical Gradients

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From 12.09.2005 to 14.09.2005

**Summer School:** Biomonitoring, bioavailability and microbial transformation of pollutants

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**Poster:** Assessment of natural attenuation of chlorinated ethenes using stable isotope techniques, microcosm studies and molecular markers.  
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**Club of Rome Seminar:** Waste Management and Sewage Disposal in Hamburg and Pinneberg  
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## LANGUAGES

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- **German:** good

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- Traveling, discovering new places and new cultures, skiing





## DECLARATION

I hereby declare that I completed this work without any improper help from a third party and without using any aids other than those cited. All ideas derived directly or indirectly from other sources are identified as such. In the selection and use of materials and in the writing of the manuscript I received support from the following persons:

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