Triple-element compound-specific stable isotope analysis of 1,2-dichloroethane for characterization of the underlying dehalogenation reaction in two *Dehalococcoides mccartyi* strains

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One sentence summary: Triple-element compound-specific stable isotope analysis provides evidence that 1,2-dichloroethane dehalogenation involves a dihaloelimination mechanism with concerted chlorine removal that avoids formation of toxic vinyl chloride.

Keywords: Compound-specific stable isotope analysis, 1,2-Dichloroethane, Dehalococcoides, reductive dehalogenation, dihaloelimination

Abstract

Chlorinated ethanes belong to the most common groundwater and soil contaminants. Of these, 1,2-dichloroethane (1,2-DCA) is an man-made, persistent and toxic contaminant, released due to improper waste treatment at versatile production sites. This study investigated the anaerobic transformation of 1,2-DCA by *Dehalococcoides mccartyi* strain 195 and strain BTF08 using triple-element compound-specific stable isotope analysis (CSIA) of carbon, chlorine and hydrogen for the first time. Isotope fractionation patterns for carbon (ε^{C}_{BTF08} = -28.4 ± 3.7 ‰; ε^{C}_{195} = -30.9 ± 3.6 ‰) and chlorine (ε^{Cl}_{BTF08} = -4.6 ± 0.7 ‰; ε^{Cl}_{195} = -4.2 ± 0.5 ‰) within both investigated *D. mccartyi* strains, as well as the dual-element analysis (Λ_{BTF08} = 6.9 ± 1.2; Λ_{195} = 7.1 ± 0.2), supported identical reaction mechanisms for dehalogenation of 1,2-DCA. Hydrogen isotope fractionation analysis revealed dihaloelimination as prevalent reaction mechanism. Vinyl chloride as major intermediate could be excluded by performing the experiment in deuterated aqueous media. Furthermore, evaluation of the derived apparent kinetic isotope effects ($AKIE^{C}_{BTF08}$ = 1.029/$AKIE^{C}_{195}$ = 1.031; $AKIE^{Cl}_{BTF08}$ = 1.005/$AKIE^{Cl}_{195}$ = 1.004) pointed towards simultaneous abstraction of both involved chlorine-substituents in a concerted matter. It was shown that *D. mccartyi* strain BTF08 and strain 195 are capable of complete, direct dihaloelimination of 1,2-DCA to ethene.

Introduction

Halogenated organic compounds occur widespread in the environment, as they are originating naturally from many organisms (e.g. marine sponges) or are released by forest fires, volcanic activities or geothermal processes (Häggblom & Bossert, 2003, Tas *et al.*, 2010). Nonetheless,

industrial chemicals and their widespread use (e.g. as solvents, precursors) are known as particularly relevant causes of severe contamination of sediments, soils and groundwater aquifers (Tas *et al.*, 2010). As prominent cause of groundwater aquifer contaminations especially 1,2-dichloroethane (1,2-DCA) has been confirmed, due to its extensive use as lead scavenger (Hageman *et al.*, 2006), where its physicochemical properties pose a threat to biota (Williams *et al.*, 2001).

Today, few bacterial strains have been described which reductively dechlorinate 1,2-DCA, including different strains of *Dehalococcoides mccartyi* (Maymó-Gatell, 1999, Kaufhold *et al.*, 2013, Schmidt *et al.*, 2014) as well as *Desulfitobacterium dehaloeliminans* (De Wildeman *et al.*, 2003), *Dehalogenimonas spp.* (Maness *et* al., 2012, Palau *et* al. 2014) and *Dehalobacter* sp. (Grostern & Edwards, 2009). While numerous strains are capable of incomplete dehalogenation, only microorganisms belonging to the class *Dehalococcoidetes* can completely dehalogenate higher halogenated organic compounds to non-toxic ethene (Smidt & de Vos, 2004, Tas *et al.*, 2010). *D. mccartyi* strain 195 (formerly known as *Dehalococcoides ethenogenes* strain 195) and strain *D. mccartyi* BTF08 were the microorganisms chosen for this study. Former studies showed dechlorination of 1,2-DCA to mainly ethene and only minor amounts of vinyl chloride (VC) in *D. mccartyi* strain BTF08 (Kaufhold *et al.*, 2013) whereas strain 195 was reported to metabolically produce VC and to co-metabolically transform it to ethene (Maymó-Gatell *et al.*, 1997).

The genomes of *D. mccartyi* strain BTF08 and strain 195 comprise 20 and 17 homologous reductive dehalogenase (rdh) genes, respectively, based on the presence of two iron-sulfur cluster-binding motifs and a twin-arginine signal motif for membrane translocation (Seshadri *et al.*, 2005, Hug *et al.*, 2013, Pöritz *et al.*, 2013). The isolated TceA (TCE reductive dehalogenase) of *D. mccartyi* strain 195 catalyzes multiple reactions as conversion of TCE and *cis*-dichloroethene (cDCE) to vinyl chloride and ethene as well as 1,2-DCA to minor

amounts of VC but mainly ethene (Magnuson *et al.*, 1998, Magnuson *et al.*, 2000). Halogenated ethanes such as 1,2-DCA are, in principle, amenable to two different pathways (Figure 1) when being dehalogenated under anoxic conditions, usually occurring within contaminated groundwater aquifers (Hunkeler *et al.*, 2002, Kaufhold *et al.*, 2013, Schmidt *et al.*, 2014). Reductive dihaloelimination to ethene may directly cleave off both chlorine atoms. Alternatively, non-reductive dehydrodechlorination to VC may occur followed by reductive hydrogenolysis to ethene. Furthermore, also both pathways could occur simultaneously, resulting in VC and ethene as parallel products, with probable ongoing dehalogenations of VC.

CSIA for evaluation of reactions

CSIA allows to investigate *in situ* biodegradation and characterizing underlying reaction mechanisms (Hunkeler *et al.*, 2002, Hirschorn *et al.*, 2004, Elsner *et al.*, 2005, Hunkeler *et al.*, 2005, Hirschorn *et al.*, 2007, Elsner *et al.*, 2008, VanStone *et al.*, 2008, Schmidt *et al.*, 2014). Gas chromatography coupled to isotope-ratio mass-spectrometry (GC-IRMS) permits determining exact isotopic compositions of separated single organic compounds at natural abundance levels (Elsner *et al.*, 2012). During a reaction, light isotopes are depleted within organic substrates as a consequence of the different zero-point energies of isotopes which result in energetically more favorable transformation of light-isotope containing bonds. (Bio)chemical reactions of contaminants are therefore associated with kinetic stable isotope fractionation (KIE) (Elsner, 2010). These changes can be described by the Rayleigh model which links the measurable changes of bulk isotopic composition to concomitant changes in concentrations by the reaction-specific enrichment factor ε (Elsner *et al.*, 2005, Elsner, 2010). Nevertheless, as the enrichment factor does not take into account unreactive positions or intramolecular competitions within the molecule, the apparent kinetic isotope effect (AKIE) is calculated to evaluate whether rate-limiting steps are masking the reaction (Northrop, 1981,

Elsner *et al.*, 2005). Most commonly determined primary KIE imply breakage or formation of isotope-bearing bonds as rate-determining step. If, however, the isotopic substitution is remote from the bond being broken, the reaction rate is changed as the remote atom influences the internal vibration states and zero-point energies, resulting in a secondary KIE (Hennig *et al.*, 2006).

CSIA to investigate 1,2-DCA dehalogenation

Different studies have targeted aerobic degradation of 1,2-DCA using carbon and chlorine isotope fractionation to investigate the predominant reaction mechanism (Table 1) (Janssen, 1985, van den Wijngaard *et al.*, 1992, Klečka *et al.*, 1998, Hage & Hartmans, 1999, Hunkeler & Aravena, 2000, Hirschorn *et al.*, 2004, Palau *et al.*, 2014, Palau *et al.*, 2017). While carbon isotope fractionation analysis is well established for decades, only recently developed methods enable chlorine and hydrogen analysis (Shouakar-Stash *et al.*, 2005, Renpenning *et al.*, 2015, Nijenhuis *et al.*, 2016). A recent study of Palau et al. (2017) presented two-element analysis for anaerobic dehalogenation of 1,2-DCA and highlighted differences in isotope effects between *Dehalococcoides*- and *Dehalogenimonas*-containing enrichment cultures. In comparison, thus far solely a single-element study has targeted reductive 1,2-DCA dehalogenation by *Dehalococcoides* spp. (Schmidt *et al.*, 2014), where dihaloelimination was proposed as major pathway with a minor contribution of hydrogenolysis. This study aimed to investigate the 1,2-DCA dehalogenation in *D. mccartyi* strain 195 and strain BTF08 for the first time with triple-element CSIA to explore whether the reaction is proceeding with intermediate production of toxic VC or direct dehalogenation to ethene (Figure 1). Therefore CSIA focused on carbon, chlorine and hydrogen fractionation patterns of 1,2-DCA. The novel approach of hydrogen isotope analysis was applied to distinguish between two possible reactions: i) conversion of 1,2-DCA directly to ethene where no primary hydrogen isotope fractionation is expected, as there is no C-H-bond cleavage and ii) reaction via intermediate

formation of toxic VC, which includes C-Cl and C-H bond cleavages, as well as addition of water-derived hydrogen (Figure 1). This would lead to a strong, primary hydrogen isotope fractionation within the product ethene. Furthermore, hydrogen isotope analysis was conducted using deuterated medium when investigating 1,2-DCA and VC as substrates for *D. mccartyi* strain BTF08, respectively. If deuterium was incorporated in ethene when using 1,2-DCA as substrate, intermediate formation of VC could be assumed. Reaction-specific apparent kinetic isotope effect (AKIEs) values were furthermore evaluated and compared to oxic and anaerobic reaction mechanisms.

Materials and Methods

Cultivation and isotope fractionation experiments

D. mccartyi strain 195 was cultivated as described elsewhere with addition of the 'butyrate pellet' from a *Dehalococcoides*-containing mixed culture (Maymó-Gatell *et al.*, 1997, Cichocka *et al.*, 2008). The cultivation of *D. mccartyi* strain BTF08 was done as described elsewhere (Cichocka *et al.*, 2008, Schmidt *et al.*, 2014). Both strains were pre-cultivated using 100 mL mineral media in 240 mL serum bottles and 1 mM 1,2-DCA or VC as substrate.

For the fractionation experiment mineral salt medium (25 mL) was transferred to 50 mL serum bottles, flushed with N_2 and CO_2 (70/30%), crimp closed with Teflon^{TM} lined grey butyl rubber stoppers and sterilized for 40 min at 120°C. Bottle sets were amended with 500 µM 1,2-DCA or VC for carbon and chlorine isotope analysis, and with 1.5 mM each for hydrogen isotope analysis. Per set, three non-inoculated bottles were prepared with substrate as negative control. Inoculation occurred with 5 % (v/v) of a culture grown on 1,2-DCA (strain 195 and BTF08) or VC (strain BTF08). Hydrogen as electron donor was added at 0.5 bar overpressure after inoculation. Cultures were incubated in the absence of light without shaking, for sets containing *D. mccartyi* strain BTF08 at 20°C and for *D. mccartyi* strain 195 at 30°C. The dehalogenation progress was determined by gas-chromatography with flame ionization detection (FID). Bottles were sacrificed at different extents of dehalogenation, described as C_t/C_0 (where C_t is the residual substrate concentration and C_0 the starting concentration) ranging between 0.1 and 0.9, by addition of 1 mL of acidic sodium sulfate solution (280 $g \cdot L^{-1}$, pH 1) as described elsewhere (Cichocka *et al.*, 2008, Schmidt *et al.*, 2014).

Isotope fractionation experiment with D_2O

An isotope fractionation experiment was conducted as described above using 1.5 mM 1,2-DCA for *D. mccartyi* strain 195 and BTF08 or VC for strain BTF08. Prior to addition of hydrogen gas, 1% (v/v) D_2O was added to the solution (sterilized and made anoxic by sparging with N_2).

Analysis of concentrations by gas chromatography-flame ionization detection (GC-FID)

Concentrations of 1,2-DCA, VC and ethene were determined by gas chromatography with FID (Varian Chrompack CP-3800, Middleburg, the Netherlands) equipped with a GS-Q-column (30 m x 0.53 mm, J&W Scientific, Waldbronn, Germany). The temperature program was started from 100°C (held constant for one minute) followed by a temperature gradient of 50°C/min to 225°C (held constant for 5 minutes).

Analysis of isotope ratios by gas chromatography – isotope ratio mass spectrometry

For determination of compound-specific stable isotope ratios, isotope ratio mass spectrometry was coupled to gas chromatography. The organic mixture was separated by gas chromatography and baseline-separated compounds were chemically converted into the respective analyte gases CO_2 for $^{13}C/^{12}C$ analysis (Elsner *et al.*, 2012), and H_2 for $^{2}H/^{1}H$ analysis (Renpenning *et al.*, 2015), or measured without conversion for $^{37}Cl/^{35}Cl$ analysis (Shouakar-Stash *et al.*, 2005, Elsner *et al.*, 2012, Palau *et al.*, 2014).

Carbon isotope analysis

Carbon isotope fractionation was determined in triplicates by injection into the gas chromatograph (Agilent 6890, Palo Alto, USA) using a CP-PoraBond column (50 m x 0.32 mm, 5 µm inner diameter, J&W Scientific, Germany). Therefore 0.3 to 0.6 mL headspace sample were injected manually with a split ratio of 1:3. For chromatographic separation a temperature program modified from Schmidt et al. (2014) was used, starting from

30 °C (held for 10 min) followed by a 20 °C · min^{-1} gradient to 250 °C (held for 5 min) with 2 mL · min^{-1} flow and an injector temperature of 280°C (Schmidt *et al.*, 2014). Carbon isotope ratios were determined at the IRMS (MAT 235, Thermo Scientific, Germany) relative to the laboratory reference gas (CO_2, calibrated against Vienna Pee Dee Belemnite standard V-PDB, IAEA Vienna, (Coplen *et al.*, 2006)).

Chlorine isotope analysis

For chlorine isotope analysis samples were diluted in Milli-Q-water to 200 µmol· L^{-1} in a total volume of 1 mL in closed 10 mL crimp-top vials wherefrom 1 mL headspace was injected by an automated Concept GC Autosampler (PAS Technology) to a Trace GC Ultra gas chromatograph (Thermo Scientific, Germany). For separation a temperature program starting with 65°C (held for 2 min), followed by a 10°C· min^{-1} gradient to 90°C followed by a second 60°C· min^{-1} gradient to 175°C, was used. Without conversion 1,2-DCA was subsequently introduced into the IRMS (MAT 235, Thermo Scientific, Germany) for isotope analysis. Chlorine isotope ratios were determined relative to the laboratory standards using a 2-point normalization (Bernstein *et al.*, 2011).

Hydrogen isotope analysis

Hydrogen isotope analysis was conducted using chromium-based high-temperature conversion as described by (Renpenning *et al.*, 2015, Renpenning *et al.*, 2017). For analysis 1 mL of headspace was injected manually with a split ratio of 1:5 to the gas chromatograph (Trace 1310, Thermo Fisher Scientific, Germany) using a CP-PoraBond column (50 m x 0.32 mm, 5 µm inner diameter, J&W Scientific, Germany). For chromatographic separation a temperature program for ethane and 1,2-DCA, starting from 30°C (held for 10 min) subsequently raising to 250°C with 20°C· min^{-1} (held for 5 min), both with a flow of 2 mL· min^{-1} and an injector temperature of 280°C, was used. Hydrogen isotope fractionation

was determined relative to laboratory standards, previously calibrated against VSMOW-SLAP (Gehre *et al.*, 2015).

Evaluation of isotopic data

Compound specific stable isotope analysis enables us to measure the isotopic composition of compounds reported in δ-notation (‰) relative to an international standard to harmonize reports of experimental data (Coplen *et al.*, 2006, Elsner, 2010, Renpenning *et al.*, 2015). The calculation of carbon, chlorine and hydrogen isotope enrichment factors ε^X (where X represents the analyzed element, i.e., C, Cl or H, respectively) was accomplished according to the Rayleigh-Equation (1) (Hoefs, 1997, Elsner *et al.*, 2005, Elsner & Hunkeler, 2008, Elsner, 2010, Elsner *et al.*, 2012). The concentrations at different time points (t) of the reaction and the starting concentration (0) are represented as C_t and C_0, respectively, whereas $R=1+\delta$ (where $\delta = \delta\,^{13}C$, $\delta\,^{37}Cl$, or $\delta\,^{2}H$) (Coplen *et al.*, 2006).

$$(1) \qquad \ln\left(\frac{R_t}{R_0}\right) = (\varepsilon^X) \times \ln\left(\frac{C_t}{C_0}\right)$$

Based on the slope of the regression a two-tailed T-test was uses to calculate the 95 % confidence interval (data evaluation in Micosoft EXCEL).

Apparent kinetic isotope effect (AKIE)

The previously calculated bulk enrichment factor ε^X_{bulk} describes the isotopic fractionation of the whole molecule. To determine position-specific kinetic isotope effects one has to take intra-molecular competition and non-reacting positions into account (Elsner *et al.*, 2005). The AKIE values were determined for 1,2-DCA according to Eq. (2) (Elsner *et al.*, 2005). The number of carbon atoms is described by n, the number of atoms at the reactive position by x, whereas z takes the number of indistinguishable reactive sites into consideration.

$$(2) \qquad AKIE = \frac{1}{1+\left(z \times \left(\frac{n}{x}\right) \times \left(\varepsilon^X_{bulk}\right)\right)}$$

Results and Discussion

Stable isotope fractionation was investigated using two distinct *Dehalococcoides mccartyi* cultures, strain BTF08 and strain 195, capable of complete dehalogenation of 1,2-dichloroethane to ethene.

D. mccartyi strain BTF08 dehalogenated 1,2-dichloroethane to ethene in 34 days (to an extent of 77 %), whereas dechlorination in *D. mccartyi* strain 195 proceeded faster within 22 days to an extent of 75 %. Traces of VC were detected (< 1%) in both cultures, however it was not clear whether VC was an intermediate product and was subsequently dehalogenated to ethene, or whether 1,2-DCA was directly transformed to ethene and VC represented the product of an alternative pathway.

Carbon stable isotope analysis

Carbon isotope values $\delta^{13}C$ of 1,2-DCA in *D. mccartyi* strain BTF08-mediated dehalogenation changed from initially -29.0 ± 0.14 to +24.4 ± 0.26 ‰ (77 % dehalogenation) resulting in $\varepsilon^{C}_{1,2\text{-DCA}}$ = -28.4 ± 3.7 ‰ (R^2=0.94). The results for dehalogenation of 1,2-DCA by strain 195 were similar: carbon isotope values $\delta^{13}C$ changed from initially -28.9 ± 0.26 ‰ to +16.7 ± 1.22 ‰ (75 % dehalogenation) resulting in $\varepsilon^{C}_{1,2\text{-DCA}}$ = -30.9 ± 3.6 ‰ (R^2=0.95) (Figure 2A). With both *D. mccartyi* strains VC was detected at levels that were too low (< 40 µmol · L^{-1}) to be quantifiable in GC-FID and to be detectable with GC-IRMS. Those results correspond with previous analysis for carbon isotope fractionation by Schmidt et al. (2014) for *D. mccartyi* strain BTF08 and strain 195 (Table 1). Carbon isotope values $\delta^{13}C$ of ethene changed from initially -60.7 ± 0.4 to -41.0 ± 0.56 ‰ for strain BTF08 and 60.1 ± 0.1 to -42.6 ± 0.11 ‰ for strain 195. It was shown that both strains differ significantly within their putative reductive dehalogenase genes and substrate spectrum (Maymó-Gatell *et al.*, 1997, Maymó-Gatell, 1999, Kaufhold *et al.*, 2013, Pöritz *et al.*, 2013) as strain BTF08 can

dehalogenate VC metabolically, whereas for strain 195 slow co-metabolic transformation from VC to ethene was observed. Though, both contain genes coding for TceA which was found to mediate 1,2-DCA dehalogenation in strain 195 (Magnusson et al. 2000), identification of the reductive dehalogenase responsible for 1,2-DCA dehalogenation in strain BTF08 is still missing. However, similar values for carbon isotope fractionation were determined for *D. mccartyi* strain BTF08 and strain 195 as previously described by (Schmidt *et al.*, 2014). Furthermore, there are detailed investigations for different pathways describing oxic 1,2-DCA degradation reactions which lead to similar carbon isotope fractionation patterns. When comparing carbon isotope fractionation of 1,2-DCA for nucleophilic substitution (C-Cl-bond cleavage) in *X. autotrophicus* GJ10 (ε^{C}_{bulk} = -31.9 ± 0.9 ‰) and *A. aquaticus* AD20 (ε^{C}_{bulk} = -32.0 ± 0.7 ‰) isotope fractionation values are similar to the ones determined here. However, differentiation from monooxygenase-reaction (C-H-bond cleavage) in *Pseudomonas* spp. (ε^{C}_{bulk} = -3.5 ± 0.1 ‰.) is possible (Palau *et al.*, 2014). That leads to the assumption that the rate determining step for reductive dehalogenation in *D. mccartyi* is a carbon-chlorine bond cleavage. This does not exclude one or the other reaction pathway for 1,2-DCA within reductive dehalogenation, since formation of either ethene or VC are both based on C-Cl-bond breakage.

Chlorine stable isotope analysis

When *D. mccartyi* strain BTF08 dehalogenated 1,2-DCA, the chlorine isotope values δ^{37}Cl changed from -0.2 ± 0.04 to 7.7 ± 0.16 ‰ which is similar to fractionation observed during dehalogenation by *D. mccartyi* strain 195 (1.1 ±0.1 to 8.5 ±0.1 ‰). The determined enrichment factors (Figure 2A) were similar to each other, with $\varepsilon^{Cl}_{1,2\text{-DCA}}$ = -4.6 ± 0.7 ‰ (R^2=0.91) for strain BTF08 and $\varepsilon^{Cl}_{1,2\text{-DCA}}$ = -4.2 ± 0.5 ‰ (R^2=0.94) for strain 195, respectively. These enrichment factors are also similar to those described for oxic S_N2-reactions in *A. aquaticus* AD20 (ε^{Cl}_{bulk} = -4.4 ± 0.2 ‰) and *X. autotrophicus* GJ10 (ε^{Cl}_{bulk} = -

4.2 ± 0.1 ‰), as well as for oxic monooxygenase reactions of *Pseudomonas* spp. (ε^{Cl}_{bulk} = -3.8 ± 0.2 ‰) (Palau *et al.*, 2014). A recent study (Palau *et al.*, 2017) with a *Dehalococcoides*-containing enrichment culture showed a high similarity in chlorine isotope fractionation (ε^{Cl}_{bulk} = -5.1 ± 0.1 ‰) to the values observed in this study for pure cultures, but contrasted with anaerobic dihaloelimination by a *Dehalogenimonas*-containing enrichment culture, where much stronger chlorine isotope fractionation of ε^{Cl}_{bulk} = -12.0 ± 0.8 ‰ was observed (Table 1).

Dual element stable isotope analysis

In a two-element isotope fractionation plot (Figure 2B) that relates carbon and chlorine isotope fractionation for *D. mccartyi* strain 195 and strain BTF08, the resulting Λ-values of 6.9 ± 1.2 for strain BTF08 and 7.1 ± 0.2 for strain 195, are indistinguishable from each other. They are also very similar to those determined for aerobic biodegradation involving an S_N2-reaction (7.7 ± 0.2 (Palau *et al.*, 2014)) and dihaloelimination in a *Dehalococcoides*-containing enrichment culture (Λ = 6.8 ± 0.2), but significantly different from a dihaloeliminating *Dehalogenimonas*-containing enrichment culture (1.89 ± 0.02 (Palau et al. (2017)) (Table 1).

Dehalogenation of 1,2-DCA in D_2O: involvement of hydrogen from water in the reaction

While carbon and chlorine isotope fractionation pointed towards a C-Cl-bond cleavage as rate determining step in the major pathway to ethene, still the influence from another reaction pathway (1,2-DCA conversion to VC followed by hydrogenolysis to ethene) was possible and needed to be excluded. To this end, a novel chromium-based hydrogen isotope analysis approach was put to use (Renpenning *et al.*, 2015). Hydrogenolysis with intermediate formation of VC leads to insertion of hydrogen (protonation) from the surrounding liquid media whereas for dihaloelimination no change in the hydrogen isotope composition is

expected when ethene is formed. Therefore, clear results are expected from using D_2O in the cultivation media. 1% D_2O was added to the mineral medium (i) using 1,2-DCA as substrate for *D. mccartyi* strain BTF08 and strain 195 and (ii) vinyl chloride as substrate for *D. mccartyi* strain BTF08, respectively $^2H/^1H$ values were expected to increase in ethene when formed as a product of VC (case ii) because one of the hydrogen atoms in ethene is introduced by protonation from surrounding water molecules. This enrichment was indeed observed when using VC as substrate (Figure 4). Whereas without D_2O the δ^2H values in ethene remained below -200 ‰ (data not shown), with D_2O the δ^2H values reached up to 4000 ‰. In contrast, ethene formed from 1,2-DCA by *D. mccartyi* strain BTF08 and strain 195 (case i), had δ^2H of up to -50 ‰ and was, therefore not enriched in ^2H relative to ^1H (Figure 4). Hence, we conclude that no VC is formed as intermediate of 1,2-DCA dehalogenation and the reaction proceeds via dihaloelimination.

Hydrogen stable isotope analysis

To further constrain the reaction pathway in reductive dehalogenation for 1,2-DCA hydrogen isotopic fractionation was determined using non-labeled medium. As our results from using deuterated water were pointing towards dihaloelimination for dechlorination of 1,2-DCA, weaker hydrogen isotope fractionation was expected resulting from secondary isotope effects, that derive from the influence of carbon-chlorine bond breakage on the molecular vibrations of carbon-hydrogen bonds. As carbon-carbon single-bonds harbor less flexibility than double-bonds, they are preferred by heavy isotope substituents such as deuterium, favoring more rigid bonds and less space for oscillation due to their lower zero-point-energies (Aelion *et al.*, 2010). If this C-C sp^3 single bond is converted towards a sp^2-hybridized double bond, the system gathers more flexibility, less substituents and therefore more space for oscillation of the substituents favoring lighter isotopes as hydrogen (Aelion *et al.*, 2010). This finding is in analogy to secondary hydrogen isotope effects observed in naphthalene degradation (Kümmel

et al., 2016). Over the time course of dehalogenation hydrogen isotope fractionation was observed for 1,2-DCA (Figure 3A), resulting in an ε^{H}_{bulk} = -62.1 ± 19.5 ‰ for strain BTF08 and ε^{H}_{bulk} = -26.8 ± 8.0 ‰ for strain 195 (Figure 3B), respectively. Since primary kinetic isotope effects for hydrogen are expected to be much higher ($KIE_{C\text{-}H,\ 2H/1H}$ = 6.4), resulting in enrichment factors of approx. -210 ‰ for 1,2-DCA (Elsner *et al.*, 2005, Elsner *et al.*, 2012), this demonstrates that 1,2-DCA dehalogenation did not involve C-H bond cleavage and supports a direct conversion from 1,2-DCA to ethene via dihaloelimination. Furthermore, this is confirmed by a closed isotope balance, since the final hydrogen isotope composition of ethene was similar to the initial value for 1,2-DCA (Figure 3A) whereas for dehalogenation mechanisms including VC the hydrogen isotope signature would be influenced by the hydrogenation of the reaction product. Also, the difference in hydrogen isotope values between the initially determined δ-values of 1,2-DCA and ethene was equivalent to the determined enrichment factors pointing towards direct conversion to ethene without external hydrogen sources. Still there is the possibility of simultaneously occurring pathways with major production of ethene from 1,2-DCA and minor amounts of VC which cannot be resolved based on the determined enrichment factors. Even if formation of VC was associated with a high fractionation, this contribution from a minor pathway would influence the overall enrichment factors for 1,2-DCA to only minor extent.

Apparent kinetic isotope effect for 1,2-DCA dehalogenation

Apparent kinetic isotope effects (AKIE) can be used to determine whether dihaloelimination of 1,2-DCA is taking place via a concerted or a stepwise reaction mechanism. Therefore a simplified reaction model can be used to estimate the appropriate maximum kinetic isotope effects being referred to as semiclassical Streitwieser limit (Elsner et al., 2005). The stepwise reaction mechanism leads to abstraction of one chlorine after the other, whereas the concerted

mechanism describes simultaneous dechlorination at both reactive sites. As additional slow, non-fractionating steps occur which preceed the bond cleavage, the determined AKIE is generally lower than the Streitwieser limit, assuming completely broken bonds in the transition state.

For *D.* mccartyi strain BTF08 and strain 195 the calculation for a stepwise and concerted reaction mode yielded $AKIE^{C}_{BTF08} = 1.059 \pm 0.008$ and $AKIE^{Cl}_{BTF08} = 1.009 \pm 0.001$ as well as $AKIE^{C}_{195} = 1.066 \pm 0.008$ and $AKIE^{C}_{195} = 1.009 \pm 0.001$. For concerted reactions AKIEs were $AKIE^{C}_{BTF08} = 1.029$ and $AKIE^{C}_{195} = 1.031$ as well as $AKIE^{Cl}_{BTF08} = 1.005$ and $AKIE^{Cl}_{195} = 1.004$. These values are in accordance to those previously described for *D. mccartyi* strain BTF08 and strain 195 by Schmidt *et al.*, 2014 (Table 2). Concerning theoretical Streitwieser limits for C-Cl-bond breakage, $AKIE^{C} = 1.057$ and $AKIE^{Cl} = 1.013$ (Elsner *et al.*, 2005), calculated values for stepwise reaction mechanisms in this study are above the limit for carbon but below for chlorine. As our determined values are close to the theoretical value, and therefore quite high, it is more likely that the reaction proceeds via a concerted mechanism, involving both C-Cl-bonds within the initial transformation step. Furthermore, calculated stepwise-mode AKIEs in this study exceed AKIEs for stepwise dihaloelimination by far (Elsner et al., 2007, Hofstetter et al., 2007) (Table 2).Furthermore, similarities for aerobic nucleophilic substitution (S_N2) of 1,2-DCA, $AKIE^{C}$ of 1.68 and $AKIE^{Cl}$ 1.009 (Palau *et al.*, 2014) were observed. AKIEs calculated for *D. mccartyi* strain BTF08 and strain 195 in concerted reaction mode are similar to Vitamin B_{12} related reduction described for PCE in *D. mccartyi* strain KB-1 $AKIE^{C} = 1.033/1.034$ (Elsner *et al.*, 2005) and below the described Streitwieser limits. Furthermore, determined values agreed well with reported concerted $AKIE^{C}_{concerted}$ of 1.04 to 1.05 for abiotic dihaloelimination of 1,2-DCA by Zn(0) (VanStone *et al.*, 2008). This supports the assumption for a concerted reaction mechanism for dihaloelimination of 1,2-DCA as AKIE for abiotic reactions are generally closest to intrinsic isotope effects (Lollar *et al.*, 2010). AKIE for a *Dehalococcoides*-

containing enrichment culture resulting in $AKIE^{C}_{stepwise}$ = 1.0707 and $AKIE^{C}_{concerted}$ = 1.034 (Palau *et al.*, 2017) and $AKIE^{C}$ = 1.068 and $AKIE^{Cl}$ = 1.0087 for oxic S_N2-reactions described by Palau et al, 2014 are in analogy as well. From the data derived in this study and by comparison to described biotic and abiotic values dihaloelimination reactions can be proposed as concerted mechanisms.

Conclusion

In this study we investigated the dehalogenation of 1,2-DCA using two distinct *Dehalococcoides mccartyi* spp., strain BTF08 and strain 195, using, for the first time, triple-element compound-specific stable isotope analysis to resolve whether dehalogenation of 1,2-DCA is taking part via dihaloelimination or intermediate vinyl chloride-forming hydrogenolysis.

Considering carbon isotope fractionation for both investigated strains, no significant differences in comparison to previously described (Schmidt *et al.*, 2014) values could be obtained. Chlorine isotope analysis revealed similarity to a *Dehalococcoides*-containing enrichment culture, described by Palau et al. (2017). Furthermore, *Dhc mccartyi* strain BTF08 and strain 195 showed similar Λ-values for the two-dimensional isotope plot, which led to the conclusion that both are following the same reaction pathway when utilizing 1,2-DCA. Finally, hydrogen isotope analysis revealed hydrogen isotope fractionation which could be classified as secondary hydrogen isotope effects after deuterium incorporation experiments. These results suggest that 1,2-DCA is dehalogenated to ethene directly via a dihaloelimination reaction. Calculation of the apparent kinetic isotope effects lead to the assumption that the dihaloelimination is taking place in a concerted reaction mechanism.

What remained unclear in this study is the source of the vinyl chloride which can be found in traces within the *Dehalococcoides mccartyi* strain BTF08 and strain 195 containing cultures

when dechlorinating 1,2-DCA. It can be excluded that it is formed as an intermediate of dehalogenation when producing ethene and might be resulting from abiotic processes involving the reducing agent sodium sulfide from the mineral media (Barbash & Reinhard, 1989, Jeffers *et al.*, 1989) which has to be verified. Furthermore, a subordinate malfunction of the responsible reductive dehalogenase as well as a minor expressed additional dehalogenase cannot be excluded. Further studies have to be conducted to investigate and identify the dominant reductive dehalogenase(s).

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Figure legends

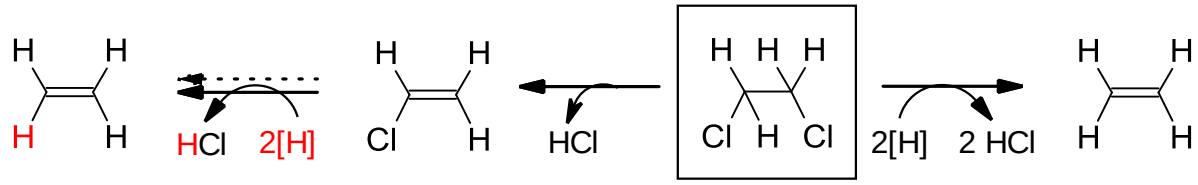

Figure 1: Possible reaction pathways for anaerobic 1,2-DCA dehalogenation in *Dehalococcoides mccartyi* strains 195 and BTF08, including direct conversion of 1,2-DCA to ethene (right pathway) and intermediate formation of toxic VC with final transformation to ethene (left pathway). Dotted arrows represent co-metabolic transformation for *Dehalococcoides mccartyi* strain 195.

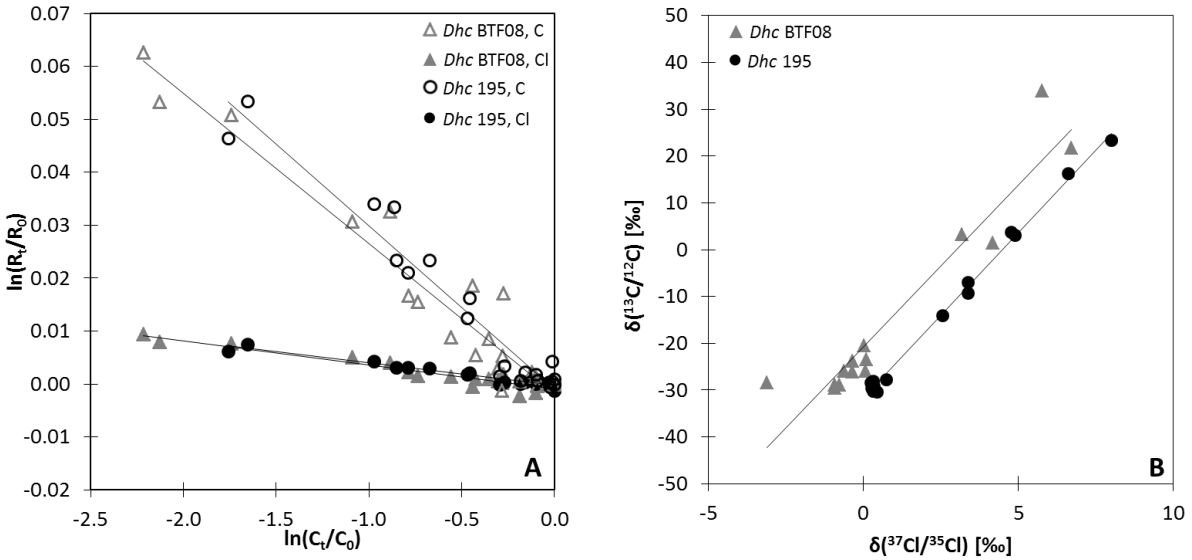

Figure 2: Rayleigh-plot for carbon and chlorine stable isotope fractionation (A) and 2-dimensional carbon-chlorine plot (B) for *Dehalococcoides mccartyi* strain BTF08 and strain 195 using 1,2-DCA as substrate. No significant difference between both *Dehalococcoides mccartyi* strains was observed.

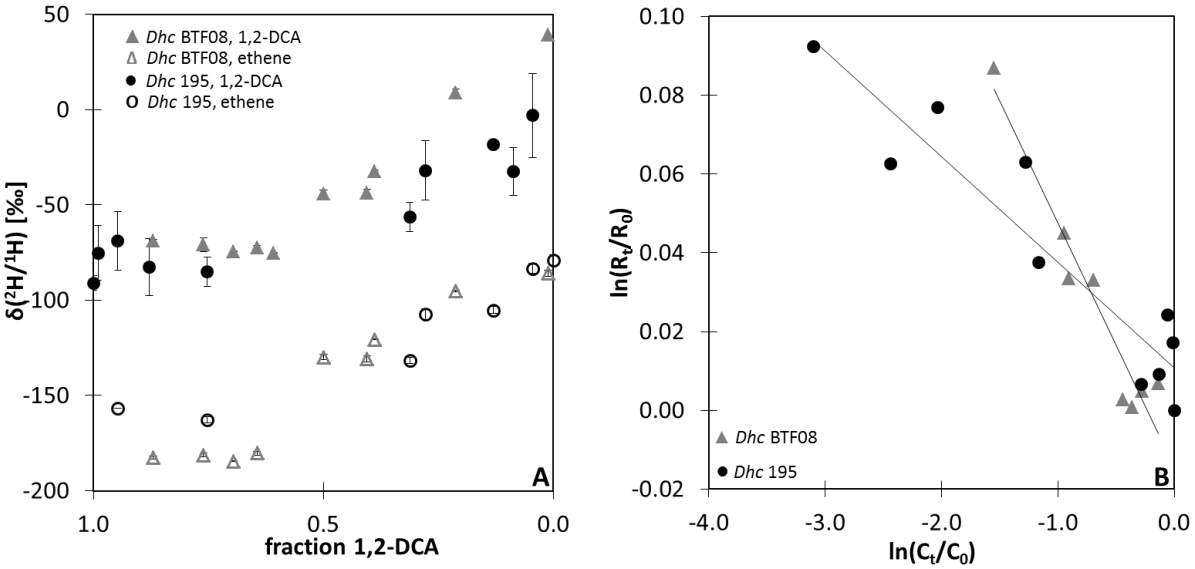

Figure 3: Hydrogen isotope fractionation for *Dehalococcoides mccartyi* strain BTF08 and strain 195 (A) and appropriate Rayleigh-Plot (B) using 1,2-DCA as substrate. No significant difference between both *Dehalococcoides mccartyi* strains was observed.

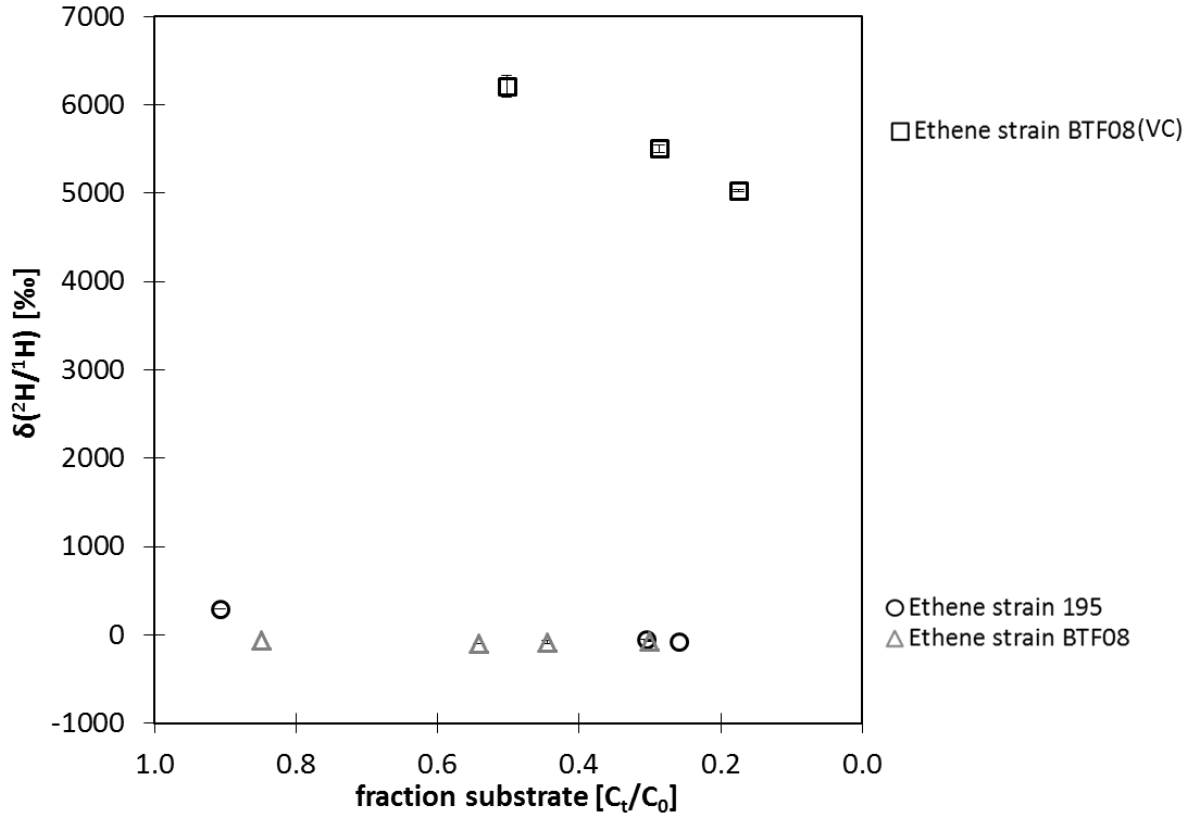

Figure 4: Hydrogen isotope fractionation for *Dehalococcoides mccartyi* strain 195 using 1,2-DCA and strain BTF08 using VC or 1,2-DCA as substrate in the presence of 1% D_2O. Significant enrichment of ethene produced from reactions using VC as substrate, compared to ethene resulting from 1,2-DCA, could be observed. Note that since deuterated water comprises high background and memory effects within the Cr-reactor and the GC-column the isotopic ratios of 1,2-DCA and VC could not be determined reliably and are not reported here.

Table 1: Isotope fractionation data determined for 1,2-DCA degradation described for carbon (ε^{C}_{bulk}), chlorine (ε^{Cl}_{bulk}), hydrogen (ε^{H}_{bulk}) and two dimensional carbon-chlorine ($\Lambda_{C/Cl}$) isotope fractionation. ec – enrichment culture. References: [a] Hunkeler & Aravena, 2000; [b] Hirschorn et al., 2004; [c] Palau et al., 2014; [d] Hirschorn et al., 2007; [e] Palau et al., 2017; [f] Schmidt et al., 2014

	Reaction mechanism	microorganism	ε^{C}_{bulk} [‰]	ε^{Cl}_{bulk} [‰]	$\Lambda_{C/Cl}$	ε^{H}_{bulk} [‰]	reference
aerobic	Nucleophilic substitution S_N2	*Xanthobacter autotrophicus* GJ10 *Amylobacter aqaticus* AD20	-31.9±0.7 to -32.2±1.8	-4.2±0.1 to -4.4±0.2	7.7±0.2	-	[a],[b],[c]
	Oxidative cleavage by monooxygenase	*Pseudomonas sp.* Strain DCA1	3.0±0.2 to -3.5±0.1	-4.2±0.1 to -4.4±0.2	0.78±0.03	-	[b],[c]
Nitrate-reducing	Hydrolytic degradation	microcosm	-25.8±3.5	-	-	-	[d]
anaerobic	Dihaloelimination	*Dehalococcoides*-containing ec	-33.0±0.4	-5.1±0.1	6.8±0.2	-	[e]
		Dehalogenimonas-containing ec	-23.0±2.0	-12.0±0.8	1.89±0.02	-	[e]
		Dehalococcoides mccartyi strain 195	-29.0±3.0	-	-	-	[f]
			-30.9±3.6	-4.2±0.5	7.1±0.24	-26.8±8.0	this study
		Dehalococcoides mccartyi strain BTF08	-30.8±1.3	-	-	-	[f]
			-28.4±3.7	-4.6±0.7	6.9±1.24	-62.1±19.5	this study

Table 2: Summary of apparent kinetic isotope effects (AKIE) for carbon and chlorine for described concerted and stepwise reaction mechanisms. ec – enrichment culture.

reaction	microorganism	AKIE carbon	AKIE chlorine	reference
oxic nucleohilic substitution (S_N2)	*A. aquaticus*	1.68	1.0089	Palau et al., 2014
	X. autotrophicus	1.68	1.0085	
oxic C-H-cleavage	*Pseudomonas sp.*	1.007	1.0038	
Vitamin B_{12} related reduction (PCE)	*Dhc* strain KB-1	1.033/1.034	-	Elsner et al., 2005

		AKIE carbon		AKIE chlorine		
		stepwise	concerted	stepwise	concerted	
dihaloelimination	abiotic (Zn(0))	-	1.04-1.05	-	-	VanStone et al., 2008
	abiotic (Cr(II), Fe(0))	1.0212-1.037	-	-	-	Elsner et al., 2007; Hofstetter et al., 2007
	Dhc strain BTF08	1.059	1.029	1.009	1.005	This study
		1.066	1.032	-	-	Schmidt et al., 2014
	Dhc strain 195	1.066	1.031	1.009	1.004	This study
		1.062	1.030	-	-	Schmidt et al., 2014
	Dhc-containing ec	1.071	1.034	-	-	Palau et al., 2017
	Dgm-containing ec	1.048	1.024	-	-	