Mechanistic dichotomy in bacterial trichloroethene dechlorination revealed by carbon and chlorine isotope effects

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Abstract: Tetrachloroethene (PCE) and trichloroethene (TCE) are significant groundwater contaminants. Microbial reductive dehalogenation at contaminated sites can produce non-toxic ethene, but often stops at toxic *cis*-1,2-dichloroethene (*cis*-DCE) or vinyl chloride (VC). The magnitude of carbon relative to chlorine isotope effects – as expressed by $\Lambda_{C/Cl}$, the slope of $\delta^{13}C$ vs. $\delta^{37}Cl$ regressions – was recently recognized to reveal different reduction mechanisms with Vitamin B_{12} as model reactant for reductive dehalogenase activity. Large $\Lambda_{C/Cl}$ values for *cis*-DCE reflected cob(I)alamin addition followed by protonation, whereas smaller $\Lambda_{C/Cl}$ values for PCE evidenced cob(I)alamin addition followed by Cl^- elimination. This study addressed dehalogenation in actual microorganisms and observed identical large $\Lambda_{C/Cl}$ values for *cis*-DCE ($\Lambda_{C/Cl}$ = 10.0 to 17.8) that contrasted with identical smaller $\Lambda_{C/Cl}$ for TCE and PCE ($\Lambda_{C/Cl}$ = 2.3 to 3.8). For TCE, the trend of small $\Lambda_{C/Cl}$ could even be reversed when mixed cultures were precultivated on VC or DCEs and subsequently confronted with TCE ($\Lambda_{C/Cl}$ = 9.0 to 18.2). This observation provides explicit evidence that substrate adaptation must have selected for reductive dehalogenases with different mechanistic motifs. The patterns of $\Lambda_{C/Cl}$ are consistent with practically all studies published to date, while the difference in reaction mechanisms offers a potential explanation to the long-standing question of why bioremediation frequently stalls at *cis*-DCE.

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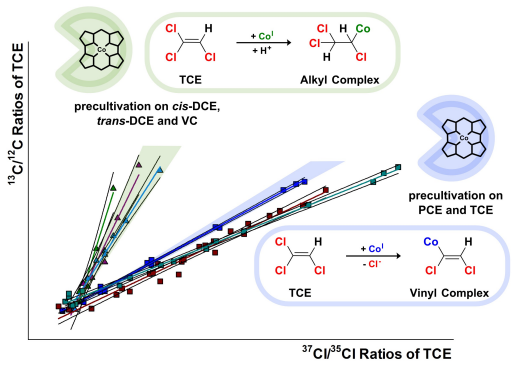


Introduction

Chlorinated ethenes like tetrachloroethene (PCE) and trichloroethene (TCE), are among the most frequent groundwater pollutants at contaminated sites worldwide[1]. Under anoxic conditions they may be reductively dechlorinated by microorganisms in a process known as organohalide-respiration. Chloroethenes act as electron acceptors so that their C-Cl bonds are reduced to C-H bonds (sequential hydrogenolysis) leading to non-toxic ethene as final product (see Figure 1)[2]. While this reaction stoichiometry is straightforward, the exact nature of the underlying biochemical reaction mechanism has been elusive.

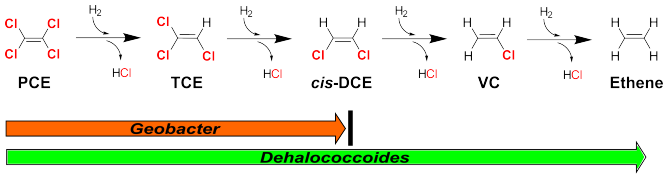
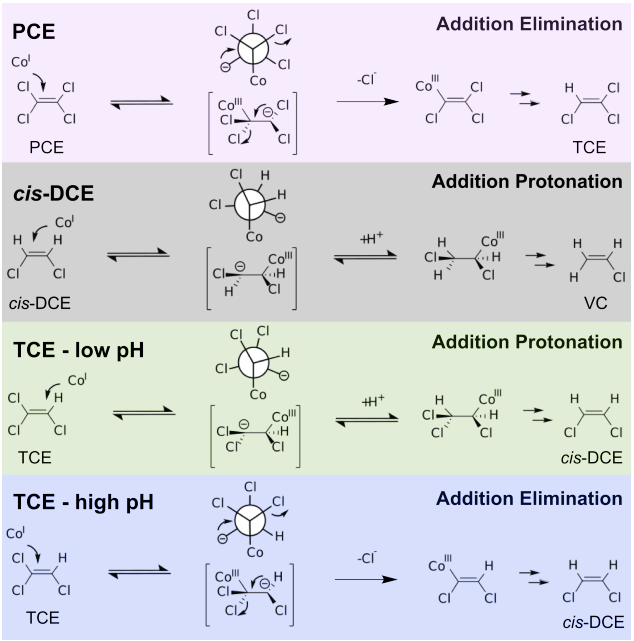


Figure 1. Reductive dechlorination of PCE to ethene with different end-points for two bacterial cultures.

Transformation frequently stalls at the stage of *cis*-1,2-dichloroethene (*cis*-DCE) or vinyl chloride (VC) constituting one of the long-standing barriers to successful bioremediation of these ubiquitous priority pollutants. Only specialized degrader strains – bacteria belonging to the class *Dehalococcoidia* (e.g., certain *Dehalococcoides mccartyi* and *Dehalogenimonas* strains) – were found to be capable of complete dechlorination to harmless ethene[3-10]. In contrast, other microorganisms, such as *Geobacter lovleyi*, cannot dechlorinate beyond *cis*-DCE[4] (see Figure 1). Pinpointing the underlying mechanistic reasons, however, has remained an elusive goal. Even though the catalytic site of all known reductive dehalogenases (RDases) contains cobalamin as an essential Co(I)-containing corrinoid cofactor, these enzymes occur in a great structural variety[2, 4, 11-13]. Very few dehalogenase protein structures have been solved yet[11, 12], and no reductive dehalogenase has been uniquely characterized for its underlying biochemical transformation mechanism (i.e., bond cleavage and formation). Consequently, critical research gaps in the chemistry of reductive dechlorination exist: Is the mechanism the same for all substrates? Does the mechanism

correlate with a given substrate? Or do mechanisms vary with the observed variety of reductive dehalogenases and organisms?

With reduced Vitamin B_{12} as a chemical model system, we recently achieved a breakthrough in understanding reaction mechanisms *in vitro*[14]. Our evidence suggests that cob(I)alamin acts as a supernucleophile and adds to the double bond in chlorinated ethenes so that a carbanion complex is formed. If the free electron pair of this complex faces two vicinal Cl substituents (as in the reaction of PCE) one of them will be in *anti*-position, leading to fast elimination of Cl^- and producing a cobalamin chlorovinyl complex as short-lived intermediate (Scheme 1). In contrast, if there is only one vicinal Cl substituent (as in the reaction of *cis*-DCE) the molecular conformation is unfavorable for subsequent elimination so the carbanion is protonated instead. This results in a slower reaction pathway involving an intermediate chloroalkyl complex (Scheme 1). If there can be either one or two vicinal Cl substituents (as in the reaction of TCE) the addition-protonation pathway is favored at low pH, whereas the addition-elimination pathway is favored at high pH (Scheme 1). In contrast, the number and position of geminal Cl substituents does not seem to have an effect on the reaction mechanism.



Scheme 1. Reaction mechanisms for reductive dehalogenation of chlorinated ethenes via addition protonation or addition elimination (adapted from Heckel et al.[14]).

Both TCE dechlorination pathways eventually produce *cis*-DCE as the respective hydrogenolysis product (Scheme 1) so that the different mechanisms are difficult to distinguish from product analysis alone. Additional experimental evidence is, therefore, warranted to determine whether the mechanistic dichotomy identified with Vitamin B_{12} is also at work in reductive dehalogenases or in dehalogenating organisms.

Compound-specific stable isotope effect measurements offer precisely such a complementary line of evidence. Gas-chromatography (GC) coupled to isotope ratio mass spectrometry (IRMS) measures carbon ($^{13}C/^{12}C$)[15, 16] and chlorine ($^{37}Cl/^{35}Cl$) isotope ratios at natural isotopic abundance[17-19]. Measured isotope ratios are expressed in the δ-notation, for example for carbon:

$$\delta^{13}C = [(^{13}C/^{12}C)_{Sample} - (^{13}C/^{12}C)_{Reference}] / (^{13}C/^{12}C)_{Reference} \quad (1)$$

where $(^{13}C/^{12}C)_{Reference}$ is the isotope ratio of an international reference material to ensure comparability between laboratories[20, 21]. An analogous equation applies to chlorine. When correlating these isotope values of two elements relative to each other the regression slope

$$\Lambda_{C/Cl} = (\delta^{13}C - \delta^{13}C_0) / (\delta^{37}Cl - \delta^{37}Cl_0) \approx \varepsilon_C/\varepsilon_{Cl} \quad (2)$$

reflects the magnitude of the underlying compound-specific isotope effects during a reaction[22]. Here δ^hE and δ^hE_0 are the isotope ratios of an element E (^{h} = mass number of the heavy isotope) at a given time and at the beginning of the reaction, respectively. Carbon and chlorine enrichment factors (ε_C, ε_{Cl}) reflect compound-specific isotope effects[22] that express by how much molecules with heavy isotopes react slower than molecules with light isotopes[20, 23]. A value of. ε = -10 ‰, for example, corresponds to a compound-specific isotope effect of $^{12}k/^{13}k$ = 1.01 (for the experimental evaluation of ε see Eq. 3 below). Our Vitamin B_{12} study demonstrated that the slope $\Lambda_{C/Cl}$ can provide a sensitive indicator of the underlying reaction mechanisms in reductive chlorinated ethene dehalogenation with Vitamin B_{12}[14]. Values of $\Lambda_{C/Cl}$ were much larger in the addition-protonation mechanism, reflecting the fact that no C-Cl bond was cleaved in the initial step so that chlorine isotope effects were small. In contrast, values of $\Lambda_{C/Cl}$ were smaller in the addition-elimination mechanism,

reflecting the larger chlorine isotope effect associated with C-Cl bond cleavage. When compared to single element ε values, $\Lambda_{C/Cl}$ values have the additional advantage that the slope $\Lambda_{C/Cl} = \varepsilon_C/\varepsilon_{Cl}$ remains remarkably constant even when intrinsic isotope effects show variations.

The objective of this study was to analyze carbon and chlorine isotope effects during reductive dehalogenation of chloroethenes with different bacterial cultures. The resulting $\Lambda_{C/Cl}$ values were compared to the $\Lambda_{C/Cl}$ values of two mechanistic trends recently observed in a Vitamin B_{12} model system. To this end, we investigated in particular whether the isotope fractionation trends in microbial dechlorination of *cis*-DCE and PCE correlate with trends of the addition-protonation and the addition-elimination mechanism observed with Vitamin B_{12}, respectively. For TCE dechlorination both mechanisms were observed in the Vitamin B_{12} study depending on pH. To test whether evidence of both mechanisms may be observed for TCE in living bacteria as well, microbial dechlorination of TCE was studied in seven different experiments, either varying in precultivation substrate or in the type of predominant RDases inside the bacteria. Finally, isotopic data of the dechlorination experiments of this study was compared to literature data of available C/Cl isotope studies to test whether the picture of a mechanistic dichotomy is consistent with published evidence to date.

Material and Methods

***cis*-DCE and TCE dechlorinating cultures.** Dehalogenation experiments with *cis*-DCE were carried out using *Dehalococcoides mccartyi* strain 195[6] and the highly enriched *Dehalococcoides mccartyi* strain BTF08 culture[8, 24]. Dehalogenation experiments with TCE were conducted with one pure culture (*Geobacter lovleyi* strain KB-1) and six mixed cultures (KB-1/1,2-DCA, KB-1/VC, KB-1/cDCE, WBC-2/tDCE, KB-1 RF and Donna II) (see Table 1 for further details). *G. lovleyi* strain KB-1, KB-1/1,2-DCA, KB-1/VC and KB-1/cDCE and KB-1 RF were derived from KB-1, a commercially available enrichment culture, which is specialized in the dehalogenation of chlorinated ethenes. It contains *G. lovleyi* strain KB-1 and a minimum of three strains of *Dehalococcoides*, as well as non-dechlorinating bacteria

like acetogens and methanogens[25-29]. Prior to the experiment the cultures were enriched on different maintenance substrates for many years (see Table 1).

Biotic dechlorination of *cis*-DCE under anoxic conditions with *D. mccartyi* strain 195 and strain BTF08. *D. mccartyi* strain 195 was cultivated as described in Cichocka et al.[30] and Maymo-Gatell et al.[6] with addition of butyrate pellets. *D. mccartyi* strain BTF08 was cultivated following the protocol of Cichocka et al.[8] and Schmidt et al.[31]. For each strain a set of 23 serum bottles (50 ml) was filled with 25 ml anoxic medium and flushed with N_2 and CO_2 (70/30 %). After closing the bottles by crimping with Teflon® lined grey butyl rubber stoppers they were sterilized for 40 min at 120 °C. Then they were spiked with *cis*-DCE (500 µM) as electron acceptor and equilibrated overnight. On the next day the bottles were inoculated with a culture grown on *cis*-DCE (2.5 % v/v for strain 195, 5 % v/v for strain BTF08). For each set three non-inoculated bottles with substrate served as negative control. The bottles were complemented with hydrogen as electron donor (0.5 bar overpressure). All cultures were incubated in the dark without shaking at 20 °C (BTF08) or 30 °C (195). Progress of substrate dehalogenation was monitored by concentration measurements with GC-FID. At different levels of dechlorination bottles were sacrificed for analysis by stopping the dechlorination reaction with 1 ml acidic sodium sulphate solution (280 g/l, pH ≈1) following the protocol of Cichocka et al.[30]. Samples were stored at 4 °C in the dark for later carbon and chlorine isotope analysis via GC-IRMS.

Table 1. Summary of precultivation conditions, RDases and compound-specific isotope enrichment factors of carbon and chlorine.

Substrate for Dehalogenation and Isotope Analysis	Culture	Dechlorinators	Precultivation Substrate (electron donor)	Most abundant functional *rdhA* Genes* before	Most abundant functional *rdhA* Genes* after	Slope $\Lambda_{C/Cl}$**	ε_{Cl} [‰]**	ε_{C} [‰]**	Duration
***cis*-DCE**	*D. mccartyi* 195	*D. mccartyi* 195 (pure culture)	*cis*-DCE (hydrogen)	*tceA* [32]		10.0 ±0.4	-2.3 ±0.4	-23.2 ±4.1	no lag period, *cis*-DCE dehalogenation completed after one month
	D. mccartyi BTF08	*D. mccartyi* BTF08 (enrichment culture)	*cis*-DCE (hydrogen)	*tceA* [7]		17.8 ±1.0	-1.7 ±0.4	-31.1 ±6.3	
TCE	*G. lovleyi* KB-1	*G. lovleyi* KB-1 (pure culture)	PCE (acetate)	*Geo-pceA* [33]		3.1 ±0.1	-3.3 ±0.3	-10.3 ±0.8	no lag period, TCE dehalogenation completed within one day
	KB-1 RF	multiple *D. mccartyi* strains (enrichment culture; no *Geobacter*)	TCE (methanol)	*vcrA* [33]		2.7 ±0.2	-3.3 ±0.3	-9.6 ±0.5	
	Donna II	*D. mccartyi* 195 (mixed culture; only one strain of *D. mccartyi*)	TCE (butyrate)	*tceA* [34]		2.3 ±0.1	-5.7 ±0.4	-13.5 ±0.6	
	KB-1/1,2-DCA	multiple *D. mccartyi* strains (enrichment cultures)	1,2-DCA (methanol)	*tceA* [35]	*tceA, vcrA*	4.5 ±0.8	-1.2 ±0.3	-5.4 ±1.5	long lag period (30-40 days), TCE dehalogenation completed after 70-100 days
	KB-1/VC		VC (methanol)	*vcrA* [33]	*vcrA*	18.2 ±4.3	-0.5 ±0.6	-10.6 ±9.3	
	KB-1/cDCE		*cis*-DCE (methanol)	*bvcA vcrA* [33]	*vcrA (tceA)*	11.8 ±2.4	-0.7 ±0.2	-8.3 ±3.4	
	WBC-2/tDCE	*Dehalogenimonas sp., D. mccartyi* (enrichment culture)	*trans*-DCE (lactate/ ethanol)	*tdrA (Dhgm), vcrA (Dhc)* [9]	*vcrA (tceA, tdrA)*	9.0 ±1.1	-0.7 ±0.3	-7.0 ±1.9	

* the abundance of specific *rdhA* genes known to be present in the cultures was used as a way to track which of multiple strains grew in the mixed culture; *rdhA* genes in brackets were only detected in minor abundance; the KB-1 enrichments were selected because each harbored a different dominant expressed RDase initially

** ±95 % confidence intervals

Biotic dehalogenation of TCE under anoxic conditions with *G. lovleyi* strain KB-1, KB-1/1,2-DCA, KB-1/VC, KB-1/cDCE and WBC-2/tDCE. Two hundred milliliters defined mineral medium[36] and 55 µl resazurin (0.4 %) were filled in glass bottles (250 ml). Subsequently they were capped with Mininert™ valves (Supelco) and purged for 40 min with a N_2/CO_2 gas mixture (80/20 %). Each bottle of *G. lovleyi* strain KB-1 was complemented with 50 µl acetate (1 M) and 9 µl TCE, whereas each bottle of KB-1/VC, KB-1/cDCE, and KB-1/1,2-DCA was complemented with 20 µl methanol and 9 µl TCE and each bottle of WBC-2/tDCE was complemented with 22 µl lactate solution (75 g/l), 44 µl ethanol and 9 µl TCE. All substances and solutions for complementation were taken from anoxic stocks. Afterwards all bottles were continuously agitated on an orbital shaker at 60 rpm at room temperature for 24 hours for equilibration. Biotic dehalogenation started by inoculating each bottle with 20 ml of active culture. In order to eliminate carryover of volatile organic compounds the active cultures had been purged for one hour with a N_2/CO_2 gas mixture (80/20 %). The bottles were prepared in triplicates for each culture. Furthermore, for each culture non-inoculated bottles with substrate served as negative control and were monitored alongside the experimental bottles. Five minutes after inoculation the first samples were taken. The next samples were taken in intervals throughout the dehalogenation process. At each sampling point 7 ml of liquid were removed from all the bottles. The sample of 7 ml was then divided into 1 ml aliquots which were distributed into seven 1.5 ml glass vials and closed with PTFE-lined screw-top caps. All samples were fixed with 50 µl NaOH (1 M) to stop biological activity. One of the seven vials was used for instant concentration analysis which was performed on a GC-FID. The other six vials were frozen upside down for later isotope analysis of carbon and chlorine performed via GC-IRMS[37, 38]. Preparation of the cultures (except the purging with N_2/CO_2) and taking samples was conducted in a glovebox containing an anoxic atmosphere (80 % N_2, 20 % H_2).

Biotic dehalogenation of TCE under anoxic conditions with KB-1 RF and Donna II. The whole experiment was conducted in a glovebox containing an anoxic atmosphere (80 % N_2, 10 % H_2, 10 % CO_2). Glass bottles (260 ml) were filled with 200 ml (KB-1 RF) or 210 ml

196 (Donna II) defined mineral medium[36] and inoculated with 20 ml (KB-1 RF) or 10 ml
197 (Donna II) active culture. Beforehand the cultures were purged with a N_2/CO_2 gas mixture for
198 30 min to eliminate carryover of volatile organic compounds. Triplicate experimental bottles
199 were capped with Mininert™ valves (Supelco) and complemented by adding 20 µl of
200 methanol (KB-1 RF), 8.75 µl butyrate (Donna II) and 9 µl of TCE. Furthermore, for each
201 culture non-inoculated bottles with substrate and killed control bottles (sterilized before TCE
202 addition) served as negative control and were monitored alongside the experimental bottles.
203 All replicates were continuously shaken at 350 rpm at room temperature (24 °C). At each
204 time point headspace samples were removed first for concentration measurements via GC-
205 FID and then for carbon isotope analysis via GC-IRMS. Subsequently 4 ml liquid samples
206 were removed and split into 1 ml aliquots. Liquid samples were acidified to a pH of < 2 with
207 50 µl of 1 M H_2SO_4 and closed with PTFE-lined screw-top caps and then frozen upside down
208 in 1.5 ml glass vials for later chlorine isotope measurements via GC-IRMS[37, 38]. Sample
209 volumes removed were compensated with identical volumes of glovebox atmosphere to
210 maintain a constant pressure within the bottle. Septa inside the stopper of the Mininert™
211 vials were replaced after every second piercing to minimize leakage.
212 **Concentration measurements and carbon and chlorine isotope analysis.** Concentration
213 measurements via GC-FID and compound-specific isotope analysis of carbon and chlorine
214 via GC-IRMS were performed according to defined protocols (see SI).
215 **Evaluation of carbon and chlorine isotope fractionation.** Carbon and chlorine
216 enrichment factors (ε_C, ε_{Cl}) of *cis*-DCE and TCE dechlorination were calculated according to
217 the Rayleigh equation (Equation 3) using Sigma-Plot. The Rayleigh equation describes the
218 gradual enrichment of the residual substrate fraction *f* with molecules containing heavy
219 isotopes[20, 22], for example for carbon:
220 $$\ln [(\delta^{13}C+1) / (\delta^{13}C_0+1)] = \varepsilon_C \cdot \ln f \quad (3)$$
221 The isotope ratios of carbon refer to certain time points, one of them at the beginning of an
222 experiment ($\delta^{13}C_0$). By plotting values of $\delta^{13}C$ vs. $\delta^{37}Cl$ (see Equation 2), dual element
223 isotope plots were obtained. These processes are also illustrated in Figure 2. 95 %

confidence intervals (CI) show the uncertainties of the calculated slopes $\Lambda_{C/Cl}$ ($\Delta\delta^{13}C/\Delta\delta^{37}Cl$). In chemical reactions isotope effects occur predominantly at the reacting position. Therefore, in many cases a position-specific apparent kinetic isotope effect (AKIE) may be estimated under the assumption that there are no isotope effects at the other positions according to

$$AKIE_{position\text{-}specific} = 1 / (n \cdot \varepsilon_{reacting\ position} + 1) \quad (4)$$

where n is the number of atoms in intramolecular competition[22]. However, for chlorinated ethene reduction our mechanistic picture (Scheme 1) suggests that the situation is more complex since isotope effects occur in different steps of the reaction sequence, and they may occur at different positions of the molecule[14, 39]. On the other hand, from IRMS measurements alone intramolecular isotope effects are difficult to resolve. Thus, in this study we decided not to estimate position-specific isotope effects but instead to report compound-specific isotope effects in the form of ε values.

qPCR (quantitative Polymerase Chain Reaction) analysis of KB-1/1,2-DCA, KB-1/VC, KB-1/cDCE and WBC-2/tDCE. qPCR analyses were conducted after the completion of the TCE experiment. 8.5 ml sample of each culture were collected and subsequently 1.5 ml of 50 % glycerol were added. The samples were stored at -80 °C after freezing in liquid nitrogen. For qPCR analysis 8 ml of each thawed sample were filtered through a sterile 0.22 µM Sterivex filter (Millipore) using an Air Cadet Vacuum/Pressure Pump 400-1902 (Barnant Company). After filtration the Sterivex filters were immediately frozen at -80 °C. The filters were removed from the filter casing, sliced into small pieces with a sterile surgical blade and then transferred to a bead-beating tube. For DNA extraction the PowerSoil DNA isolation kit (Mo Bio Laboratories Inc.) was used. The DNA was extracted by following the manufacturer's protocol for maximum yields, except that DNA was eluted in 50 µl sterile UltraPure distilled water (Invitrogen) rather than in the eluent provided with the kit. By using a spectrophotometer (NanoDrop ND-1000; NanoDrop Technologies) the DNA concentration and quality were assessed. Afterwards the DNA samples were 10 times diluted with sterile UltraPure distilled water. All subsequent steps were performed in a PCR cabinet (ESCO Technologies). qPCR reactions were run in triplicates where each run was calibrated by

constructing a standard curve using known plasmid DNA concentrations containing the gene insert of interest. The standard curve was run with eight concentrations ranging from 10 to 108 gene copies/µl. qPCR reaction solutions (20 µl) were prepared in sterile UltraPure distilled water containing 10 µl of EvaGreen® Supermix, 0.5 µl of each primer (forward and reverse, each from 10 µM stock solutions) and 2 µl of diluted template (DNA extract or standard plasmids). The qPCR reactions were conducted using a CFX96 real-time PCR detection system with a C1000 Thermo Cycler using SsoFast™ EvaGreen® supermix (Bio-Rad Laboratories). The thermocycling program started with the initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 98 °C for 5 s, annealing for 10 s (see Table S1 in the SI for annealing temperatures), and a plate read. A final melting curve analysis was conducted at the end of the program. The following genes were targeted by qPCR using the defined primer sets (see Table S1 in the SI): the phylogenetic 16S rRNA genes of *Dehalococcoides* and *Dehalogenimonas*, the functional genes *vcrA*, *tceA*, *bvcA*, *tdrA*, as well as the 16S rRNA genes of total bacteria and total archaea.

Results and Discussion

Starkly contrasting carbon and chlorine isotope fractionation suggests that microbial dechlorination of *cis*-DCE and PCE follows different mechanisms. To take advantage of compound-specific isotope effects and evaluate whether the mechanistic dichotomy observed *in vitro* can also be identified in pure strains of living organisms, we began with a comparison between PCE and *cis*-DCE. Carbon and chlorine isotope values of *cis*-DCE were measured in dehalogenation experiments with the strictly anaerobic organism *Dehalococcoides mccartyi* strain 195[6] and the highly enriched *Dehalococcoides mccartyi* strain BTF08 culture[8, 24]. Results were compared to our previous data on reductive dechlorination of PCE by *Desulfitobacterium* sp. strain Viet1[39]. Figure 2A and B show the changes in carbon and chlorine isotope ratios with decreasing fraction of respective substrate and the corresponding enrichment factors. Combining the isotope ratios of panel A

(carbon) and B (chlorine) leads to the formation of a dual element isotope plot as illustrated in panel C.

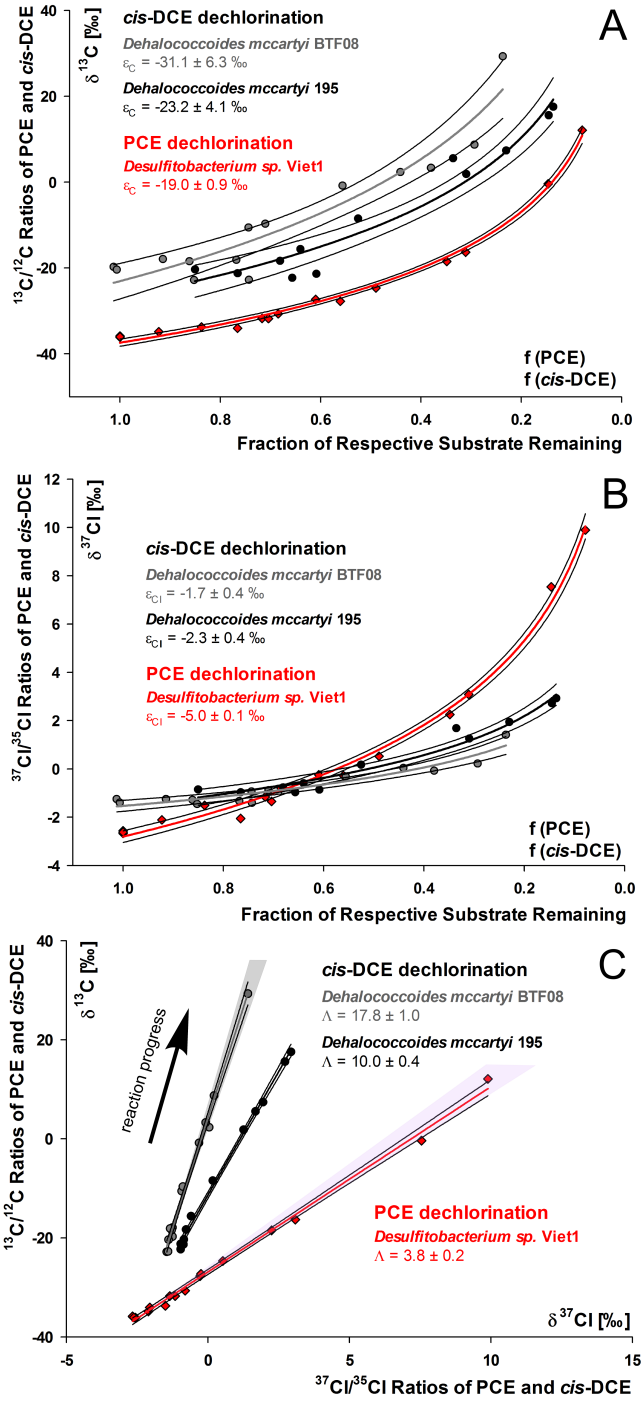


Figure 2. Carbon and chlorine isotope effects in reductive dehalogenation of *cis*-DCE by *D. mccartyi* BTF08 (grey) and *D. mccartyi* 195 (black) and PCE by *Desulfitobacterium sp.* Viet1 (red) (data from Cretnik et al.[39]) resulting in a dual element isotope plot. (95 % confidence intervals are given as values and as black lines next to the regression slopes). (A) Carbon isotope fractionation and corresponding carbon enrichment factors ε_C. (B)

Chlorine isotope fractionation and corresponding chlorine enrichment factors ε_{Cl}. (Both ε evaluated according to Eq. 3). (C) Resulting dual element isotope plots ($\delta^{13}C$ vs $\delta^{37}Cl$) indicate the occurrence of different underlying transformation mechanisms corresponding to mechanisms observed with *cis*-DCE (shaded in grey) and PCE (shaded in pink) by model reactions with Vitamin B_{12}[14].

The dual element isotope trends with bacteria reproduced the trends obtained with Vitamin B_{12}, and were reflected on the level of compound-specific carbon and chlorine isotope effects, illustrated by ε_C and ε_{Cl}. Dechlorination of *cis*-DCE was associated with large carbon and small chlorine isotope effects (*D. mccartyi* 195: ε_C = -23.2 ±4.1 ‰, ε_{Cl} = -2.3 ±0.4 ‰; *D. mccartyi* BTF08: ε_C = -31.1 ±6.3 ‰, ε_{Cl} = -1.7 ±0.4 ‰) resulting in large dual element isotope slopes Λ_{195} = 10.0 ±0.4 and Λ_{BTF08} = 17.8 ±1.0. In contrast, dechlorination of PCE was associated with pronounced isotope effects in both elements (ε_C = −19.0 ±0.9 ‰, ε_{Cl} = −5.0 ±0.1 ‰) giving rise to a smaller dual element isotope slope $\Lambda_{Desulfitobacterium}$ = 3.8 ±0.2. This large chlorine isotope effect is even more striking when one considers that it is averaged over four chlorine atoms in PCE (of which only one is cleaved off) while in *cis*-DCE the average is taken over only two chlorine atoms. Hence, kinetic isotope effects of PCE and *cis*-DCE at the reacting position (after correcting for the dilution by non-reacting chlorine atoms) would show even greater differences[40]. The same would be true for dual element isotope slopes $\Lambda_{C/Cl}$. Our results therefore provide key lines of evidence suggesting that *cis*-DCE and PCE must be dechlorinated via different mechanisms, and they exemplify the pattern observed for addition-protonation vs. addition-elimination pathways (Scheme 1 and Figure 2)[14].

Dual element isotope trends in TCE dechlorination by pure cultures are indicative of an addition-elimination mechanism. In an *in vitro* study using Vitamin B_{12} as model system TCE was recently observed to be dechlorinated via two different reaction mechanisms depending on pH (see Figure 3A and Scheme 1). In order to probe which mechanism would be observed for TCE *in vivo* with bacterial pure cultures, a *Geobacter* subculture (*Geobacter lovleyi* KB-1) of the mixed consortium KB-1 that had been cultivated

to purity was investigated and compared to previously observed trends for *Geobacter lovleyi* SZ and *Desulfitobacterium hafniense* Y51[38] (Figure 3B). The dual element isotope slopes of the pure cultures correspond to the dual element isotope slopes of the Vitamin B_{12} study at high pH values indicating that *in vivo* TCE is dechlorinated via the addition-elimination pathway.

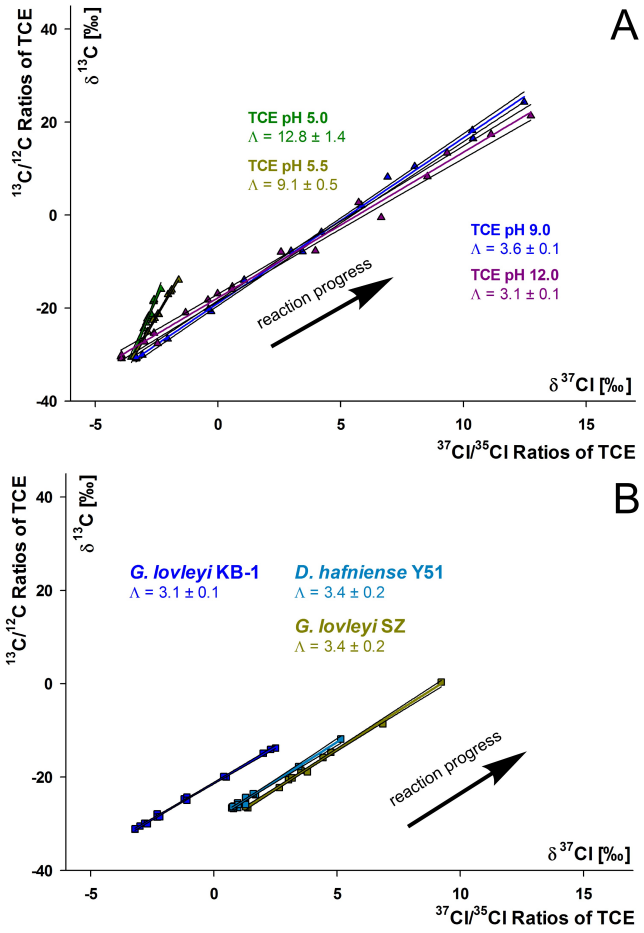


Figure 3. Carbon and chlorine isotope effects in TCE reductive dehalogenation (A) by Vitamin B_{12} at different pH values and (B) with pure cultures resulted in similar dual element isotope plots. (95 % confidence intervals are given as values and as black lines next to the regression slopes). (A) TCE reductive dehalogenation at high (green/yellow) and low (purple/blue) pH values (adapted from Heckel et al.[14]). (B) TCE reductive dechlorination with the pure culture *G. lovleyi* KB-1 (blue, this work) and the pure cultures *G. lovleyi* SZ (yellow) and *D. hafniense* Y51 (blue) (adapted from Cretnik et al.[38]).

Precultivation of bacteria on less chlorinated ethenes leads to TCE dual element isotope trends indicative of an addition-protonation mechanism. In order to investigate whether a different reaction mechanism can nonetheless be observed for TCE when using precultivation conditions to select for organisms with a different substrate preference, we conducted another set of experiments. Mixed cultures, Donna II and KB-1 RF, were precultivated on PCE or TCE for years (see Table 1), meaning that they were already adapted to TCE (substrate / daughter product of PCE dechlorination). On the other hand, we maintained another set of cultures on less chlorinated precultivation substrates: Three subcultures of the dechlorinating consortium KB-1 RF that were maintained on *cis*-DCE (KB-1/cDCE), VC (KB-1/VC) and 1,2-DCA (KB-1/1,2-DCA) for at least two years, and a fourth mixed culture that was enriched on *trans*-DCE (WBC-2/tDCE) for many years. As expected, cultures precultivated on TCE and PCE started to dechlorinate TCE immediately and the dechlorination was completed within one day (see Table 1). In contrast, the set of cultures enriched and precultivated on less chlorinated ethenes showed a lag period of 30-40 days before they started to dechlorinate TCE and dechlorination took 70-100 days for completion.

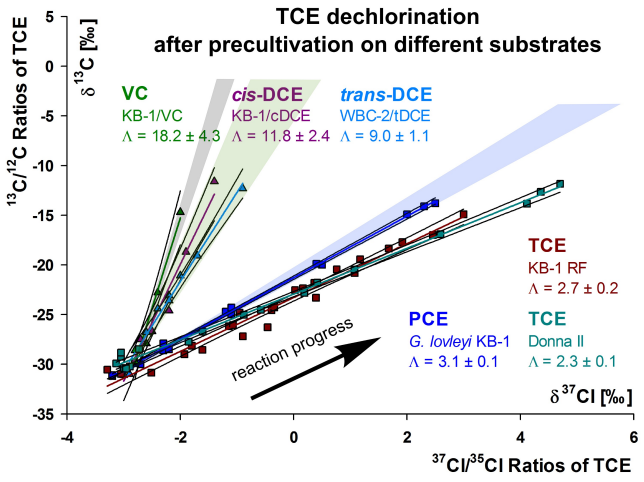


Figure 4. Dual element isotope trends indicate a mechanistic divide between TCE dechlorination by cultures precultivated on PCE (*G. lovleyi* KB-1, blue) and TCE (KB-1 RF, brown and Donna II, cyan) vs. cultures precultivated on VC (KB-1/VC, light green), *cis*-DCE (KB-1/cDCE, purple), and *trans*-DCE (WBC-2/tDCE, light blue). Shaded areas show the corresponding trends observed with *cis*-DCE (grey, pH 6.5) and TCE (green, low

pH/blue, high pH) in the Vitamin B_{12} model[14]. (95 % confidence intervals are given as values and as black lines next to the regression slopes).

Figure 4 shows that precultivation affected the carbon and chlorine isotope effects. A clear divide appears between two dual element isotope trends depending on precultivation conditions. Cultures precultivated on less chlorinated ethenes like VC (KB-1/VC), *cis*-DCE (KB-1/cDCE) and *trans*-DCE (WBC-2/tDCE) showed large carbon isotope effects in combination with small chlorine isotope effects corresponding to $\Lambda_{C/Cl}$ values between 9.0 and 18.2 ($\Lambda_{KB\text{-}1/VC}$ = 18.2 ±4.3, $\Lambda_{KB\text{-}1/cDCE}$ = 11.8 ±2.4, $\Lambda_{WBC\text{-}2/tDCE}$ = 9.0 ±1.1). In contrast the cultures *G. lovleyi* strain KB-1, KB-1 RF, and Donna II, precultivated on TCE or PCE, showed significantly smaller $\Lambda_{C/Cl}$ values of 2.3 to 3.1 ($\Lambda_{Donna\ II}$ = 2.3 ±0.1, $\Lambda_{KB\text{-}1\ RF}$ = 2.7 ±0.2, $\Lambda_{G.\ lovleyi\ KB\text{-}1}$ = 3.1 ±0.1) indicative of larger chlorine isotope effects. These results are similar to the dual element isotope slopes $\Lambda_{C/Cl}$ observed for an addition-elimination mechanism with Vitamin B_{12}. In contrast, cultures precultivated on less chlorinated substrates – *cis*-DCE (KB-1/cDCE), VC (KB-1/VC), and *trans*-DCE (WBC-2/tDCE) – resulted in $\Lambda_{C/Cl}$ values of TCE dechlorination that correspond to an addition-protonation pathway with Vitamin B_{12}.

Our observations suggest that in the bacterial cells a similar mechanistic dichotomy of cob(I)alamin addition-elimination vs. cob(I)alamin addition-protonation took place as in the model reaction with Vitamin B_{12} at different pH (Scheme 1). In experiments with bacterial cells, however, both the medium and the inside of the cells were buffered so that catalysis of the different pathways must be effectuated by functional groups inside the enzymes' catalytic sites. We therefore hypothesize that the enzyme architecture of RDases is tailored to different specific reaction mechanisms, possibly due to the presence / absence of amino acids with specific protonation functionalities.

Mechanism-specific dual element isotope trends of TCE did not correlate with RDase predominance. Given that we observed evidence of different reaction mechanisms in bacterial reductive dehalogenation of TCE, we further explored whether this mechanistic dichotomy could be correlated with the predominance of specific reductive dehalogenases.

Therefore, three different bacterial cultures, that had been adapted to TCE and for which the predominance of different RDases can be inferred (see Table 1), were compared. *G. lovleyi* strain KB-1 has been shown to harbor only one RDase, *Geo*-PceA[33]. For the mixed culture KB-1 RF, the RDase VcrA is considered to be responsible for dechlorination[33]. In the mixed culture Donna II, *D. mccartyi* strain 195 is the organism responsible for dechlorination, and the RDase TceA was identified as the most prominent dechlorinating enzyme[34].

The key outcome of this approach was that the dual element isotope plot of these three cultures shows similar regression slopes ($\Lambda_{\text{Donna II}}$ = 2.3 ±0.1, $\Lambda_{\text{KB-1 RF}}$ = 2.7 ±0.2, $\Lambda_{\textit{G. lovleyi}\ \text{KB-1}}$ = 3.1 ±0.1, see Table 1 and Figure S2) for all three experiments, indicating that TCE was dechlorinated via a similar chemical mechanism, irrespective of the type of RDase (*Geo*-PceA vs. VcrA vs. TceA). The three slopes agree with those at high pH in the Vitamin B_{12} study[14], suggesting that in all three cases a sequence of addition-elimination was the predominant reaction pathway.

Subsequently, quantitative polymerase chain reaction (qPCR) analysis was applied to detect changes in the reductive dehalogenase gene (*rdhA*) composition when cultures that had been precultivated on less chlorinated ethenes were adapting to TCE reductive dechlorination (see Table 1). qPCR analysis indicated a significant shift in the culture KB-1/cDCE after changing the electron acceptor from *cis*-DCE to TCE. Typically KB-1/cDCE is dominated by the RDase BvcA when precultivated on *cis*-DCE[33]. After the TCE dechlorination experiment, however, the *rdhA* gene *bvcA* was no longer detected in the qPCR analysis. Instead, the *rdhA* gene *vcrA* was most abundant, indicating that TCE dechlorination was likely performed by a *vcrA*-containing strain of *Dehalococcoides*. For the WBC-2/tDCE culture, only minor changes in the RDase composition were observed. Here *vcrA* and *tdrA* genes were predominant before[9] and after the experiment. WBC-2/tDCE contains *Dehalogenimonas sp.*, which expresses TdrA for the dechlorination of *trans*-DCE to VC[9]. Additionally, after the TCE dechlorination experiment a small number of *tceA* genes were detected by qPCR. In case of KB-1/VC, no changes in the *rdhA* gene composition were discernible. Before[33] and after the TCE dechlorination experiment with KB-1/VC, *vcrA* was

406 the most abundant RDase gene analyzed. The information obtained from the qPCR data
407 therefore suggests that the maintenance on one specific precultivation substrate has a
408 significant influence on the microbial community and the prevalence of RDase genes[35].
409 Nevertheless, isotope effects of all cultures still gave evidence of the same addition-
410 protonation mechanism (see Table 1 and Figure S2) suggesting that the reaction
411 mechanism was conserved in precultivated cultures even though shifts in the dominantly
412 expressed RDase were observed.
413 Finally, a comparison of Figure 4 and the qPCR data on predominant RDases (see Table 1
414 and Figure S2) suggests that there can be different mechanisms at work ($\Lambda_{KB\text{-}1\ RF}$ = 2.7 ±0.2
415 vs. $\Lambda_{KB\text{-}1/VC}$ = 18.2 ±4.3) even though the same nominal RDase (VcrA) was predominant.
416 One possibility is that the VrcA dehalogenase complex in organisms adapted to less
417 chlorinated substrates is different from those enriched on TCE. Kublik et al.[41] showed that in
418 *Dehalococcoides* the reductive dehalogenase is part of a complex containing a variety of
419 proteins. Potentially, these other electron transport proteins may affect the enzyme and its
420 isotope fractionation. Also the role of corrinoid prosthetic groups, which can affect
421 dechlorination[13, 42], has to be further investigated, since it was unclear what types of
422 corrinoids were produced in the mixed cultures. Another possibility is that the RDase
423 catalyzing the dechlorination in the non-TCE-adapted cultures is not VcrA, even though the
424 strains contained that gene. Quantitative polymerase chain reaction can only reveal that
425 genes containing *vcrA* became more abundant after switching the electron acceptor, but
426 qPCR cannot provide direct information about whether the RDase were actually expressed.
427 For example, Heavner et al.[43] pointed out that in all *Dehalococcoides*, and particularly in KB-
428 1, a specific RDase (DET 1545 homolog) shows elevated expression upon stress.
429 The observation that the predominance of nominal RDases did not correlate with isotope
430 effect trends therefore highlights the need for a complementary approach to classify
431 degradation in natural and engineered systems: not only based on the (meta)genomic
432 detection of RDase genes, but also based on dual element (C, Cl) isotope fractionation as
433 indicator of underlying (bio)chemical transformation mechanisms. For transformation of TCE

with different pure corrinoid cofactors, dual element isotope slopes between 3.7 and 4.5 were recently observed[44], which we may now interpret as indicative of an addition-elimination mechanism.

Previously observed stable isotope fractionation is consistent with the mechanistic dichotomy observed in this study.

Figure 5 shows our data in the context of previously reported dual element isotope trends $\Lambda_{C/Cl}$ in reductive dehalogenation by bacteria[38, 39, 45-50], in enzyme extracts[44], by pure cofactors[38, 44] or model systems[14, 38]. To account for the potential effect of masking, these values of $\Lambda_{C/Cl}$ are plotted against the corresponding carbon isotope enrichment factors ε_C, where pronounced negative ε_C indicate that intrinsic isotope effects are strongly expressed meaning that the influence of masking is small. Vice versa, only slightly negative ε_c values (corresponding to data points Figure 5, region shaded in green) indicate that intrinsic isotope effects were strongly masked meaning that observable $\Lambda_{C/Cl}$ values did not necessarily reflect the intrinsic biochemical reaction. Data points located in this putative masking-dominated domain are derived from microbial degradation of PCE ($\Lambda_{C/Cl}$ values of 0.7 to 2.8 and slightly negative ε_C values of -0.7 ‰ to -5.6 ‰)[44, 45, 50], as well as from the TCE dechlorinating culture KB-1/1,2-DCA (ε_C = -5.4 ±1.5 ‰, $\Lambda_{KB\text{-}1/1,2\text{-}DCA}$ = 4.5 ±0.8) of this study. These smaller dual element isotope slopes potentially do not reflect the chemical bond conversion but rather a preceding step (e.g., mass transfer into the cell, substrate-enzyme binding, etc.)[44] and are, therefore, not discussed further here.

Pronounced negative ε_C, together with moderate $\Lambda_{C/Cl}$ values (Figure 5, region shaded in yellow) are indicative of the addition-elimination mechanism[14] brought forward in this study. Indeed, microbial data in this domain[38, 39, 45-48] originate almost exclusively from dechlorination of PCE and TCE, including this study's data with cultures adapted to TCE ($\Lambda_{Donna\ II}$ = 2.3 ±0.1, $\Lambda_{KB\text{-}1\ RF}$ = 2.7 ±0.2, $\Lambda_{\textit{G. lovleyi}\ KB\text{-}1}$ = 3.1 ±0.1). Similar trends were observed in transformation of TCE with enzymatic extracts[44] and purified cofactors[38, 44] where all values fell in a rather narrow experimental range ($\Lambda_{C/Cl}$ = 3.7 - 5.3) indicating that the

predominance of an addition-elimination mechanism can be traced down to the enzyme level[14]. An exception is a former *cis*-DCE degradation study ($\Lambda_{C/Cl}$ = 4.5). The nature of this degradation with field sediment rather than bacterial cultures was, however, little constrained so that general conclusions are difficult[48].

In contrast, data points corresponding to pronounced negative ε_C, together with large $\Lambda_{C/Cl}$ values (Figure 5, region shaded in grey) are indicative of the addition-protonation mechanism[14]. Indeed, all data are derived from either *cis*-DCE dechlorination (this and previous[48, 49] studies), or from TCE reductive dechlorination by cultures precultivated on less chlorinated ethenes (this study). Taken together, the regions of Figure 5 confirm that also all dual element isotope trends reported so far are consistent with the mechanistic dichotomy observed in this study.

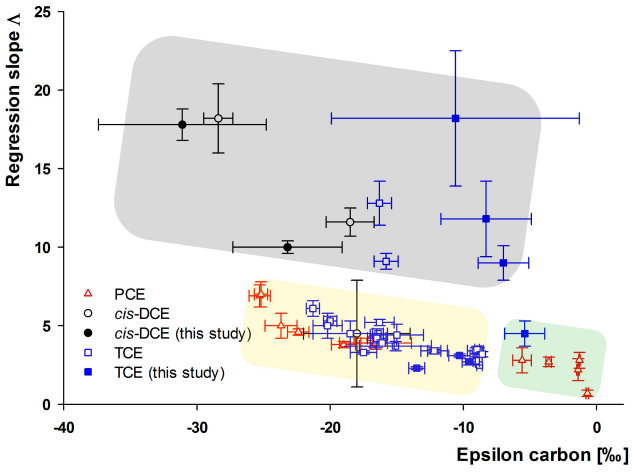


Figure 5. Carbon isotope fractionation factors ε_C and dual element isotope regression slopes $\Lambda_{C/Cl}$ in reductive chlorinated ethene dehalogenation by bacteria[38, 39, 45-50], in enzyme extracts[44], by pure cofactors[38, 44] or model systems[14, 38] observed in this study (filled symbols) and reported from previous studies (empty symbols). Reductive dechlorination of PCE is depicted by red triangles, of TCE by blue squares and of *cis*-DCE by black circles. (Error bars show 95 % confidence intervals of respective values). Shaded areas illustrate regions which indicate that intrinsic isotope effects are masked (green), that they follow an addition-elimination mechanism (yellow) or an addition-protonation mechanism (grey).

Environmental Significance

Available dual element isotope data reveal a surprising dichotomy in reductive dechlorination chemistry of microbial communities. These results suggest that for dehalogenation of chlorinated ethenes catalyzed by RDases two different reductive dechlorination mechanisms exist, which are mimicked by the addition-elimination vs. addition-protonation pathways identified in a recent Vitamin B_{12} study[14]. The evidence that reductive dehalogenases may be optimized to catalyze fundamentally different mechanisms, despite an identical net reaction (hydrogenolysis), offer an explanation why some RDases can be specialized in the dechlorination of PCE and TCE but cannot dechlorinate *cis*-DCE or VC. These results, therefore, hold promise to potentially resolve a fundamental challenge to our understanding of reductive dechlorination that has been a long-standing barrier to successful bioremediation in the field – why dechlorination of chlorinated ethenes often stops at *cis*-DCE or VC. A new RDase classification system based on catalyzed mechanisms may, therefore, represent a transformative advance to the field in the future. Finally, this study highlights the potential of dual element compound-specific stable isotope analysis as an enabling technology to overcome a long-standing dilemma of organic (bio)chemistry: to bridge the gap between *in vitro* and *in vivo*; to probe for reaction mechanisms in organisms; and to directly observe a change of the involved RDases by detecting underlying dechlorination mechanisms at contaminated sites.

Associated Content

Supporting Information

Concentration measurements via GC-FID, Figure depicting concentration vs. time for TCE reductive dehalogenation, Figure depicting dual element isotope plots for TCE dechlorination with regard to the predominant RDases, stable isotope analysis of carbon and chlorine via GC-IRMS, Tables with detailed information regarding qPCR, Table with data of previous studies used for Figure 5.

The Supporting Information is available free of charge.

Acknowledgements

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