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Multiple Dual C−Cl Isotope Patterns Associated with Reductive Dechlorination of Tetrachloroethene

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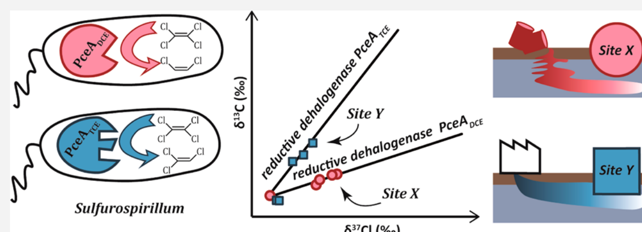
ABSTRACT: Dual isotope slopes are increasingly used to identify transformation pathways of contaminants. We investigated if reductive dechlorination of tetrachloroethene (PCE) by consortia containing bacteria with different reductive dehalogenases (*rdhA*) genes can lead to variable dual C−Cl isotope slopes and if different slopes also occur in the field. Two bacterial enrichments harboring *Sulfurospirillum* spp. but different *rdhA* genes yielded two distinct δ^{13}C to δ^{37}Cl slopes of 2.7 ± 0.3 and 0.7 ± 0.2 despite a high similarity in gene sequences. This suggests that PCE reductive dechlorination could be catalyzed according to at least two distinct reaction mechanisms or that rate-limiting steps might vary. At two field sites, two distinct dual isotope slopes of 0.7 ± 0.3 and 3.5 ± 1.6 were obtained, each of which fits one of the laboratory slopes within the range of uncertainty. This study hence provides additional insight into multiple reaction mechanisms underlying PCE reductive dechlorination. It also demonstrates that caution is necessary if a dual isotope approach is used to differentiate between transformation pathways of chlorinated ethenes.



INTRODUCTION

Chlorinated ethenes are widespread persistent groundwater contaminants. Their occurrence in groundwater results from their common industrial use as solvents.[1] Accidental spills and careless disposal led to a high number of contaminated sites.

To comply with regulations and ensure human safety, risk assessment, if not remediation, must be carried out. Among all cleanup strategies, approaches involving bacteria-mediated contaminant degradation, also denoted as bioremediation, are particularly attractive as they are cost-effective and have a low environmental impact.[2] A major drawback of this technique lies in the often incomplete dechlorination of the commonly encountered tetrachloroethene (PCE) or trichloroethene (TCE) due to the slow bioremediation of its toxic metabolites *cis*-dichloroethene (*c*DCE) or vinyl chloride (VC), resulting in an accumulation of these compounds.

Understanding the mechanisms underlying dechlorination of chlorinated ethenes is a current scientific challenge. Enzymes belonging to the reductive dehalogenase (RdhA) family which are present in virtually all chlorinated ethene-degrading anaerobic bacteria are known to contain a corrinoid and two iron−sulfur centers as cofactors.[3] For PCE, three possible reaction mechanisms (Figure 1) involving the active corrinoid cofactor have been suggested.[4−7] The first one involves the formation of a trichlorovinyl radical as a first and rate-limiting step (scenario C). The other two consist of a first nucleophilic attack of the cobalamin's cobalt center (CoL) on a carbon (C) atom. This step can then either lead to the intermediate 1,1,2,2-tetrachloroethyl complex further undergoing the elimination of a chlorine (Cl) substituent from the Co center (addition−elimination type mechanism, scenario B), or to the substitution of a Cl atom by the Co center followed by the elimination of the Co ligand and addition of a proton to yield TCE (S_N2-like mechanism, scenario A). However, there is no evidence so far to what extent each of these mechanisms are actually involved in the dechlorination process of PCE.[4]

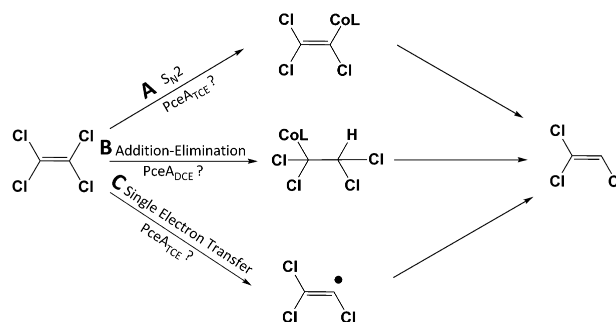


Figure 1. Proposed mechanisms for reductive dechlorination of PCE. See text for details.

Recent studies revealed the potential use of a C−Cl dual isotope approach to gain mechanistic insight in the dechlorination reaction of chlorinated ethenes.[8,9] As chemical bond breakage or formation controls the extent of isotope effects, this approach represents a valuable tool to differentiate the rate-limiting step in reaction mechanisms. In earlier studies, the sole use of C isotope data showed limitations as the magnitude of isotope fractionation not only reflects the actual reactive step but may be influenced by rate-limiting preceding steps.[10] In particular for PCE, C isotope enrichment factors have been shown to vary consequently (i.e., from 0.4 to −16.7‰[10,11]). Isotope enrichment factor corresponds to the change of the isotope ratio per unit of transformation. Conversely, the dual isotope approach is believed not to be affected by masking effects and is thus a promising tool to explore the mechanisms involved during chlorinated ethene reductive dechlorination.[9,12] Abe et al. demonstrated that C and Cl isotope ratios ($\delta^{13}C$ and $\delta^{37}Cl$) of *c*DCE and VC showed a linear correlation with different dual isotope slopes $m \approx \varepsilon_C/\varepsilon_{Cl}$ (ratio between C and Cl enrichment factors) for reductive dechlorination and aerobic oxidation, suggesting the slope to be characteristic of the reaction mechanism.[8] For PCE, the C−Cl dual isotope approach was recently investigated by Wiegert et al. at the field and laboratory scales.[13,14] At the field site undergoing PCE reductive dechlorination, two different isotope patterns occurred along two different groundwater flow paths corresponding to dual isotope slopes of 2.2 ± 2.0 and 0.9 ± 0.7 (95% confidence interval).[13] On the other hand, a unique dual isotope slope of 2.5 ± 0.8 was determined for a bacterial consortium dominated by a bacterium closely related to *Desulfitobacterium aromaticivorans* strain UKTL (mentioned as *Desulfitobacterium*-containing consortium in the present study).[14] According to the previous mechanistic assumptions, the two field slopes could be attributed to different reductive dechlorination mechanisms. However, in addition to dechlorination, isotopes can be affected by various processes in the field such as diffusion[15] and thus the isotopic data might not only reflect reactive processes. To evaluate whether different reaction mechanisms are associated with different dual isotope slopes, additional laboratory studies are necessary that yield well-constrained dual isotope slopes. In addition to providing insight into reaction mechanisms, such studies are also required to establish reference data sets for dual isotope approaches in order to differentiate between biotic (e.g., bacterial dechlorination) and abiotic (e.g., in situ chemical oxidation) processes. As RdhAs are controlling the biotic dechlorination mechanisms, the comparison of dual isotope slopes generated by bacterial populations containing different active RdhAs might bring answers to these questions.

The main objective of this study was to evaluate if (i) consortia with bacteria expressing different RdhA enzymes are associated with different dual C−Cl isotope slopes for reductive dechlorination of PCE and (ii) corresponding differences in slopes can also be observed at the field scale. For this purpose, we investigated C and Cl isotope fractionation in two bacterial consortia containing members of the *Sulfurospirillum* genus expressing different RdhA enzymes that were previously characterized.[16] In addition, we analyzed C and Cl isotope ratios of PCE in samples from two field sites, at which reductive dechlorination was expected to occur based on redox conditions.

■ MATERIALS AND METHODS

Sites Description. C and Cl isotope ratios of PCE were determined for two sites. At site X, PCE was formerly used for the processing of slaughterhouse waste. The subsurface consists of an impermeable clay silt layer overlain by a 3.5 m thick sandy gravel aquifer which is covered by a 4 m thick silt layer. The first 2.5 m of the aquifer consists of coarse gravel. On the basis of previous campaigns and a site characterization performed by consultants, five wells representing various proportions of PCE and its three dechlorination products TCE, *c*DCE, and VC were chosen in view of covering different stages of dechlorination. The wells are located along the plume central line covering a distance of 800 m (Figure S1, Table S2, Supporting Information (SI)).

At site Y, PCE was used for dry cleaning purposes. The subsurface consists of a Tertiary fine sand (Molasse) base acting as an aquitard overlain by a 2.5−25 m thick layer of unconsolidated Quaternary fluvio-glacial sediments forming the aquifer.[17] The latter includes a layer of sandy gravel (5−25 m) with a high hydraulic conductivity (10^{-3} to 10^{-4} m·s^{-1}) covered by a clay−silty sand sheet rich in organic matter located 2.5−5 m below surface which exhibits a low hydraulic conductivity (10^{-5} to 10^{-6} m·s^{-1}). The water table is located between 2.0 and 2.3 m below surface. On the basis of former sampling campaigns, wells representing various proportions of the PCE and its three dechlorination products TCE, *c*DCE, and VC were chosen in view of covering different stages of dechlorination. Samples were taken in four wells: three multilevel wells (ML13, ML14, ML15) situated on a transect perpendicular to the groundwater flow direction and located 7 m downstream of the source area, and one (P13) located 28 m downstream of the source area (Figure S2, SI). The multilevel installation allowed sampling at different depths in the well ML13. The various sampled depths are summarized in Table S2, SI.

Groundwater Sampling. Sites X and Y were sampled in June and July 2013, respectively. The wells were purged until field parameters (i.e., pH, conductivity, dissolved oxygen (DO)) were stable. Submersible pumps were used for purging and collecting samples except for multilevel wells where foot valve or peristaltic pumps were used. Teflon and PVC tubing were used for collecting samples in sites Y and X, respectively. Samples for concentration analysis were collected in 40-mL glass vials sealed without headspace with PTFE-lined silicone septa and screw caps. Nitric acid (10%) was added onsite to water samples to bring the pH to 2 in order to avoid any biodegradation during storage. Samples were transported in coolers topped with ice until their storage in a cold room at 4 °C. pH, conductivity, and DO were measured onsite (HD40Q probe, HACH LANGE).

Chemicals. The PCE used in the dechlorination experiment was purchased from Acros Organics (extra pure, 99%, Geel, Belgium) while the PCE used as a working standard for $\delta^{13}C$ and $\delta^{37}Cl$ measurements was purchased from Riedel-de-Haën (min. 99.5% purity, Seelze, Germany).

Cultivation of Bacterial Consortia, Sampling and Detection of *rdhA* Genes. Two bacterial consortia (SL2-PCEb and the subculture thereof SL2-PCEc) which harbor members of the *Sulfurospirillum* genus and were recently described for their distinct PCE dechlorination pattern[16] were used for the laboratory scale experiments.

The consortia were cultivated in serum bottles of 1000 mL (VWR international AG, Merck, Dietikon, Switzerland), sealed with butyl rubber stoppers, that contained 900 mL of anaerobic medium that had been adapted from previously described methods.[16] Briefly, the cultivation was performed in the presence of PCE in a phosphate-bicarbonate-buffered medium. Addition of 5 mL of 100 mM PCE dissolved in ethanol yielded a final PCE concentration of 560 μM in the aqueous phase of the culture. Formate and acetate were used at 20 mM and 2 mM final concentration as electron donor and C source, respectively. As inoculum, 20 mL of similarly cultivated culture was added per bottle. The cultures were incubated at 30 °C in the dark and agitated on a rotary shaker at 100 rpm.

During dechlorination of PCE to *c*DCE by SL2-PCEb, 5-mL samples were taken from one of the three replicate cultures at times when the fractions of remaining PCE were 82, 60, 20, and 0%, which were subjected to DNA extraction by using the DNA Tissue Kit according to the manufacturer's instructions (Qiagen). A terminal-restriction fragment length polymorphism (T-RFLP) analysis dedicated to the detection of *Sulfurospirillum*-specific *rdhA* genes was applied as described earlier.[16] Briefly, a fragment of the *rdhA* genes was amplified by PCR from 5 ng of DNA with primers targeting conserved regions of all *Sulfurospirillum*-specific *rdhA* genes. The fluorescently labeled PCR product was digested with *Taq*I enzyme and the obtained terminal restriction fragments (TRFs) analyzed on a capillary sequencer (Applied Biosystems) allowing to distinguish between $pceA_{DCE}$ (284-bp) and $pceA_{TCE}$ (272-bp) genes.

Quantification of Stable Isotope Fractionation. For each consortium, isotope fractionation was characterized in 3–4 replicate assays. Control experiments which were not inoculated were also included. The PCE concentration in the cultures was followed by sampling the headspace for GC-FID analysis. Aqueous samples for isotope and concentration analysis were taken at 6–10 time points per bottle, for PCE remaining fractions ranging from 100 to 5%. A total amount of 20 mL was taken at each sampling step and distributed in 2-mL glass vials closed with PTFE-lined screw caps in which 50 μL of NaOH 20 M was added to stop the dechlorination. The vials intended for concentration analysis were filled without headspace, stored at 4 °C, and analyzed within 6 days by GC-MS. The concentrations yielded by these measurements were used to determine the enrichment factors. The vials for isotope analysis were frozen upside down with a small headspace to allow for expansion during freezing.[18]

The enrichment factor ε_E of each replicate was determined according to the following form of the Rayleigh equation:

$$\ln \frac{\delta_t + 1000}{\delta_0 + 1000} = \varepsilon_E \cdot \ln f \quad (1)$$

where f is the PCE remaining fraction at time t, E is the considered element, and δ_t and δ_0 are the isotope ratios of one element at time t and time 0 reported against international standards (Vienna Pee Dee Belemnite, VPDB, or Standard Mean Ocean Chloride, SMOC, for C and Cl, respectively) according to the following expression:

$$\delta = \left(\frac{R}{R_{std}} - 1\right) \cdot 1000[‰] \quad (2)$$

where R and R_{std} are the isotope ratios of the sample and the standard, respectively.

The enrichment factors and dual isotope slopes were calculated by combining $\delta^{13}C$ and $\delta^{37}Cl$ data from all replicates and results were given with a 95% confidence interval.

Analytical Methods. Concentration analysis of the aqueous samples was performed using a Thermo-Finnigan Trace GC Ultra gas chromatograph coupled to a Thermo-Finnigan DSQ II quadrupole mass spectrometry (GC-qMS). Headspace injections of 500 μL from 10 mL were carried out using a CombiPal Autosampler (CTC Analytics, Zwingen, Switzerland), and concentration values were corrected in order to take the equilibrium between the gas and the aqueous phase into account.

C isotope ratios were determined using an Agilent 7890a gas chromatograph (GC) coupled to an Isoprime 100 isotope ratio mass spectrometer (IRMS) via an Isoprime GC5 combustion interface and a purge-and-trap (P&T) system (Stratum, Teledyne Tekmar). Before analysis, aqueous samples were diluted in 40-mL glas vials with a PTFE-lined screw cap to reach a final PCE concentration of 30 $\mu g \cdot L^{-1}$. Twenty-five mL of the diluted samples were purged with N_2 gas (40 $mL \cdot min^{-1}$) and the degassed compounds were retained on a Vocarb 3000 trap (VICI). After the purging step (10 min), the compounds were transferred into a cryogenic trap (Tekmar Dohrmann) connected to the GC column (DB-VRX, 60 m, 0.25 mm, 1.4 μm). This step was followed by a rapid temperature increase (from −100 to 180 °C, using a ramp of 15 $°C \cdot s^{-1}$) which released the concentrated compounds to the column. Helium was used as a gas carrier (1.2 $mL \cdot min^{-1}$). Samples were measured in duplicate unless their concentration was too low and enabled only one measurement. Standard deviation of the in-house reference material was 0.3‰ (n = 47). In each sequence, samples containing reference compounds with known isotope ratios (EA-IRMS measurement) were included to check the accuracy of the method.

Cl isotope ratios were determined using the method based on gas chromatography quadrupole mass spectrometry (GC-qMS) previously described.[19,20] An Agilent 78901 GC coupled to an Agilent 5975C quadrupole mass selective detector (Santa Clara, CA, USA) was used for the analysis. A recent interlaboratory study showed that the use of two standards improved the accuracy of $\delta^{37}Cl$.[21] Thus, a calibration with two external PCE standards ($\delta^{37}Cl_{EIL1}$ = 0.3‰ and $\delta^{37}Cl_{EIL2}$ = −2.5‰) which were formerly characterized by the Holt method[22] at the University of Waterloo was completed for each sequence to obtain δ values on the SMOC scale. A working PCE standard was measured after every ten samples to check the measurement stability. To reach a high precision and in order to be largely above the quantification limit of 30 $\mu g \cdot L^{-1}$, samples were diluted to a constant concentration of 100 $\mu g \cdot L^{-1}$ and analyzed 5–10 times by headspace injection using a CombiPal Autosampler (CTC Analytics, Zwingen, Switzerland). Only two samples from the field showing concentrations between 80 and 90 $\mu g \cdot L^{-1}$ were measured without dilution. A DB-5 column (30 m, 0.25 mm, 0.25 μm, Agilent) with a constant helium flow of 1.2 $mL \cdot min^{-1}$ was used to perform chromatographic separation. As chlorinated ethenes were the only contaminants present in the sites and the retention times for PCE, TCE, *c*DCE, and VC being sufficiently different, the PCE peak could be resolved without any problem (SI Figure S3). In samples from the control experiment that were stored similarly as samples from the active assays, isotope ratios remained within the range of uncertainty of the measurement.

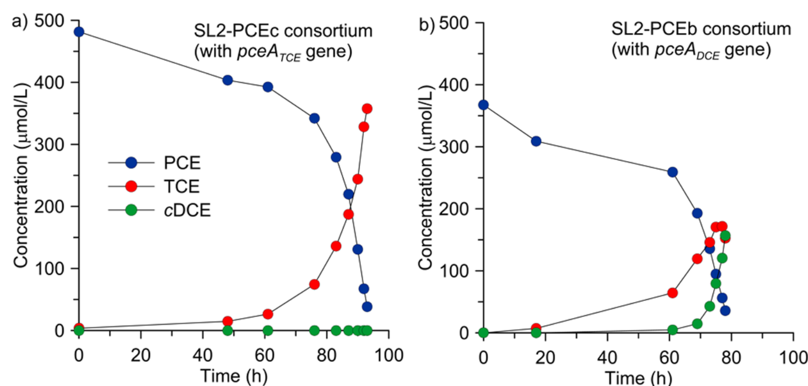


Figure 2. Concentration evolution of PCE, TCE, and *c*DCE for a representative replicate of consortium SL2-PCEc (a) and SL2-PCEb (b).

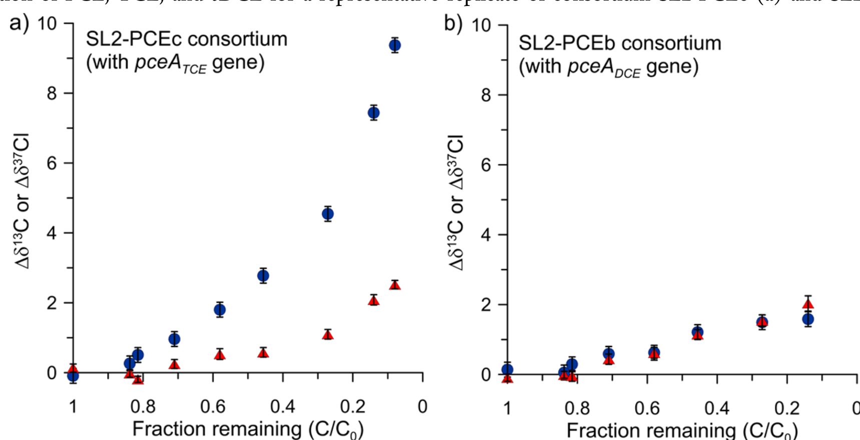


Figure 3. Change in C (blue circle) and Cl (red triangle) isotope ratio as a function of reaction progress for a representative replicate of consortium SL2-PCEc (a) and SL2-PCEb (b). Error bars correspond to the standard uncertainty.

Calculation of AKIEs and Theoretical Dual Isotope Slopes for Radical Mechanism. To gain more insight into the mechanism underlying PCE reductive dechlorination, C and Cl kinetic isotope effects (KIEs) associated with the C and Cl involved in the reaction can be compared. Enrichment factors were transformed to apparent kinetic isotope effects (AKIEs) to take into account the effect of nonreacting positions and reactive positions which are in intramolecular competition.[8,12,23,24] The following was applied to obtain AKIE values:

$$\text{AKIE}_\text{E} = \frac{1}{1 + z\frac{n}{x}\cdot\varepsilon} \tag{3}$$

where E is the considered element (C or Cl), n is the number of atoms of the considered element in the molecule, x is the number of these atoms located at reactive sites, and z is the number of atoms located at the reactive sites and being in intramolecular competition. Thus, for scenarios C (radical mechanism) and A (S_N2), $n = 2$, $x = 2$, $z = 2$, and $n = 4$, $x = 4$, $z = 4$, were applied for C and Cl, respectively, as in both cases, all C and Cl atoms are located at reactive sites and are in intramolecular competition.

For scenario B (nucleophilic addition), bond strengths to both carbons are altered during the initial step. Therefore, an average AKIE_C for the two positions was calculated although the AKIE likely varies between the positions ($n = 2$, $x = 2$, $z = 1$). The calculated AKIE_Cl in this scenario corresponds to a secondary isotope effect as Cl atoms are not involved in any bond formation or breakage during the rate-limiting step ($n = 4$, $x = 4$, $z = 1$).

RESULTS AND DISCUSSION

***Sulfurospirillum* Consortia and *rdhA* Genes Detection in Laboratory Experiment.** The consortium SL2-PCEb catalyzes the reduction of PCE to *c*DCE and contains at least two different *Sulfurospirillum* populations, each of them displaying one distinct PceA enzyme (PceA_DCE and PceA_TCE). In the present study, this consortium was completely dominated by the *Sulfurospirillum* population harboring PceA_DCE. This situation was demonstrated by T-RFLP analysis of SL2-PCEb targeting the *rdhA* genes of *Sulfurospirillum* showing that the $pceA_{DCE}$ gene amounted to ≥99.5% of the *rdhA* genes (SI Table S1). The consortium SL2-PCEc on the other hand, has been obtained from SL2-PCEb after selecting the *Sulfurospirillum* population that is dechlorinating PCE only until TCE.[16] This population displays only one PceA enzyme, namely PceA_TCE.[16]

Concentration and Isotope Ratios of Laboratory Experiments. For both consortia, a similar reaction kinetics was observed for PCE which was consumed within about 95h (SL2-PCEc) and 80h (SL2-PCEb) (Figure 2). As expected, SL2-PCEc produced TCE only, while in the SL2-PCEb bottles both TCE and *c*DCE were observed. The two consortia showed distinctly different isotope patterns as a function of reaction progress (Figure 3). For SL2-PCEc, changes in isotope ratios were about three times larger for C (maximum 9.5‰ for all replicates) than for Cl (maximum 3.6‰). In contrast, SL2-

Table 1. Summary of Enrichment Factors and Dual Isotope Slopes for Reductive Dechlorination of PCE in Various Laboratory and Field Studies

studied system (genus, enzyme)	substrate to final product	enrichment factor ε (‰)	dual isotope slope $\Delta\delta^{13}C/\Delta\delta^{37}Cl$	ref
consortium SL2-PCEc (*Sulfurospirillum*, $PceA_{TCE}$)	PCE to TCE	$\varepsilon_C = -3.6 \pm 0.2^a$	2.7 ± 0.3^a ($R^2 = 0.94$; $n = 23$)	this study
		$\varepsilon_{Cl} = -1.2 \pm 0.1^a$		
consortium SL2-PCEb (*Sulfurospirillum*, $PceA_{DCE}$)	PCE to *c*DCE	$\varepsilon_C = -0.7 \pm 0.1^a$	0.7 ± 0.2^a ($R^2 = 0.74$; $n = 29$)	this study
		$\varepsilon_{Cl} = -0.9 \pm 0.1^a$		
consortium (*Desulfitobacterium*)	PCE to *c*DCE	$\varepsilon_C = -5.6 \pm 0.7^a$	2.5 ± 0.8^a ($R^2 = 0.88$; $n = 10$)	Wiegert et al., 2013[14]
		$\varepsilon_{Cl} = -2.0 \pm 0.5^a$		
field site X	PCE to VC	NA	0.7 ± 0.3^a ($R^2 = 0.95$; $n = 5$)	this study
field site Y	PCE to VC	NA	3.5 ± 1.6^a ($R^2 = 0.94$; $n = 5$)	this study

[a]95% Confidence interval. NA: not available.

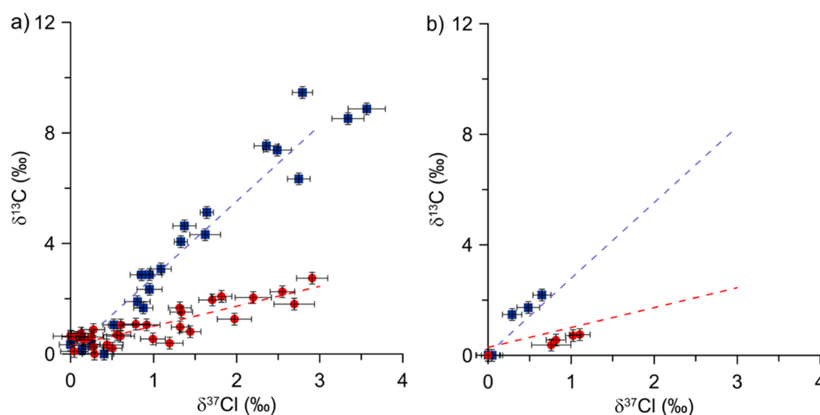


Figure 4. (a) Dual isotope plot for reductive dechlorination of PCE by consortium SL2-PCEc (blue squares) and SL2-PCEb (red circles). (b) Dual isotope plot for reductive dechlorination of PCE at field sites Y (blue squares) and X (red circles). The dashed lines represent the dual isotope slope from the laboratory experiment. Error bars correspond to the standard uncertainty.

Table 2. Apparent Kinetic Isotope Effects (AKIE) Calculated with Simplifying Assumptions[a]

studied system (genus, enzyme)	scenario	isotope	factors n/x/z	AKIE	$((A)KIE_C - 1)/((A)KIE_{Cl} - 1)$	ref
consortium SL2-PCEc (*Sulfurospirillum*, $PceA_{TCE}$)	A/C	C	2/2/2	1.007	1.39	this study
		Cl	4/4/4	1.005		
	B	C	2/2/1	1.004	2.78	
		Cl	4/4/1	1.001		
consortium SL2-PCEb (*Sulfurospirillum*, $PceA_{DCE}$)	A/C	C	2/2/2	1.001	0.39	this study
		Cl	4/4/4	1.004		
	B	C	2/2/1	1.001	0.78	
		Cl	4/4/1	1.001		
consortium (*Desulfitobacterium*)	A/C	C	2/2/2	1.011	1.40	Wiegert et al., 2013[14]
		Cl	4/4/4	1.008		
	B	C	2/2/1	1.006	2.81	
		Cl	4/4/4	1.002		
Streitwieser limit for C−Cl bond cleavage		C		1.057	4.38	
		Cl		1.013		

[a]For scenario A/C it was assumed that only a primary isotope effect during C−Cl bond cleavage occurred. For scenario B, an average AKIE over all positions was calculated. See text for more explanations.

PCEb showed a similar shift for C (maximum 2.7‰) and Cl (maximum 2.9‰). The C isotope enrichment factor for PCE was −3.6 ± 0.2‰ for the consortium SL2-PCEc harboring the enzyme $PceA_{TCE}$ and −0.7 ± 0.1‰ for the consortium SL2-PCEb dominated by $PceA_{DCE}$ (Table 1). The values are within the range of those obtained by previous authors (i.e., from −0.4‰ to −16.7‰[10,11]) and are significantly different from each other. The Cl isotope enrichment factors were −1.2 ± 0.1‰ for SL2-PCEc and −0.9 ± 0.1‰ for SL2-PCEb, which is also comparable to the value of −2.0 ± 0.5‰ recently reported for a *Desulfitobacterium*-containing consortium studied by Wiegert et al.[14] (Table 1). To date, in addition to the study from Wiegert et al., the only other Cl isotope enrichment factor determined for PCE reductive dechlorination is of −10‰,[25] which is much larger than what was obtained in this study and by Wiegert et al. The variation could be explained by the difference in methods as Numata et al. performed $\delta^{37}Cl$ analysis without separating the chlorinated ethenes.

When applying the dual isotope approach, significantly different (ANCOVA, $p < 0.05$) slopes were obtained with a

value of 2.7 ± 0.3 (95% confidence interval) for SL2-PCEc and 0.7 ± 0.2 (95% confidence interval) SL2-PCEb (Table 1 and Figure 4). The dual isotope slope obtained for SL2-PCEc agrees with the value obtained by Wiegert et al., i.e. 2.5 ± 0.8.[14]

Potential Reaction Mechanisms. To be able to relate isotope fractionation to reaction mechanisms, AKIE values were calculated making some unavoidable simplifying assumptions (Table 2).[12] The calculated values can be considered as limiting values that help to identify additional contributions that do not enter into the calculations and will be discussed accordingly. The obtained AKIE values cannot be directly compared to KIE values for specific reactions as they are often masked by rate-limiting steps.[12] An advantage of a dual isotope effect is, however, that ratios of AKIE-1 values for two elements are calculated which should correspond to ratios of KIE-1 values for reference reactions as rate-limiting effects cancel.[12] Scenario A/C were grouped as they both involve cleavage of a C−Cl bond in an initial step. For scenario A/C, it was assumed that only primary isotope effects occur during cleavage of C−Cl bond. The obtained AKIE-1 ratio for SL2-PCEc (2.78, Table 2) is closer to the KIE-1 ratio for simple C−Cl bond cleavage (4.38) than the ratio of SL2-PCEb (0.78) suggesting that mechanism A/C is more likely for SL2-PCEc. However, the ratio for SL2-PCEc (2.78) remains below the reference ratio (4.38) suggesting that either the primary Cl isotope effect is larger than given by the Streitwieser limit or secondary Cl isotope effects occur. Both explanations are plausible. A recent study has suggested that primary Cl isotope effect as high as 1.028 and secondary isotope effects as high as 1.005 might occur.[26] For SL2-PCEb, scenario B is more likely as C and Cl AKIE in the same range are more plausible than a Cl AKIE four times that of the C. However, the difference in dual isotope slope could also be related to a shift of the rate-limiting step within a reaction sequence rather than two distinct reaction mechanisms.

Correlation between RdhA Protein Sequence and Reaction Mechanism. Furthermore, despite their highly similar protein sequences,[16] SL2-PCEc ($PceA_{TCE}$) and SL2-PCEb ($PceA_{DCE}$) each seem to catalyze PCE reductive dechlorination according to a different mechanism as suggested by the distinct dual isotope slopes. Conversely, SL2-PCEb and the *Desulfitobacterium*-containing consortium[14] seem to catalyze this reaction according to the same reaction mechanism, although the RdhA protein sequence of this consortium is expected to be significantly different from $PceA_{TCE}$ and $PceA_{DCE}$ as RdhA sequences observed so far in *Sulfurospirillum* are different from those observed in *Desulfitobacterium*.[3] In previous studies, different C isotope enrichments within the same genera have been measured.[10,11] However, if only one element is analyzed, it remains unclear to what extent the variability in isotope fractionation is due to rate-limiting step preceding that actual fractionating step or due to different reaction mechanisms. Our study demonstrates for the first time that some of the variability can be explained by different reaction mechanisms occurring even within the same genus. Finally, the results suggest that the reaction mechanism is not related to the level of RdhA sequence identity. This goes in the same direction as what was previously shown regarding similarities between RdhA protein sequences and their substrate specificity (i.e., between different chlorinated compounds): RdhAs showing comparable substrate specificity do not necessarily show high sequence identity and highly similar sequences do not imply equivalent substrate specificity.[3,16,27]

Reductive Dechlorination of PCE at Two Field Sites. At site X (SI Figure S1), PCE concentrations vary from 83 to 1500 $\mu g \cdot L^{-1}$ at site Y (SI Figure S2) from 88 to 321 $\mu g \cdot L^{-1}$ (SI Table S2). At both sites, the presence of the dechlorination products TCE, *c*DCE, and VC is a clear sign of ongoing PCE dechlorination (SI Table S2). There is no correlation between PCE concentrations and isotope ratios as concentrations are likely also influenced by difference in screen length and geological heterogeneity. The dual C−Cl isotope plots obtained from five wells in each site (SI Figures S1 and S2) yielded two significantly different (ANCOVA, $p < 0.05$) dual isotope slopes of 0.7 ± 0.3 (95% confidence interval) and 3.5 ± 1.6 (95% confidence interval) for site X and site Y, respectively (Table 1 and Figure 4). The slope for site X is statistically similar (ANCOVA, $p < 0.05$) to the slope of SL2-PCEb, and the slope of site Y is similar to that of SL2-PCEc. This suggests that PCE is degraded by different mechanisms at the two sites, analogously as in the laboratory study. Similarly as for *c*DCE at another site,[28] a single linear trend, and thus a single mechanism, per site were identified. Conversely, Wiegert et al.[13] identified a wider range of slopes for a single site reaching from 0.8 to 2.2 suggesting that different reaction mechanisms might occur at a given site. Indeed, bioremediation in the field generally occurs in a system which usually involves several bacterial genera[29,30] each harboring various RdhAs possibly involved in different reaction mechanisms.

Although the collection of dual isotope slopes is still limited, the dual isotope approach potentially helps to select an appropriate value (or range of values) for calculating the degree of contaminant transformation. For example, at site Y, a maximal shift in $\delta^{13}C$ of 2.2‰ is obtained (SI Table S2). Using the C isotope enrichment factors of SL2-PCEc (−3.6‰, Table 1), SL2-PCEb (−0.7‰), and the consortium of Wiegert et al., 2013[14] (−5.6‰), fractions remaining of 0.54, 0.04, and 0.68, respectively, are calculated using the Rayleigh equation. Based on the dual isotope slope of site Y, the second value can be discarded and thus the fraction remaining narrowed to 0.54 to 0.68.

Advances in Understanding the Mechanisms Underlying PCE Reductive Dechlorination and Implications for Environmental Studies. The study provides for the first time evidence that reductive dechlorination of PCE can be associated with different dual isotope slopes likely due to different reaction mechanisms. This has an incidence on the use of dual isotope measurements to distinguish abiotic from biotic dechlorination processes in the field as the dual isotope slope for reductive dechlorination covers an interval rather than a unique value. It is yet to be seen if this interval is significantly different from slopes for other processes such as in situ chemical oxidation. For process quantification using isotope data, an appropriate isotope enrichment factor has to be chosen,[12,31] which is challenging when a compound is degraded by multiple mechanisms. Previous studies on 1,2-DCA have shown that mechanisms with different dual isotope slopes also show distinctly different absolute values of enrichment factors helping to choose appropriate enrichment factors.[32] In the case of PCE, it seems that enrichment factors partition into two groups (Table 1) although additional data is required to confirm this pattern. As shown by the example given above, a dual isotope approach can potentially help to narrow the calculated fraction remaining for a specific site.

Pinpointing two distinct dual isotope slopes associated with RdhAs with similar protein sequence also constitutes an additional step to the understanding of fundamental aspects of RdhAs activity. Our results show that similarities in RdhA protein sequences do not necessarily imply similarities in reductive dehalogenation mechanisms. Thus, enzymes that show less sequence similarities might use the same mechanism while others that share high sequence similarities might catalyze different reaction mechanisms.

Yet, further investigations are required to confidently link field slopes to their relative reaction mechanism. Determining dual isotope slopes obtained for chemical models mimicking the three conjectured dechlorination mechanisms might, for example, allow associating reaction mechanisms to slopes.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Data on relative abundance of *rdhA* genes, schematic descriptions of sites X and Y, tables of PCE concentration, and isotope data for field. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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