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Reductive Dechlorination of TCE by Chemical Model Systems in Comparison to Dehalogenating Bacteria: Insights from Dual Element Isotope Analysis ($^{13}C/^{12}C$, $^{37}Cl/^{35}Cl$)

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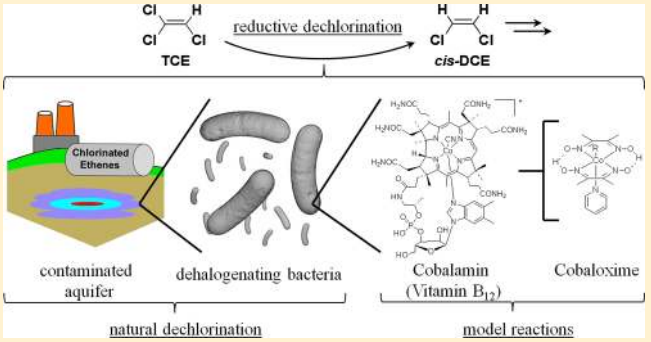
Supporting Information

ABSTRACT: Chloroethenes like trichloroethene (TCE) are prevalent environmental contaminants, which may be degraded through reductive dechlorination. Chemical models such as cobalamine (vitamin B_{12}) and its simplified analogue cobaloxime have served to mimic microbial reductive dechlorination. To test whether in vitro and in vivo mechanisms agree, we combined carbon and chlorine isotope measurements of TCE. Degradation-associated enrichment factors ε_{carbon} and $\varepsilon_{chlorine}$ (i.e., molecular-average isotope effects) were −12.2‰ ± 0.5‰ and −3.6‰ ± 0.1‰ with *Geobacter lovleyi* strain SZ; −9.1‰ ± 0.6‰ and −2.7‰ ± 0.6‰ with *Desulfitobacterium hafniense* Y51; −16.1‰ ± 0.9‰ and −4.0‰ ± 0.2‰ with the enzymatic cofactor cobalamin; −21.3‰ ± 0.5‰ and −3.5‰ ± 0.1‰ with cobaloxime. Dual element isotope slopes $m = \Delta\delta^{13}C/\Delta\delta^{37}Cl \approx \varepsilon_{carbon}/\varepsilon_{chlorine}$ of TCE showed strong agreement between biotransformations (3.4 to 3.8) and cobalamin (3.9), but differed markedly for cobaloxime (6.1). These results (i) suggest a similar biodegradation mechanism despite different microbial strains, (ii) indicate that transformation with isolated cobalamin resembles in vivo transformation and (iii) suggest a different mechanism with cobaloxime. This model reactant should therefore be used with caution. Our results demonstrate the power of two-dimensional isotope analyses to characterize and distinguish between reaction mechanisms in whole cell experiments and in vitro model systems.



INTRODUCTION

Chloroethenes such as trichloroethene (TCE) are commonly used industrial solvents, and are among the most ubiquitous groundwater contaminants.[1] In order to reduce the risk of exposure through groundwater, remediation techniques aim to remove these compounds under anoxic conditions through reductive dechlorination. However, such efforts do not always lead to the desired detoxification. Biotransformation sequentially replaces the chlorine substituents by hydrogen (hydrogenolysis), but only few organisms are capable of complete dechlorination.[2,3] This leads to accumulation of toxic degradation products such as *cis*-dichloroethene (*cis*-DCE) at contaminated sites.[4]

Ultimately, product formation is determined by the underlying reaction chemistry. For chlorinated ethenes, cyanocobalamin (vitamin B_{12}), which is the active cofactor of dehalogenase enzymes (Figure 1), has been investigated in detail.[5,6] Nucleophilic substitution by Co(I), nucleophilic addition of Co(I),[7] and single electron transfer have been brought forward as possible initial mechanisms.[8,9] Investigations used a broad set of approaches including enrichment cultures,[10] pure microorganisms,[11] studies with purified dehalogenases,[12,13] and on the most fundamental level, with chemical model reactants to mimic putative dehalogenation mechanisms.[8,14,15] Among the most prominent model systems for dehalogenase enzymes are cobaloxime and isolated cyanocobalamin. Cobaloxime is a synthetic cobalt complex with a similar but simplified ligand structure compared to

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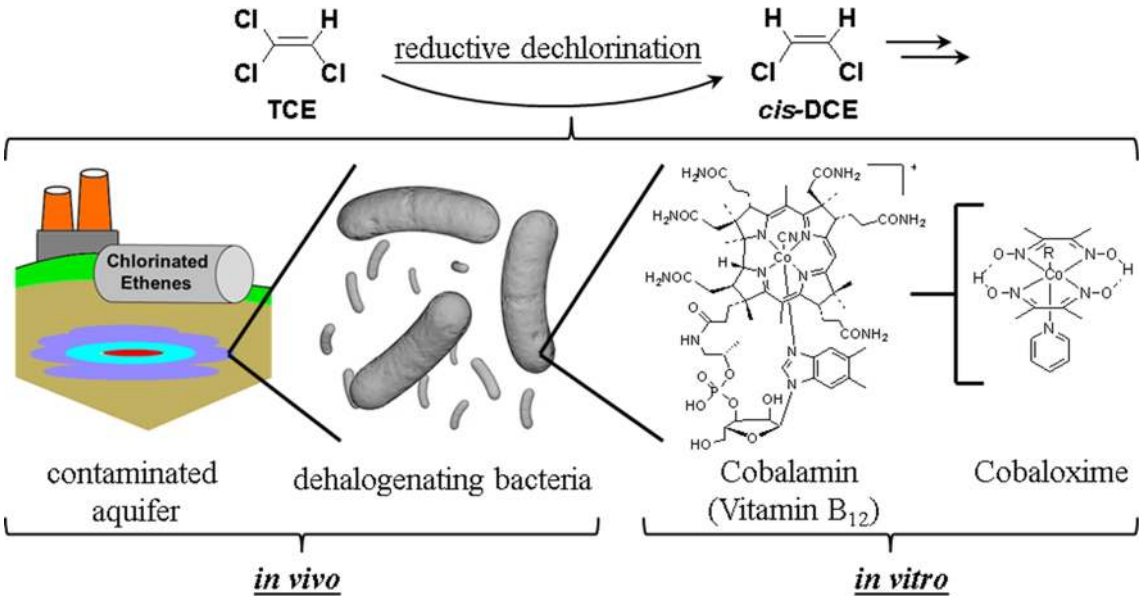

Figure 1. Reductive dechlorination of TCE to *cis*-DCE in different experimental systems, reaching from environmental scenarios (left) to chemical model reactants (right).

cyanocobalamin (Figure 1). Products formed by reaction of TCE in protic solvents are *cis*-DCE as the major product with cobaloxime as the reactant, while cyanocobalamin yields a variety of DCE isomers, vinylchloride and further dechlorination to hydrocarbons. Despite this difference, cobaloxime is a relevant model to mimic biotic systems of dechlorination, since it has been successfully applied to synthesize chlorovinyl-Co complexes,[8] which are proposed as putative intermediates based on mass spectrometric evidence from reaction of cobalamine with TCE.[5] As a result, the properties of Co−C bonding in dichlorovinylcobaloxime complexes could be studied,[50] and a vinyl-cobalt species has been brought forward as a putative reaction intermediate also for cobalamin. However, in order to understand the authenticity of a model for the reaction chemistry in the actual system, a more robust indicator is needed to compare underlying mechanisms of the systems.

The work presented in this study aims to compare the mechanisms of the respective systems through measurements of dual element ($^{13}C/^{12}C$, $^{37}Cl/^{35}Cl$) kinetic isotope effects. Isotope effects serve as a direct indicator for different initial reaction mechanisms, since the magnitude of isotope effects depends on the order and manner (i.e., transition state structure) of chemical bond breakage or bond formation. Position-specific isotope effect studies with labeled substrate have a long tradition in chemistry, but require dedicated synthesis.[16] In comparison, recent developments in gas chromatography coupled to isotope ratio mass spectrometry (GC-IRMS) have made it possible to measure compound-specific isotope effects of environmental contaminants with much greater ease. Measurements can be conducted at natural isotopic abundance meaning that no label is required and the analysis can be conducted on environmental samples. In exchange, isotope effects are not position-specific, but reflect the compound average. On the other hand, compound-specific isotope effects can be measured for multiple elements. When changes in compound-specific isotope values (e.g., $^{13}C/^{12}C$ versus $^{2}H/^{1}H$) are visualized in dual element isotope plots, the given slopes correspond to characteristic combinations of compound-specific isotope effects, which relate to the respective mechanisms.

Such dual element isotope representations have the additional advantage that their slopes are remarkably insensitive toward masking. Masking occurs when observable (apparent) kinetic isotope effects (A)KIE decrease because other steps become rate-determining (e.g., mass transfer, substrate binding to enzymes ("commitment to catalysis").[17,18] While the effect can be dramatic for isotope effects of one element, slopes in dual element isotope plots often remain constant. The reason is that the additional steps often do not show element-specific isotope effects themselves so that KIE of both elements decrease in the same proportion.[19] If these steps do show isotope effects, (A)KIE is the weighted average of them and depends on respective relative rates.[20] Therefore, dual isotope plots have the potential to bridge the gap between model and real life systems, since (i) identical transition states result in the same slope, whereas (ii) different slopes are indicative of different mechanisms, or may reflect a change in kinetics toward other rate-determining steps.[20] Such information has the potential to demonstrate similarities, and to uncover differences, in the (bio)chemical reaction mechanisms in different experimental systems.

Contrasting with the insight obtained for other environmental contaminants,[21−23] such an approach has not been possible for chloroethenes until recently. While routine compound-specific isotope analysis of chlorinated ethenes has been well established for carbon,[24] it had yet to be achieved for chlorine and hydrogen. An analytical breakthrough has been brought about by the latest developments in compound-specific chlorine isotope analysis.[25−27] By now, online measurements of compound-specific chlorine isotope signatures,[27] chlorine isotope fractionation[28,29] and dual isotope plots of carbon and chlorine are within reach.[30,31]

The objective of this study was, therefore, to measure dual element ($^{13}C/^{12}C$, $^{37}Cl/^{35}Cl$) isotope fractionation of TCE in some of the most relevant experimental systems for reductive dehalogenation. We investigated biodegradation with different microbial strains (i) *Geobacter lovleyi* strain SZ and (ii)

Desulfitobacterium hafniense Y51, (iii), transformation by the enzymatic cofactor cobalamin and (iv) by the simplified chemical model system cobaloxime.[15,32] Differences in dual element isotope slopes are discussed with respect to the questions (a) if microbial strains with substantial biological differences employ similar reaction mechanisms for degradation of TCE, and (b) how authentic in vivo transformations are reflected by their corresponding in vitro model systems.

MATERIALS AND METHODS

Chemicals. Cyanocobalamin (Acros), dimethylglyoxime (Alfa Aesar), pyridine (Alfa Aesar), zinc (20−30 mesh; Sigma Aldrich), $TiCl_3$ (15% in 10% HCl; Merck), $Co(OAc)_2{\cdot}4H_2O$ (Alfa Aesar), dimethoxyethane (Alfa Aesar), sodium citrate (Sigma Aldrich) and tris(hydroxymethyl)aminomethane (Sigma) were used as received. Trichloroethene was purchased from Dow, PPG California and Merck.

Biodegradation with *Geobacter lovleyi* Strain SZ. Biodegradation experiments were carried out using the microbial strain *Geobacter lovleyi* strain SZ, purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). This strain reductively dechlorinates TCE to the final product *cis*-DCE. A growth medium was prepared according to DSMZ instructions, medium 732, with the exception that neither hexadecane nor perchloroethene were added to the medium. The growth medium for the experiment was prepared in glass bottles (250 mL), equipped with Mininert valves (Supelco, Bellefonte, PA), and filled with 150 mL of medium, leaving a headspace of 40%. The bottles were amended with 10 μL of neat TCE and constantly shaken on a horizontal shaker at 120 rpm for four days. Inoculation was carried out by adding 14 mL of active culture, which was previously grown in a similar medium. To eliminate carry-over of the degradation product (*cis*-DCE) to the fresh medium, the medium with the culture that was used for inoculation was flushed with N_2/CO_2 gas stream (80/20%) for 5 h prior transferring to the fresh medium. A complete removal of chloroethenes after degassing was controlled by GC-FID measurements. This procedure was followed for three biological replicates in the main degradation experiment ("experiment 2"). Abiotic control batches were prepared similarly, but without inoculation of the active culture. In preliminary experiments, a similar procedure was followed in three biological replicates with the exception that they were conducted in serum bottles closed with Viton stoppers ("experiment 1"). Sampling was carried out 20 min after inoculation for the initial sample, and at given time points along the degradation. For each time point, a total sample volume of 7 mL was taken with a glass syringe (Hamilton), which was distributed in portions of 1 mL each into seven amber vials with an active volume of 1.6 mL. In order to stop biological activity, the vials were spiked with 50 μL of NaOH (1M) and closed with PTFE-lined screw caps. All vials were frozen upside down to preserve them for subsequent isotope analysis, except for one vial, which was used immediately for concentration analysis.

Biodegradation with *Desulfitobacterium hafniense* Strain Y51. In biodegradation experiments with the microbial strain *Desulfitobacterium hafniense* Y51, the substrate TCE was degraded to the final product *cis*-DCE. A growth medium was prepared according to modified DSMZ instructions medium 720. The growth medium for the experiment was prepared in glass serum bottles (560 mL) filled with 500 mL of medium, leaving a headspace of 11%. The bottles were closed with butyl stoppers. The bottles were amended with 25 μL of neat TCE and constantly shaken on a horizontal shaker at 120 rpm for 12 h. Inoculation was carried out by adding 10 mL of active culture, which was previously grown on a similar medium with pyruvate as electron donor and TCE as electron acceptor. To eliminate carry-over of the degradation products (TCE and *cis*-DCE) to the fresh medium, the medium with the culture that was used for inoculation was flushed with N_2/CO_2 gas (80/20%) for 1 h prior to transferring to the fresh medium. Complete removal of chloroethenes after degassing was controlled by GC-MS measurements. Three biological replicates were set up in the degradation experiments. One abiotic control batch was prepared similarly, but without inoculation of any culture. Sampling was carried out before inoculation for the initial concentration and at given time points along the degradation. At each of the 5 samplings during the course of the experiment a total volume of 10.5 mL aqueous sample was withdrawn from the microcosms. For concentration analysis, 500 μL of aqueous sample were taken with a gastight glass syringe (Hamilton), distributed into 10 mL crimp vials (amended with 5 mL Millipore water and 100 μL H_3PO_4) and crimped with aluminum crimp caps with PTFE septum. For isotope analysis a total sample volume of 10 mL was taken with a gastight glass syringe (Hamilton), which was distributed in portions of 1 mL each into ten 1.9 mL amber vials. In order to stop biological activity, the vials were spiked with 50 μL of NaOH (10M) and closed with PTFE-lined screw caps. All vials were frozen upside down until subsequent isotope analysis. Two vials were shipped from Tübingen (where the *Desulfitobacterium hafniense* experiments were conducted) to Helmholtz Zentrum München on dry ice for chlorine isotope analysis (all other experiments were conducted in München).

Abiotic Degradation with Cyanocobalamin. Aqueous stock solution of Ti(III)citrate solutions was prepared from tris(hydroxymethyl)aminomethane (8 g), sodium citrate (16 g) and $TiCl_3$ (25 mL; 10% in HCl) under anoxic conditions in 50 mL of degassed ultrapure H_2O.[33] Addition of Na_2CO_3 followed to adjust to pH 9. Cyanocobalamin (100 μM, 0.027 g) and tris(hydroxymethyl)aminomethane (90 mM, 2.18 g) were weighed into a glass bottle of 250 mL, and transferred into an anoxic chamber. Here aqueous TCE stock solution (190 mL, 0.58 mM) was added. The reaction solution had a pH of 7.6. The bottle was closed with a mininert valve (Supelco) and constantly shaken on a horizontal shaker at 120 rpm for 4 h. To initiate the reaction, 10 mL of the Ti(III)citrate stock solution were added. In total 13 time points were sampled per bottle with sampling volumes of 1 mL that were subsequently diluted 1:10 in oxygenated H_2O. From these solutions, headspace vials were prepared directly for concentration analysis; the remaining volume was frozen for isotope analyses. Two experimental replicates were performed and measured according to this preparation. A similar procedure, but without the addition of cobalamin, was followed as a negative control.

Abiotic Degradation with Cobaloxime. A stock solution of dimethylglyoxime (dmgH; 4.7 g, 81 mM) and pyridine (py, 4.85 mL, 120 mM) was prepared in 500 mL of dimethoxyethane (glyme). In total, ten 100 mL sealed reaction vessels were evacuated, backfilled with N_2 and charged with 25 mL of the dmgH/py stock solution under N_2 flow. $Co(OAc)_2{\cdot}4H_2O$ (0.25 g; 1.0 mmol) and granular Zn (2.0 g; 30.6 mmol) were added using a glass funnel. The vessels were then sealed under N_2 flow, and placed in an oil bath for 60 min at 40 °C in order

to synthesize reduced cobaloxime in situ (40 mM). After reduction, the vessels were cooled in an ice−water bath prior to the addition of neat TCE. In total ten individual reactions were performed with varying molar ratios of TCE relative to cobalt of 10, 6.7, 5.0, 3.0, 2.0, 1.8, 1.5, 1.3, 1.2, and 1.1 respectively in each vessel, which represent time point samples at the respective remaining fractions of TCE. Subsequently, the reaction was heated in a closed vessel at 40 °C in an oil bath for 16 h with constant stirring. A color change to orange indicated the formation of the chlorovinylcobalt complex as reported in Follett et al. 2007.[50] After cooling, the vessel was transferred into an anoxic glovebox. Samples were taken with a glass syringe by filling two amber glass vials with 1.6 mL of liquid without headspace.

From these samples 20 μL were dissolved in 2 mL of aqueous Ti(III)citrate (40 mM) solution present in headspace vials with a total volume of 10 mL. The vials were quickly closed after addition and shaken for 1 h. In total, nine vials were prepared in this way. Two of them served for concentration measurements, the others were frozen for isotope analysis. A similar procedure was followed in the absence of cobalt, as a negative control to exclude any other processes of TCE reduction.

Concentration Measurements. TCE and *cis*-DCE concentrations in the degradation experiments were measured by a gas chromatograph equipped with flame ionization detector (GC-FID, Hewlett-Packard 5890 series II) equipped with a 30 m VOCOL column (Supelco) 0.25 mm inner diameter, with a film thickness of 1.5 μm and operated with nitrogen as carrier gas at 1.6 mL/min. Automated headspace injections of 1 mL from 10 mL headspace vials were carried out using a CombiPal Autosampler (CTC Analytics), and an injector temperature on the GC of 200 °C. For cobaloxime experiments, the temperature program started at 40 °C (14 min) and increased at 60 °C/min to 200 °C (2 min). For cobalamin experiments the temperature program started at 45 °C, increasing at 25 °C/min to 90 °C (5 min) and increasing at 60 °C/min to 180 °C (1 min). For the biodegradation experiments, the temperature program started at 85 °C (0.3 min), increasing at 40 °C/min to 140 °C (2.70 min), and at 40 °C/min to 180 °C (1 min). Calibration curves were obtained using TCE solutions with defined concentrations between 0 and 242 mg/L. Along each sequence of sample measurements, one standard concentration was repeatedly introduced as a quality control. The resulting total relative error in concentrations was estimated as ±10%.

TCE and *cis*-DCE concentrations in experiments with *D. hafniense* were measured by a GC-MS system in SIM mode. An Agilent 7890A GC coupled to an Agilent 5975C quadrupole mass selective detector (Santa Clara, CA) equipped with a 60 m RTX-VMS column (Restek, Bellefonte, PA) 0.25 mm inner diameter, with a film thickness of 1.4 μm and operated with helium as carrier gas at 1 mL/min was used for measurements of TCE and *cis*-DCE. Automated headspace injections of 500 μL from 10 mL headspace vials were performed using an automatic multipurpose sampler (Gerstel, Muelheim an der Ruhr, Germany), and an injector temperature on the GC of 200 °C. The temperature program started at 40 °C (2 min), increased at 25 °C/min to 110 °C (0 min) and further increased at 15 °C/min to 200 °C (5 min). Calibration curves were obtained using TCE solutions with defined concentrations between 0 and 1000 μg/L. Concentrations were corrected for distribution in the microcosm bottle according to Henrys law. Then the number of moles of TCE removed at each sampling per bottle was calculated, cumulatively summed up and added to the Henry-corrected concentration of the respective time point, as described in Fletcher et al. (2011).[34]

Stable Carbon Isotope Analysis. Compound Specific Isotope Analysis (CSIA) for carbon was conducted by injection of headspace samples on a GC-IRMS system (Thermo Fisher Scientific, Waltham, MA) consisting of a Trace GC with a Pal autosampler (CTC Analytics), coupled to a MAT 253 IRMS through a GC/C III combustion interface. This setup was used for experiments with *Geobacter lovleyi,* cobalamin and cobaloxime.

For samples from experiments with *Geobacter lovleyi* and cobaloxime, the gas chromatograph was equipped with a 30 m VOCOL column (Supelco), 0.25 mm inner diameter, with a film thickness of 1.5 μm and operated with He carrier gas at 1.4 mL/min. The GC program started at 85 °C (8 min) and increased at 60 °C/min to 205 °C (1 min).

For cobalamin samples a 60 m DB624 column was used, 0.32 mm inner diameter (Agilent, Santa Clara, CA). The GC program started at 70 °C (2 min), increasing at 30 °C/min to 120 °C (9 min), and increasing at 30 °C/min to 220 °C (0 min). The analytical uncertainty 2σ of carbon isotope measurements was ±0.5‰. An internal standard of TCE was used along the measurements with a carbon isotope signature ($\delta^{13}C$) of −27.1‰ ± 0.2‰. The given delta notation refers to the Vienna Pee Dee Belemnite international standard (VPDB).[35]

Compound-specific carbon isotope analyses in experiments with *D. hafniense* were performed using a different GC/IRMS system, consisting of a Trace GC Ultra Thermo Finnigan, Milan, Italy) coupled to a DeltaPLUS XP (Thermo FinniganMAT, Bremen, Germany) via a combustion interface (GC Combustion III, Thermo Finnigan MAT) operated at 940 °C. Headspace samples were enriched with SPME using a StableFlex-Fiber, covered with 85 μm Carboxen/Polydimethylsiloxan (Supelco). After a sorption time of 20 min at 35 °C the compounds were desorbed for 30 s. A 60 m x 0.32 mm RTX-VMX capillary column (Restek) with a film thickness of 1.8 μm was used. The following temperature program was applied: 4 min at 40 °C, 7 °C/min to 180 °C, held for 3 min, total time 27 min. A laboratory standard of TCE was used along the measurements with a carbon isotope signature ($\delta^{13}C$) of −26.7‰ ± 0.1‰

Stable Chlorine Isotope Analysis. Chlorine isotope analysis of TCE was performed according to a method adapted from Shouakar-Stash et al. (2006).[26] In this new approach for GC/IRMS the TCE is directly transferred in the gas phase to the IRMS through the He carrier stream, where TCE is ionized and fragmented for isotope ratio measurements. The measurements were conducted at masses m/z = 95, 97 on a GC-IRMS system (Thermo Scientific) consisting of a Trace GC that was connected to a MAT 253 IRMS with dual inlet system via a heated transfer line. The gas chromatograph was equipped with a 30 m VOCOL column (Supelco) with 0.25 mm inner diameter, a film thickness of 1.5 μm and operated with a He carrier gas at 1.4 mL/min. The GC program used started at 50 °C (7 min), increasing at 60 °C/min to 70 °C (2.70 min) and at 80 °C/min to 140 °C (0.10 min). External standards were measured daily for calibration of $\delta^{37}Cl$ values according to Bernstein et al.[27]

Briefly, a reference gas of TCE was introduced via a dual inlet system at the end of each measurement. The conversion to

delta values relative to the international reference Standard Mean Ocean Chloride (SMOC) was performed by an external two-point calibration analyzing TCE-standards "Eil-1" and "Eil-2" with a chlorine isotope signature (δ^{37}Cl) of +3.05‰ and −2.7‰, respectively,[27] as previously characterized in the Department of Earth Sciences, University of Waterloo.[26] Each of these standards was added in triplicates before, during and at the end of each sequence, in order to calibrate the obtained values of the samples with respect to SMOC. The analytical uncertainty 2σ of chlorine isotopic measurements was ±0.2‰.

Evaluation of Carbon and Chlorine Isotope Fractionation. Isotope enrichment factors of carbon (ε_C) in TCE were evaluated using Sigma-Plot with curve fittings ($r^2 > 0.96$) according to the Rayleigh equation:

$$\delta^{13}C = \delta^{13}C_0 + [\varepsilon_C \cdot \ln f] \quad (1)$$

where $\delta^{13}C_0$ and $\delta^{13}C$ are carbon isotope values in the beginning and at a given time (t) respectively, and f is the fraction of substrate remaining at time t. Elsner and Hunkeler demonstrated that chlorine isotope fractionation also follows a Rayleigh trend despite the high abundance of ^{37}Cl compared to ^{13}C.[36] Thus chlorine isotope data was treated similarly using the Rayleigh equation:

$$\delta^{37}Cl = \delta^{37}Cl_0 + [\varepsilon_{Cl} \cdot \ln f] \quad (2)$$

where $\delta^{37}Cl_0$ and δ^{37}Cl are chlorine isotope ratios in the beginning and at a given time (t), respectively. An apparent kinetic chlorine isotope effect (AKIE) may be estimated under the assumption of negligible secondary isotope effects from the following equation:[36]

$$AKIE_{Cl} = \frac{1}{1 + (n \cdot \varepsilon_{Cl}/1000)} \quad (3)$$

where n is the number of three chlorine atoms for TCE.

Dual element isotope fractionation can be compared by (a) either considering the ratio of $\varepsilon_C/\varepsilon_{Cl}$ or (b) by plotting isotope values of δ^{13}C vs δ^{37}Cl (as shown in Figure 3). Uncertainties were obtained from 95% confidence intervals (CI).

RESULTS AND DISCUSSION

Isotope Fractionation According to the Rayleigh Equation. An overview of the obtained data is presented in Figure 2. It reflects the results of combined experimental replicates, which were highly consistent for each experimental system (individual results are given in the SI). Pronounced carbon isotope effects of TCE were observed in all experiments. Biodegradation experiments with *Geobacter lovleyi* strain SZ and *Desulfitobacterium hafniense* Y51 showed enrichment factors of ε_{carbon} = −12.2‰ ± 0.5‰ and −9.1‰ ± 0.6‰, respectively. Although the value for *Geobacter lovleyi* is higher than reported by Cichocka et al. (ε_{carbon} = −8.5‰ ± 0.6‰), both values fall in the range of previously reported values for reductive biodegradation of TCE.[37−40] The obtained value for cobalamin reactions of −16.1‰ ± 0.9‰ agrees with previously reported data of Slater et al. (−16.5‰ ± 0.6‰).[41] These data compare to an enrichment factor of the cob(I)aloxime reaction of −21.3‰ ± 0.5‰, to our knowledge the first reported for this reaction.

The obtained enrichment factors from this study represent a total range of apparent kinetic isotope effects (AKIE) in carbon reaching from 1.018 to 1.043. These significant carbon isotope effects indicate that the rate-limiting step of all investigated

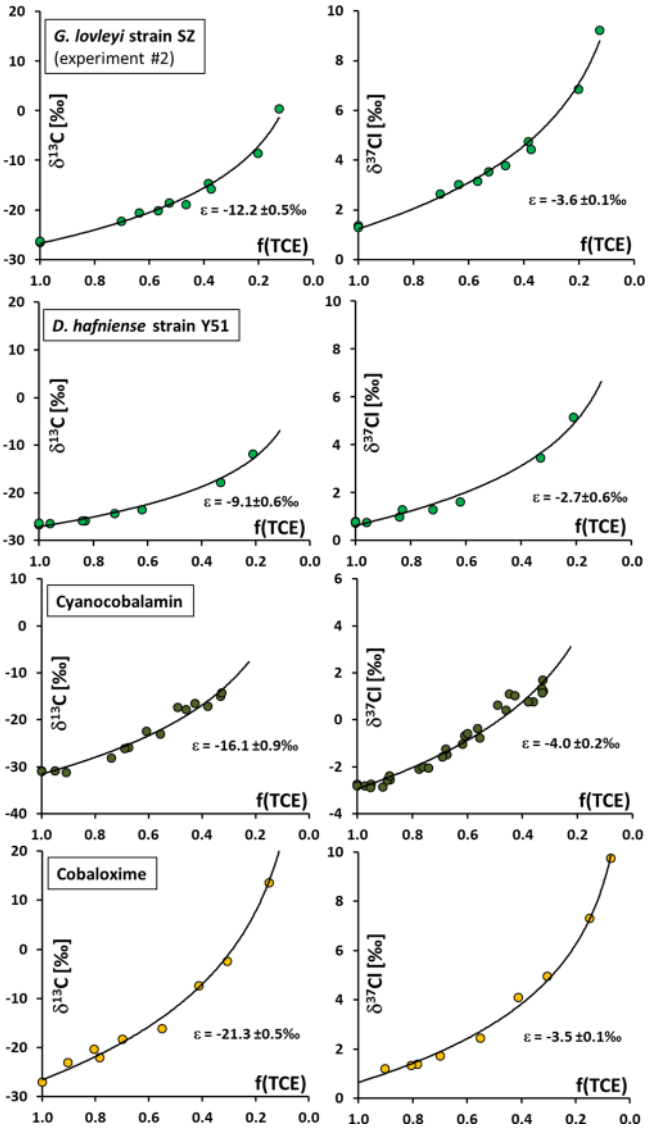

Figure 2. Isotope fractionation patterns in TCE of δ^{13}C (left) and δ^{37}Cl (right) measured during degradation in the different experimental systems. Data points are from triplicate batches per biodegradation experiment, from duplicate cyanocobalamin experiments and from ten sacrificial cobaloxime batches. The fraction of remaining TCE is presented as f(TCE) on the x-axis. Enrichment factors were extracted from curve fittings according to the Rayleigh equation (eq 1 and eq 2). Error Ranges in given isotope data are smaller than the displayed points, and uncertainties for given ε are 95% CI.

reactions involves the carbon atoms in TCE to some extent. However, a statement on the individual pathways remains elusive because masking effects potentially may decrease enrichment trends to an unknown degree. Consequently, the individual enrichment factors of one element may not be representative for the intrinsic isotope effects of the transformation, particularly biotic systems. With these constraints, underlying mechanisms cannot be compared between experimental systems using isotope effects of only one element. However, this obstacle may potentially be overcome by including isotope information from a second element: chlorine.

In Figure 2 we report pronounced chlorine isotope fractionation of TCE in all performed reactions with a range of $\varepsilon_{chlorine}$ = −2.7‰ ± 0.6‰ to −4.0‰ ± 0.2‰. When

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converting these bulk isotope effects of chlorine into apparent kinetic isotope effects under the assumption of negligible secondary isotope effects (eqs 1 and 2), they result in AKIE values between 1.008 and 1.012, and therefore reach to the upper end of Streitwieser limits in C−Cl bonds.[42] This indicates that primary isotope effects are present in the investigated systems and that C−Cl bond cleavage is at least partially rate-limiting.

Dual Isotope Approach. While the individual C and Cl isotope data alone do not allow a conclusive comparison of the given reactions of TCE, a different picture is given in the dual isotope plot in Figure 3. Slopes of biodegradation experiments

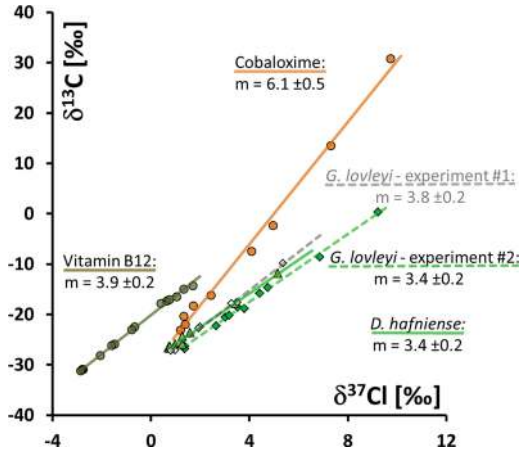

Figure 3. Dual isotope plots of $\delta^{13}C$ versus $\delta^{37}Cl$ during degradation of TCE in the investigated experimental systems. The figure includes data from three biological replicates in each of the biodegradation experiments, two replicates for reactions with cyanocobalamin, and 10 individual reactions with cobaloxime. The displayed points of the data are larger than the respective error ranges.

(3.4 ± 0.2, 3.4 ± 0.2 and 3.8 ± 0.2) and reaction with cobalamin (3.9 ± 0.2) are essentially indistinguishable, within the given 95% CI. In contrast, these results differ significantly from the trend observed with cobaloxime (6.1 ± 0.5). In the following, the implications of this result are discussed for the different experimental systems in decreasing order of similarity towards environmental biodegradation.

Biodegradation Experiments. The study involves biodegradation experiments with two different strains of dehalorespiring bacteria, *Geobacter lovleyi* strain SZ and *Desulfitobacterium hafniense* strain Y51. Both microorganisms feature a metabolic pathway of degrading TCE strictly to *cis*-DCE as the final product. When comparing isotope fractionation during this reaction, a difference in ε_C of 3.1‰ and in ε_{Cl} of 0.9‰ was obtained between the two strains (Figure 2). Previous studies have addressed differences in carbon isotopic enrichment factors of TCE biodegradation in the context of possible variations in enzyme structures or kinetic processes such as transport and enzyme−substrate binding prior to the cleavage of the carbon-chlorine bond. Since such parameters may mask the kinetic isotope effect (KIEs) of a reaction, the observed variations are plausible but an interpretation from enrichment factors of only one element can be problematic.[38,18,37]

In contrast, the good agreement of the slopes in their dual element isotope plots suggests that the δ-proteobacterium *Geobacter lovleyi* strain SZ and *Desulfitobacterium hafniense* Y51 share the same TCE degradation mechanism, despite their biological differences: The two strains belong to different branches of the phylogenetic tree, and have low similarities in the genome sequence coding for the reductive dehalogenases.[43] However, an important common feature is their dependency on cobalamin.[44] The dehalogenases contain a cobalamin reactive center,[45] but the structure of the PceA corrinoid cofactor has not been identified so far.[46] The identical dual element isotope plots suggest that the rate limiting step in the C−Cl cleavage is likely to be similar in the two strains, which emphasizes once more the role of this cofactor in microbial dehalogenation.[7,43] In this context, the value of the presented dual isotope approach becomes evident: While the observable KIEs of one element may decrease dramatically with the influence of masking effects, the ratio of KIEs from two elements remained constant, most likely because KIEs of both elements decreased in the same proportion.

Abiotic Degradation with Cyanocobalamin. The individual carbon and chlorine isotope effects of TCE in the dechlorination mediated by cobalamin are larger than during biodegradation. This observation may potentially indicate different mechanisms. Alternatively, it may indicate that the same mechanism prevailed but that masking simply decreases—and isotope effects increase—with the absence of a cell membrane so that effects became greater in the chemical reaction with cobalamin.[18,47] The comparison of dual isotope plots of cobalamin in vitro to the biotic reactions gave evidence for the second hypothesis. In this case the respective slopes match within their range of errors, suggesting that the transformation with isolated cobalamin occurs in a similar fashion as it does in vivo when it is incorporated in the dehalogenase enzyme.[7,48] This may serve as an important reference in the use of this model system, since it is the first experimental confirmation that directly refers to the rate limiting step of dechlorination.

A remarkable difference between the two systems, however, lies in the product formation: Whereas in biodegradation *cis*-DCE is selectively formed as final and only product, a more diverse set of products is found with cobalamin. Besides the major product *cis*-DCE, also 1,1-DCE; *trans*-DCE and others were observed here and in previous studies under identical conditions.[49,7] Despite these differences, the rate limiting step in the reaction between TCE and the cobalt center appears to be the same in both experimental systems. This indicates that the formation of toxic chlorinated compounds versus harmless hydrocarbons is not yet determined in the initial rate-determining step, but rather in subsequent reactions of short-lived intermediates. Here, another strength of the dual element isotope approach becomes evident compared to product studies: Isotope effects are diagnostic of the initial rate limiting step, allowing it to disentangle this step from subsequent steps of product formation. Based on these findings we propose that a putative initial intermediate formed from cobalamin and TCE has similar properties in vitro compared to the species formed at the active site of the enzyme. For this, different possible, but as yet unresolved, reaction mechanisms have been brought forward in the past.[8] See discussion below.

Abiotic Degradation with Cobaloxime. The performed reactions of cobaloxime with TCE yield selective formation of *cis*-DCE, similar to the presented biodegradation experiments.[32,50] Irrespective of this similarity, the presented isotope data allow a more fundamental distinction of the underlying reaction chemistry.

From the individual isotope effects in Figure 2 a notably high carbon isotope enrichment factor of −21.3‰ ± 0.5‰ was observed, while the fractionation in chlorine stayed in the range of previously discussed systems with $\varepsilon_{Cl} = -3.5‰ \pm 0.2‰$. As mentioned before, a comparison of the reactions based on the individual isotope effects from Figure 2 can be problematic. A different picture arises if isotope effects of both elements are considered together in the dual element isotope plot (Figure 3). Contrasting with the slopes of $\Delta = \varepsilon_C/\varepsilon_{Cl}$ between 3.4 ± 0.2 and 3.9 ± 0.2 observed with cobalamin and the two bacterial strains, a significantly higher slope of 6.1 ± 0.5 was obtained for the degradation of TCE with cobaloxime. This result points out a hitherto unrecognized difference in the rate-determining step of these reactions and indicates that the use of cobaloxime to understand product formation in biotic dechlorination of TCE is likely problematic.

The observed difference in $\varepsilon_C/\varepsilon_{Cl}$ can be pinpointed to the high enrichment factor of carbon, which indicates that the reaction pathway with cobaloxime involves a strong participation of carbon in the rate-limiting step. It is a particular property of the cobaloxime reagent that it forms stable chlorovinylcobaloxime species.[15] For this reaction, direct S_N2-like substitution of a Cl substituent by the nucleophilic Co center has been suggested as possible reaction pathway.[8] For cobalamin, in contrast, previous studies have indicated a different mechanism involving single electron transfer and radical intermediates.[33,51] Potentially, the observed differences in the dual element isotope slopes may reflect these different pathways.

Along the same lines, a comparison of the observed dual element isotope slope for anaerobic TCE biodegradation ($\varepsilon_C/\varepsilon_{Cl}$ = 3.4 to 3.8) with earlier data for anaerobic *cis*-DCE and VC biodegradation ($\varepsilon_C/\varepsilon_{Cl}$ = 11.6)[30] shows a surprising difference in chlorine isotope effects (much smaller in *cis*-DCE and VC). This difference may again be attributable to a different reaction mechanism suggested for *cis*-DCE and VC: a nucleophilic addition mechanism involving formation of a C−Co bond.[7] Our study, therefore gives an exciting glimpse on the potential to distinguish different dehalogenation mechanisms from carbon and chlorine isotope data, provided that these trends can be confirmed in future studies.

■ ENVIRONMENTAL SIGNIFICANCE

An exciting feature of the presented dual isotope approach is the possibility to directly compare transformation mechanisms of environmental scenarios, biotic transformations, and their putative chemical lab scale systems. Our results suggest that different microbial strains, as well as in vitro reactions with cobalamin, all share a common initial reaction step of TCE degradation. For cobalamin, previous studies have brought forward evidence for single electron transfer as the initial mechanism.[33,51] This suggests that the same mechanism may be active in biodegradation. Since these strains are only two among a large variety of dehalogenating micro-organisms, however, different mechanisms in strains with other dehalogenase enzymes cannot be excluded. Neumann et al. hypothesized that the degradation with a purified dehalogenase enzyme involved nucleophilic attack.[6] With dual element isotope analysis there is now a tool available to test such hypotheses and investigate if different microbial strains involve different dechlorination mechanisms, despite using the same cofactor cobalamin. In addition, our approach even allows comparing in vitro model reagents with natural transformations. Such information is not only important for process understanding from a fundamental scientific point of view, it is also essential when using the dual isotope approach to assess the fate of chlorinated compounds in the environment.[3,28]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

An additional figure is available. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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