Chlorinated Ethene Reactivity with Vitamin B12 Is Governed by Cobalamin Chloroethylcarbanions as Crossroads of Competing Pathways

Author(s):
Heckel, Benjamin; McNeill, Kristopher (iD); Elsner, Martin

Chlorinated Ethene Reactivity with Vitamin B_{12} Is Governed by Cobalamin Chloroethylcarbanions as Crossroads of Competing Pathways

Benjamin Heckel,[†,§] Kristopher McNeill,[‡] and Martin Elsner*,[†,§]

[†]Institute of Groundwater Ecology, Helmholtz Zentrum München, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany

[‡]Institute of Biogeochemistry and Pollutant Dynamics (IBP), ETH Zurich, CH-8092 Zurich, Switzerland

[§]Chair of Analytical Chemistry and Water Chemistry, Technical University of Munich, Marchioninistrasse 17, D-81377 Munich, Germany

S Supporting Information

ABSTRACT: Chlorinated ethenes are toxic groundwater contaminants. Although they can be dechlorinated by microorganisms, reductive dehalogenases, and their corrinoid cofactor, biochemical reaction mechanisms remain unsolved. This study uncovers a mechanistic shift revealed by contrasting compound-specific carbon (ε^{13}C) and chlorine (ε^{37}Cl) isotope effects between perchloroethene, PCE (ε^{37}Cl = −4.0‰) and *cis*-dichloroethene, *cis*-DCE (ε^{37}Cl = −1.5‰), and a pH-dependent shift for trichloroethene, TCE (from ε^{37}Cl = −5.2‰ at pH 12 to ε^{37}Cl = −1.2‰ at pH 5). Different pathways are supported also by pH-dependent reaction rates, TCE product distribution, and hydrogen isotope effects. Mass balance deficits revealed reversible and irreversible cobalamin-substrate association, whereas high-resolution mass spectrometry narrowed down possible structures to chloroalkyl and chlorovinyl cobalamin complexes. Combined experimental evidence is inconsistent with initial electron transfer or alkyl or vinyl complexes as shared intermediates of both pathways. In contrast, it supports cobalamin chlorocarbanions as key intermediates from which Cl⁻ elimination produces vinyl complexes (explaining rates and products of TCE at high pH), whereas protonation generates less reactive alkyl complexes (explaining rates and products of TCE at low pH). Multielement isotope effect analysis holds promise to identify these competing mechanisms also in real dehalogenases, microorganisms, and even contaminated aquifers.

KEYWORDS: outer-sphere single-electron transfer, reductive dehalogenation, kinetic isotope effect, groundwater contamination, chlorinated ethenes, trichloroethene, mechanistic study, cobalamin
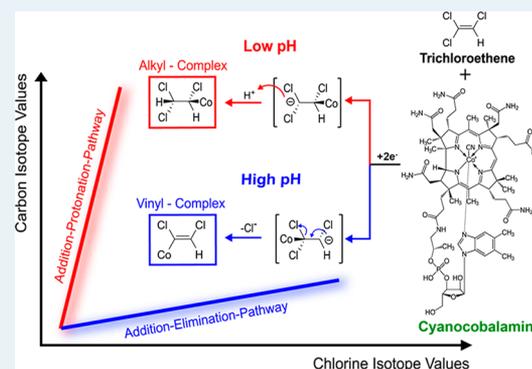
Chlorinated ethenes are important chemical intermediates in degreasing and dry-cleaning agents, and they rank among the most frequent groundwater contaminants.[1] They can enter the subsurface through accidents and improper handling. Because of their high density and low water solubility, they tend to accumulate as pure-phase pools at the confining layer of aquifers leading to long-lasting groundwater contaminations. Highly chlorinated ethenes are rather persistent under oxic conditions but are conducive to biotic reductive dehalogenation in anoxic zones of aquifers.[2] Natural or engineered biodegradation does not always achieve complete dehalogenation, however.[3] Biodegradation involves sequential replacement of chlorine by hydrogen so that perchloroethene (PCE) and trichloroethene (TCE) are transformed to more problematic *cis*-dichloroethene (*cis*-DCE) and vinyl chloride (VC), before harmless ethene is formed. While some microorganisms of the genus *Dehalococcoides* are able to achieve complete dehalogenation, most microorganisms stop at the stage of *cis*-DCE or VC.[4] This raises the question for the underlying (bio)chemical degradation mechanism and why transformation frequently stops at the stage of these more problematic daughter compounds. The key must lie in the reaction chemistry of cobalamin (vitamin B_{12}) because this enzymatic cofactor lies at the heart of practically all reductive dehalogenases (Rdases) identified to date.[5] A possible role of the lower axial ligand at the Co center was highlighted in dechlorination with *Dehalococcoides mccartyi*, where amendment with a 5,6-dimethylbenzimidazole base resulted in complete cis-DCE, whereas amendment with other ligands did not.[6] In contrast, crystal structures of Rdases from *Sulfurospirillum multivorans* and *Nitratireductor pacificus* suggest that this base is not even ligated with the Co center.[7−9] Despite this evidence and much research on putative reaction intermediates of cobalamin and its functional mimics

Scheme 1. Suggested Reaction Mechanisms for Reductive Dehalogenation of TCE: (1) Nucleophilic Addition; (2) Single-Electron Transfer; (3) Nucleophilic Substitution[a]
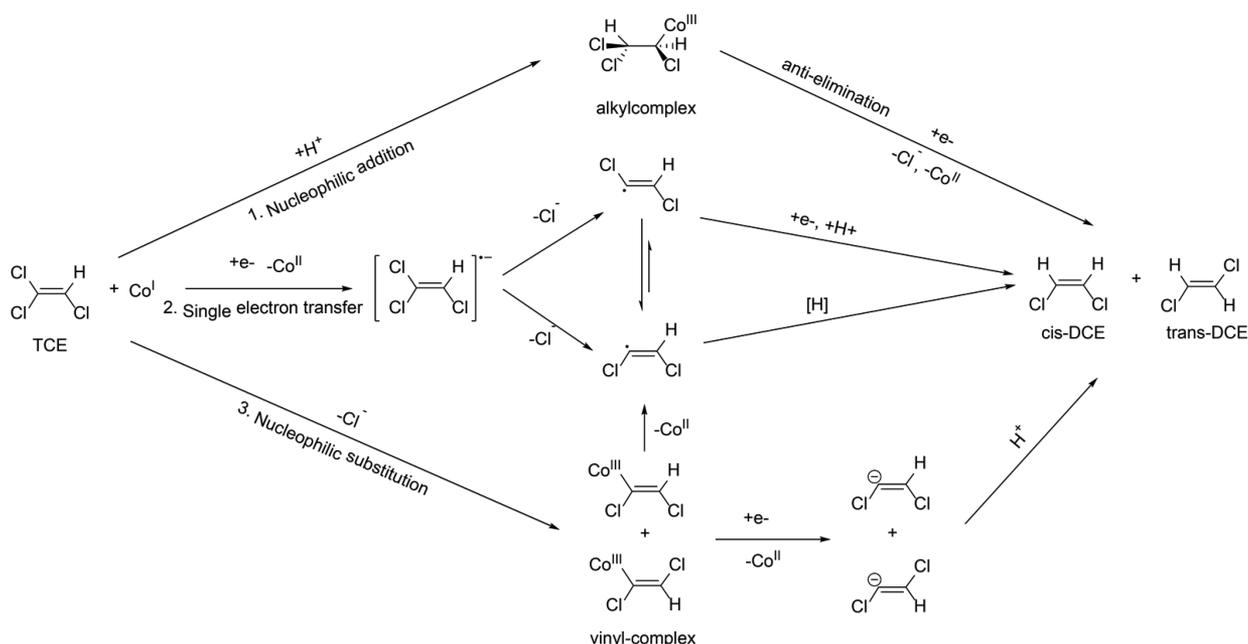
[a]Here, "-e⁻, + H⁺" indicate an electron transfer followed by protonation, whereas "[H]" indicates abstraction of a hydrogen radical.

(cobaloxime, tetraphenylporphyrin cobalt (TPP)Co),[10−17] and despite even the first elucidation of reductive dehalogenase enzyme structures,[7−9] a consistent picture of the underlying reaction biochemistry of chlorinated ethenes has still not been determined. Three different reductive dechlorination mechanisms have been brought forward:[10] 1. (inner-sphere) nucleophilic addition;[18] 2. electron transfer (either inner-[8,19−21] or outer-sphere;[7,9,22,23] 3. (inner-sphere) nucleophilic substitution.[10,24]

The nucleophilic addition and substitution pathways are motivated by the observation of pH-dependent reaction kinetics,[18] by the detection of alkyl and vinyl vitamin B_{12} complexes from (low resolution) mass spectrometry,[22,25] and by the observation that typical product spectra are obtained from synthesis and subsequent reaction of such intermediates.[10,16,22,25,26] Conversely, the hypothesis of single-electron transfer is based on the detection of trichlorovinyl radicals during the reaction of vitamin B_{12} with TCE.[15,22,23,26,27] Furthermore, studies of the reductive dehalogenase PceA from *Sulfurospirillum multivorans*[9,24] favor an outer-sphere single-electron transfer (OS-SET) based on a Co(II)-to-TCE distance of 5.8 Å. EPR evidence for a direct interaction of the halogen atom and Co in reductive dehalogenation of aromatic phenols by the reductive dehalogenase NpRdhA of *Nitratireductor pacificus* supports an inner-sphere SET mechanism;[8] DFT-based computational calculations simulated this mechanistic scenario also for chlorinated ethenes.[21] Conversely, the absence of such EPR observations in reductive dehalogenation of PCE by PceA from *Sulfurospirillum multivorans* was interpreted as evidence for an outer-sphere SET mechanism.[7] An inner-sphere two-electron transfer via a halogenophilic substitution, finally, has been proposed for halogenated aromatic compounds (not shown in Scheme 1).[19]

The difficulty in coming up with a coherent mechanistic picture for even the simplest model of reductive biotransformation—chlorinated ethene reduction by vitamin B_{12}—

presents, therefore, a major obstacle in advancing our understanding of reaction mechanisms in dehalogenases, or even whole organisms. The research need exists for additional experimental evidence to integrate existing observations in a coherent mechanistic picture, to subsequently transfer such insight from model systems to real environmental systems and to demonstrate, thereby, that the same reaction mechanisms prevail in enzymes, or real organisms.

Multiple element compound-specific isotope effect analysis (CSIA) of chlorinated ethenes offers an underexplored opportunity from geochemistry[28] to answer both questions and to close the gap between mechanistic insight from model systems, enzymes and organisms.[29] Isotope ratios of carbon ($^{13}C/^{12}C$), chlorine ($^{37}Cl/^{35}Cl$) and hydrogen ($^{2}H/^{1}H$) can be measured at their natural isotopic abundance (i.e., without isotope label) in samples taken along a (bio) chemical reaction and can be expressed as difference relative to an international reference.

$$\delta^{13}C = \frac{\left(^{13}C/^{12}C_{Sample} - {}^{13}C/^{12}C_{Reference}\right)}{^{13}C/^{12}C_{Reference}} \quad (1)$$

$$\Delta\delta^{13}C = \delta^{13}C - \delta^{13}C_{initial} \quad (2)$$

Here, ($^{13}C/^{12}C_{Reference}$) and sample ($^{13}C/^{12}C_{Sample}$) are the isotope ratios of an international reference and of the sample, and $\delta^{13}C$ and $\delta^{13}C_{initial}$ are isotope values of samples taken at a given time point and at the beginning of the reaction, respectively. Recent analytical advances[30−32] have made it possible to analyze such isotope ratios for chlorine ($^{37}Cl/^{35}Cl$) and hydrogen ($^{2}H/^{1}H$) in chlorinated ethenes; analogous equations apply for these elements. Compound-specific isotope effects are subsequently evaluated for each element according to[33,34]

$$\delta^{13}C = \delta^{13}C_{initial} + [\varepsilon^{13}C \cdot \ln f] \quad (3)$$
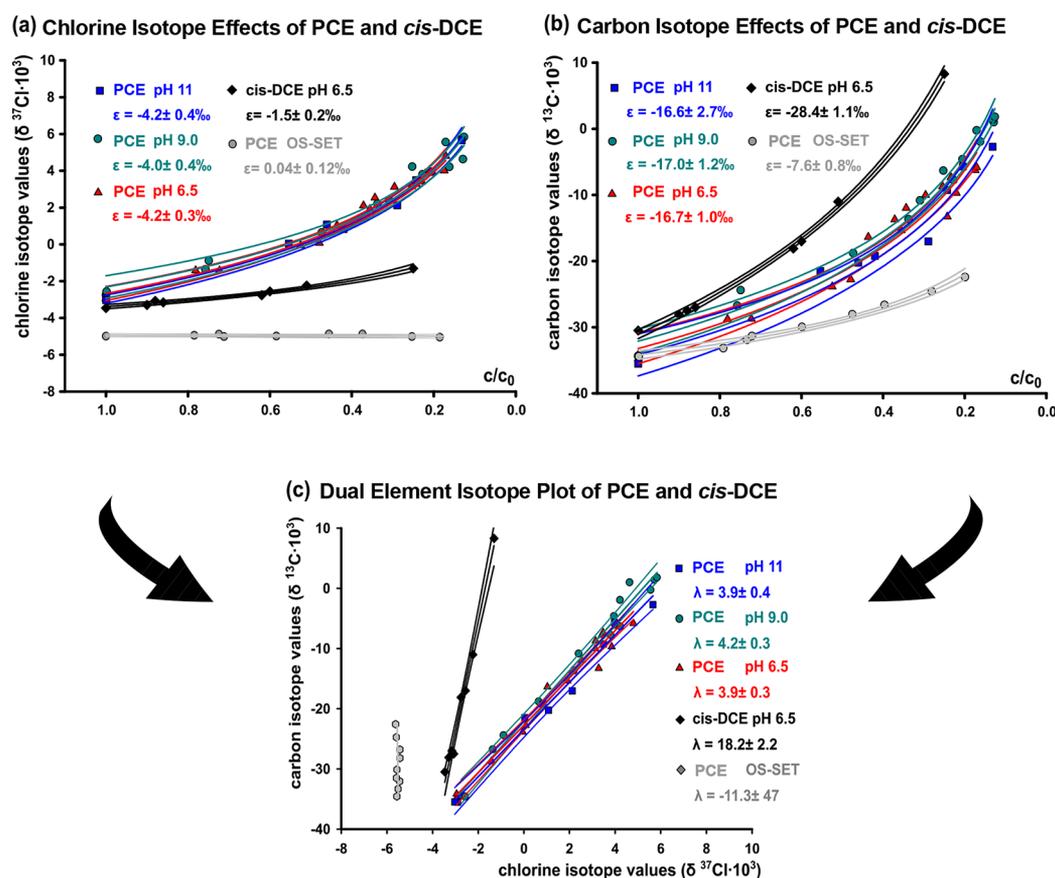
Figure 1. Different carbon vs chlorine isotope effects in reaction of PCE and *cis*-DCE with vitamin B_{12}. Isotopic enrichment factors ε of chlorine (a) and carbon (b) for PCE and *cis*-DCE in reaction with vitamin B_{12} were obtained according to eq 3 by fitting changes in isotope values against the remaining fraction of substrate. (c) The different trends in isotope effects are represented in the dual-element isotope plot which combines carbon and chlorine isotope values from the reaction of vitamin B_{12} with PCE and *cis*-DCE.

where f is the fraction of reactant remaining and $\varepsilon^{13}C$ is the so-called enrichment factor which expresses the difference in transformation rates of light and heavy isotopologues and which is equivalent to a compound-specific kinetic isotope effect $^{12}k/^{13}k$. Specifically, a value, for example, of $\varepsilon^{13}C = -10‰$, means that molecules containing ^{13}C react by 10 per mille—or one percent—slower than those with ^{12}C corresponding to a compound-average kinetic isotope effect of $^{12}k/^{13}k = 1.01$. For chlorine and hydrogen, analogous expressions are valid.[35] When plotting changes in isotope ratios of two elements against each other, a dual-element isotope plot is obtained. Its slope $\lambda = \Delta\delta^{13}C/\Delta\delta^{37}Cl \approx \varepsilon^{13}C/\varepsilon^{37}Cl \approx (^{12}k/^{13}k - 1)/(^{35}k/^{37}k - 1)$ expresses the magnitude of isotope effects of different elements relative to each other and is, therefore, a sensitive tool to distinguish different reaction mechanisms.[36−40] Indeed, a recent computational study brought forward in this journal suggests that dual-element isotope analysis may provide the key evidence necessary to distinguish different mechanisms of chlorinated ethene reduction by cob(I)alamin.[24] However, even though several studies have recently determined compound-specific isotope effects of carbon,[41−44] chlorine,[41−44] and even hydrogen[45] for chlorinated ethenes in reductive dehalogenation by vitamin B_{12}, enzymes, and bacteria,[41−44,46−48] interpretations remain inconclusive: the variability of isotope effects has not been systematically investigated; observed isotope effects have not been combined with "classical" mechanistic probes such as reactivity trends and detection of intermediates. Finally, the

approach has not been pursued to rationalize product formation (e.g., why reactions often stop at the stage of *cis*-DCE).

With the aim to derive a consistent mechanistic picture for the conflicting mechanisms of Scheme 1, this study, therefore, combines all available lines of evidence: reactivity trends and multielement (C, H, Cl) isotope effects of chlorinated ethenes with different molecular structures (PCE, TCE, *cis*-DCE); detection of intermediates by high-resolution mass spectrometry, radical trap experiments, and mass balance deficit analysis; and changes in the relative contribution of various pathways upon changes in reaction conditions (pH between 5 and 12). The mechanistic insights gained from this study are then explored for their ability to explain reactivity trends and product formation and to answer why biodegradation of chlorinated ethenes often stops at *cis*-DCE or VC.

■ MATERIALS AND METHODS

Chemicals. A list of all chemicals, including sources and purification procedures, is provided in the Supporting Information.

Kinetics, Isotope Effects, and Product Formation in Dehalogenation of Chlorinated Ethenes by Vitamin B_{12} at Different pH. For a detailed description, see the Supporting Information. Briefly, a pH-specific buffer solution (pH between 5 to 12) was prepared, degassed with nitrogen, and PCE, TCE, or *cis*-DCE were added in an anoxic glovebox. In parallel, titanium(III)chloride was dissolved in water, the solution was
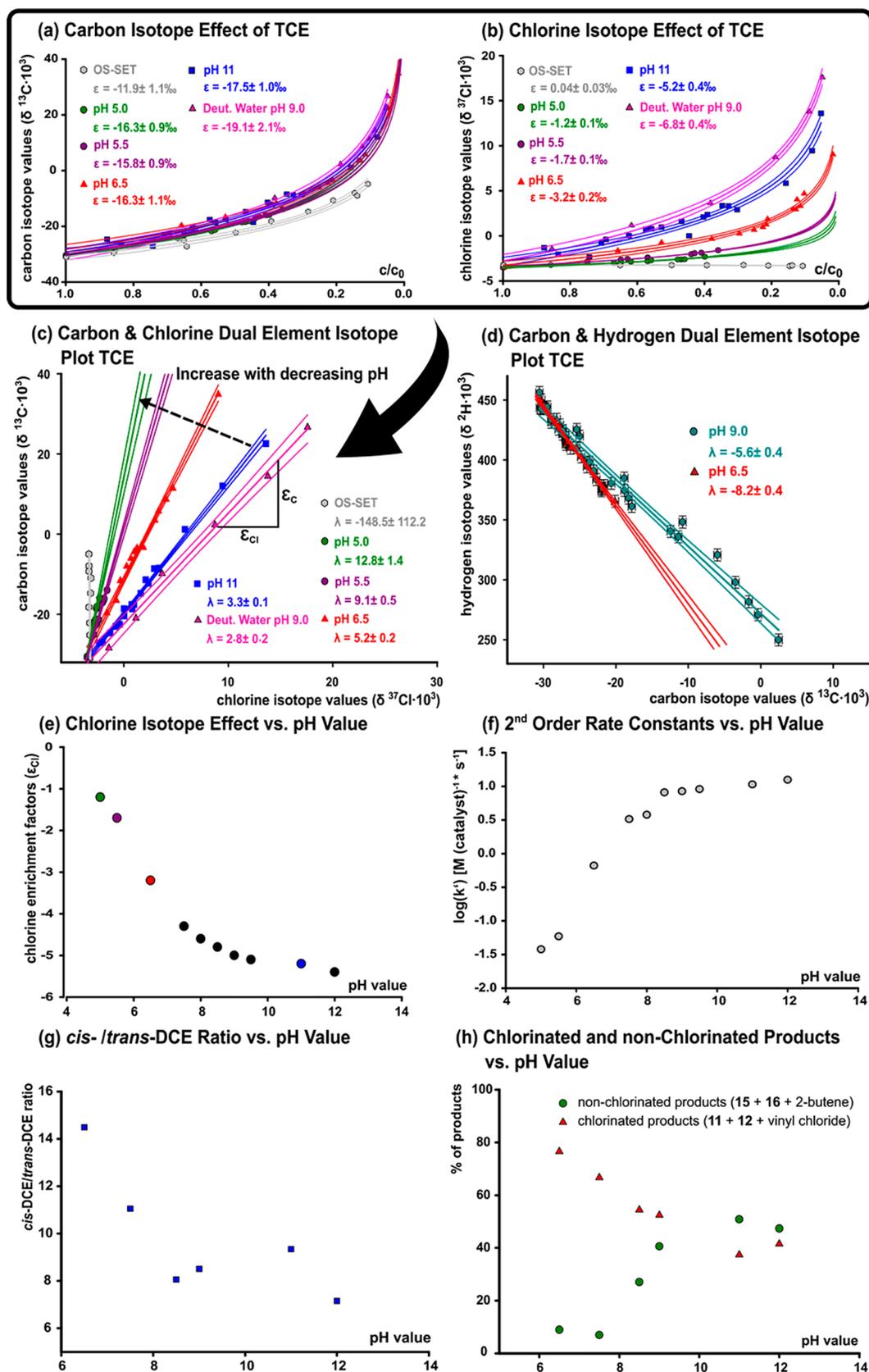
Figure 2. Trends in TCE chlorine isotope effects and observed product ratios indicate a change in the dechlorination mechanism with vitamin B_{12}. Isotope enrichment factors of carbon (a) and chlorine (b) for TCE in reaction with vitamin B_{12} were obtained by fitting changes of isotope values against the remaining fraction of TCE substrate according to eq 3. (Data on OS-SET are from ref 59.) (c) pH-dependent variation in carbon and chlorine isotope effects of TCE represented in a carbon/hydrogen dual-element isotope plot, (d) pH-dependent variation of carbon and hydrogen isotope effects of TCE represented in a carbon/hydrogen dual-element isotope plot. (e) Changes of chlorine isotope enrichment factors of TCE with pH (note that a more negative value indicates a stronger isotope effect). (f) Changes in second-order rate constants of TCE with pH. (g) cis-DCE-

Figure 2. continued

to-*trans*-DCE ratio versus pH. (h) Formation of chlorinated and nonchlorinated products versus pH (where numbers (11, 12, 15, and 16) correspond to structures in Scheme 2).

degassed with nitrogen, sodium citrate was added, and the pH adjusted. The reaction was started by combining the chlorinated ethene solution, the Ti(III)citrate solution, and vitamin B_{12} to give concentrations of about 1 mM chlorinated ethene, between 15 μM and 325 μM vitamin B_{12} and about 25 mM Ti(III) citrate. Reactions were conducted in the dark. Samples for concentration, as well as carbon- and chlorine isotope measurements were removed at selected time-points either by headspace sampling with a pressure lock syringe or by taking liquid samples in which the reaction was stopped through hydrogen peroxide addition.

Experiments To Detect Complex Formation between Vitamin B_{12} and TCE by Direct Injection-Mass Spectrometry (DI-MS) and Analysis of Mass Balance Deficits. To capture intermediates, experiments at different pH were conducted with stoichiometric amounts of prereduced vitamin B_{12} and without Ti(III) as bulk reductant. Mass balance deficits were determined as the difference between (a) initial TCE; (b) remaining TCE; (c) volatile products (not observed under stoichiometric conditions); and (d) reversibly associated TCE that could be recovered by exhaustive sparging of the solution. A detailed description is provided in the Supporting Information.

Analytical Methods. A detailed description of concentration analysis, isotope analysis (carbon, chlorine, and hydrogen), and high-resolution mass spectrometry is provided in the Supporting Information. Briefly, concentrations were analyzed by gas chromatography–mass spectrometry (GC-MS), and isotope values by gas chromatography coupled to an isotope ratio mass spectrometer (GC-IRMS). $^{13}C/^{12}C$ and $^{2}H/^{1}H$ ratios were determined after peak separation and combustion to CO_2[49] or reduction to H_2,[50] respectively, whereas $^{37}Cl/^{35}Cl$ ratios were determined after gas chromatographic separation by direct analysis of intact CE isotopologue molecules.[30,51]

■ RESULTS AND DISCUSSION

Isotope Effects and Reactivity Trends Indicate Different Reaction Mechanisms for PCE and *cis*-DCE. To probe for the occurrence of different transformation mechanisms as hypothesized in Scheme 1 and brought forward in previous studies,[18,22] vitamin B_{12}-catalyzed dehalogenation was investigated for the chlorinated ethenes PCE and *cis*-DCE with titanium citrate as bulk reductant at different pH (6.5 to 11). Increasing pH values resulted in opposing reactivity trends for PCE and *cis*-DCE, where second-order rate constants were derived for "S" = PCE, TCE, *cis*-DCE and "catalyst" = vitamin B_{12} according to $d[S]/dt = -k \cdot [S] \cdot [\text{catalyst}]$. These second-order rate constants increased for PCE (pH 6.5: $k = 115 \pm 15$ (M catalyst)$^{-1} \cdot$s^{-1}; pH 9: $k = 176 \pm 14$ (M catalyst)$^{-1} \cdot$s^{-1}; pH 11: $k = 270 \pm 41$ (M catalyst)$^{-1} \cdot$s^{-1}) whereas they decreased for *cis*-DCE (from pH 6.5: $k = 0.03 \pm 0.006$ (M catalyst)$^{-1} \cdot$s^{-1} over pH 9: $k = 0.0008 \pm 0.0002$ (M catalyst)$^{-1} \cdot$s^{-1} to pH 11: hardly any degradation at all). The greater rate constants of PCE compared to *cis*-DCE are in perfect agreement with those of previous studies (e.g., PCE pH 9.0 k= 155 ± 21 M$^{-1} \cdot$s^{-1}; *cis*-DCE pH 9.0 $k = 0.0006 \pm 0.0001$ M$^{-1} \cdot$s^{-1}).[18] Conversely, the

different nature of *relative* reactivity (trends of decreasing rates of PCE at lower pH vs increasing rates of *cis*-DCE) indicate the occurrence of different underlying reaction mechanisms.

In agreement with these opposing reactivity trends, also carbon and chlorine isotope effects showed pronounced differences between the two compounds. Large carbon and small chlorine isotope effects were detected for *cis*-DCE ($\varepsilon^{13}C$ = $-28.4‰ \pm 1.1‰$; $\varepsilon^{37}Cl = -1.5‰ \pm 0.2‰$; Figure 1a,b), whereas reaction of vitamin B_{12} with PCE resulted in smaller carbon but much greater chlorine isotope effects ($\varepsilon^{13}C$ = $-16.6‰ \pm 2.7‰$ to $-17.0‰ \pm 1.2‰$; $\varepsilon^{37}Cl = -4.0‰ \pm 0.4‰$ to $-4.2‰ \pm 0.4‰$; Figure 1a and b). (Note that these differences cannot be explained by the number of chlorine atoms in the molecules: when taking into account a "dilution" by nonreacting positions,[34] the discrepancy in intrinsic chlorine isotope effects ($2 \cdot (-1.5‰) = -3‰$ vs $4 \cdot (-4‰) = -16‰$) would be even more pronounced). The trend is also represented in the dual-element isotope plot of Figure 1c, where the small chlorine isotope effects in reaction of *cis*-DCE resulted in a steep slope of $\lambda = \Delta\delta^{13}C/\Delta\delta^{37}Cl = 18.2 \pm 2.2$, whereas the larger chlorine isotope effects in PCE gave rise to a much flatter slope ($\lambda = 3.9 \pm 0.4$ to 4.2 ± 0.3). The pH value of the reaction solution had no influence on the carbon and chlorine isotope effects of PCE. Taken together, both lines of evidence point to different reaction mechanisms in the reductive dehalogenation of PCE vs *cis*-DCE. The increasing reaction rates of PCE with increasing pH can be explained with the increasing reduction potential of titanium(III) citrate[52] and—consequently—a higher steady-state concentration of Co^I (where we have considered that the redox potential of Co^I does not change above pH 4.7[53,54]). The opposite trend for *cis*-DCE, in contrast, indicates that its reactivity must be governed by a different underlying mechanism and that the reaction rate of this mechanism is accelerated by the presence of H$^+$. Further, the strong chlorine isotope effect in PCE indicates that a C–Cl bond must be cleaved in the rate-determining step of PCE dehalogenation, whereas the small chlorine isotope effect (in conjunction with a large carbon isotope effect) in *cis*-DCE indicates that a different chemical step involving carbon, but not chlorine atoms was rate-determining in reduction of *cis*-DCE.

Isotope Effects and Reactivity Trends Indicate a pH-Dependent Mechanistic Shift in Reaction of TCE. To further probe for the observed pH-dependency in reductive chlorinated ethene dehalogenation by vitamin B_{12}, experiments were conducted with TCE between pH 5 and 12. TCE contains one dichlorovinylidene group (=CCl$_2$) like in PCE and one chlorovinylidene group (=CHCl) like in *cis*-DCE, and its redox potential (CHCl=CCl$_{2(aq)}$ + 2e$^-$ + H$^+$ → CHCl= CHCl$_{(aq)}$ + Cl$^-_{(aq)}$; -0.67 V vs the standard hydrogen electrode) has been computed to lie between PCE (CCl$_2$= CCl$_{2(aq)}$ + 2e$^-$ + H$^+$ → CHCl = CCl$_{2(aq)}$ + Cl$^-_{(aq)}$; -0.60 V) and *cis*-DCE (CHCl=CHCl$_{(aq)}$ + 2e$^-$ + H$^+$ → CH$_2$= CHCl$_{(aq)}$ + Cl$^-_{(aq)}$;; -1.012 V).[55] The reactivity trend of TCE was analogous to PCE: a decrease in pH resulted in a reduction of TCE reaction rates, where the decline was particularly drastic below pH 8 (Figure 2f). Remarkably, this trend was also accompanied by a change in observable TCE chlorine isotope
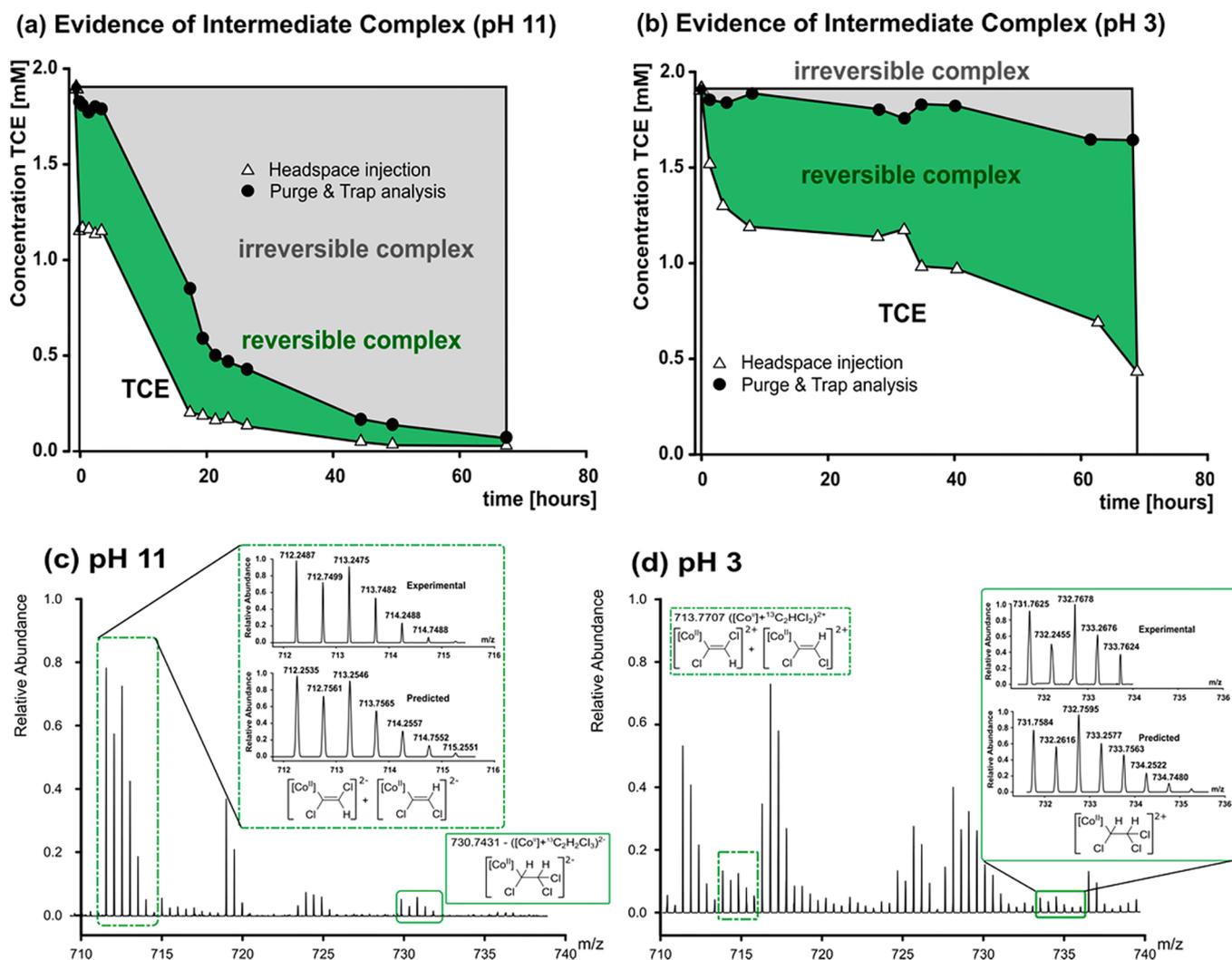
Figure 3. Evidence of reversible and irreversible complex formation (a,b) and complementary evidence of Cob(II)alamin chlorovinyl- and chloroalkyl-complexes high-resolution mass spectra (c,d). Stoichiometric amounts of TCE and prereduced Cob(I)alamine were brought to reaction at pH 11 (panel a) and pH 3 (panel b). The mass balance deficit between equilibrium TCE concentrations (from headspace analysis) and exhaustive extraction of TCE (from purge and trap analysis) gives the concentration of reversibly formed TCE-Cob(I)alamin complexes. The remaining mass balance deficit is interpreted as evidence of irreversibly formed TCE−cobalamin complexes. High-resolution mass spectra of the reaction products from doubly ^{13}C-labeled TCE with vitamin B_{12} at pH value 11 (panel c) and 2 (panel d). Note that the spectra are recorded in double-negative (panel c) or double-positive mode (panel d) so that a difference in m/z of 0.5 corresponds to a difference in one atomic mass unit. Complete mass spectra are provided in the Supporting Information (S4a,b).

effects, where the change was again particularly pronounced at pH 8 (Figure 2e). While carbon isotope effects showed only a small variation ($\varepsilon^{13}C = -15.8‰ \pm 0.9‰$ to $-17.5‰ \pm 1.0‰$; Figure 2a) chlorine isotope effects of TCE decreased dramatically from $\varepsilon^{37}Cl = -5.2‰ \pm 0.4‰$ at pH 11 to $\varepsilon^{37}Cl = -1.2‰ \pm 0.1‰$ at pH 5 (Figure 2b,e). The observed carbon isotope enrichment factors $\varepsilon^{13}C$ are consistent with those of previous studies.[41,42,56−58] Variation of chlorine isotope enrichment factors, $\varepsilon^{37}Cl$, in contrast, was much greater than previously observed,[41,42,56−58] indicating a hitherto unrecognized shift to a different reaction mechanism in the reduction of TCE. This shift is also visualized in the dual-element isotope plot of Figure 2e, where the slope $\lambda = \Delta\delta^{13}C/\Delta\delta^{37}Cl$ increased with decreasing pH (from $\lambda = 3.3 \pm 0.1$ to 12.4 ± 1.4; Figure 2e).

The large chlorine isotope effect at high pH is indicative of a mechanism that involves C−Cl bond cleavage. In contrast, the small chlorine isotope effect at low pH indicates a different

mechanism which, like in the case of *cis*-DCE, does not involve C−Cl bond cleavage in the rate-determining step and which is accelerated by the presence of [H$^+$]. The role of protonation in this mechanistic shift is emphasized by an additional experiment conducted with vitamin B_{12} in deuterated water at pH 9.0, which resulted in the greatest chlorine isotope effect ($\varepsilon^{37}Cl = -6.8‰ \pm 0.4‰$; Figure 2b) and the smallest slope ($\lambda = 2.8 \pm 0.2$; Figure 2c) of all observations. Hence, the lower reaction rate of D$^+$ ions slowed down the alternative pathway to the same, or an even greater extent, than a smaller concentration of H$^+$ ions imposed by the change of pH. Such a change in mechanism is further supported by carbon/hydrogen dual-element isotope fractionation trends (Figure 2d) where also different slopes with changing pH are detected (for further discussion see below).

The results of the two sets of experiments with PCE/*cis*-DCE (Figure 1) and with TCE (Figure 2) are strikingly consistent. Both give evidence of different reaction mechanisms which are

reflected by a different magnitude of chlorine isotope effects, and both have in common that the mechanistic path that is associated with a small chlorine isotope effect is accelerated by H^{+}.

pH-Dependent Product Distribution Supports a Mechanistic Shift in TCE Transformation. Figure 2g,h demonstrate that this mechanistic dichotomy is also reflected in the pattern of TCE product distribution. Identical products, but drastic changes in their quantity, were found between high and low pH values (Figure S2a and b), which is consistent with, but exceeds previous observations by Glod et al.[22] Degradation of TCE at pH 6 resulted in around 80% chlorinated products, whereas less than 15% nonchlorinated products were formed (remaining TCE 2%). In contrast, over 50% nonchlorinated products, mainly ethene, and less than 40% chlorinated products were produced at high pH (remaining TCE 5%). Figure 2g further shows that even the *cis*- to *trans*-DCE ratio changed significantly with pH. It has been recognized that the preference of *cis*- over *trans*-DCE may derive from different intermediates[26,46,59,60] and that the exact ratio of *cis*- over *trans*-DCE may serve as indicator of competing mechanisms.[60] Hence, our data suggests that different intermediates were involved as precursors to these isomers depending on pH. Experiments with d_7-isopropanol as radical trap,[27] finally, yielded more deuterated *cis*- and *trans*-DCE at pH 6.5 than at pH 9, revealing a greater steady-state concentration of dichlorovinyl radicals at pH 6.5 (Figure S2c). While such radicals have previously been taken as evidence of an initial SET mechanism,[22,23] they may alternatively also derive from decomposition of intermediate cobalamin vinyl complexes (see Scheme 1). Therefore, even though we reproduced in our study the observation of previous studies and were able to trap such radicals (Figure S2c), we conclude that they cannot provide conclusive evidence about the initial reaction step at this point.

Mass Balance Deficits Indicate Formation of Reversible and Irreversible Vitamin B₁₂−TCE Complexes. To probe for reaction intermediates associated with either mechanism, experiments were conducted in the absence of Ti(III) citrate so that putative cob(I)alamin−TCE complexes would not be further reduced but could be captured by targeted analysis. To this end, cob(III)alamin was prereduced with zinc, and the pure cob(I)alamin was subsequently added to stoichiometric amounts of TCE in water at pH 3 and 11. These extreme pH values were chosen to probe for the putative endmember mechanisms. Besides TCE, no other volatile compounds were detected. To probe for reversibly formed complexes from cob(I)alamin and TCE, the mass balance deficit was determined between TCE concentrations sampled from the headspace of reaction vials and TCE concentrations sampled through exhaustive extraction of aqueous samples by Purge and Trap (P&T) at the same time point. Whereas headspace analysis gave the concentration of TCE in equilibrium with any reversible complexes, P&T analysis determined the maximum amount of TCE that could be extracted from these equilibria. In both cases, measurements were calibrated with standards treated in an identical way. In the presence of vitamin B₁₂, calibrated P&T concentrations were much higher than headspace concentrations (Figure 3a,b). This reveals the formation of reversibly formed TCE-cobalamine complexes in which TCE was captured and from which it could be extracted. Besides this reversible complex formation, an additional mass balance deficit was observed. Because no other products were detected, any mismatch must be

attributable to the presence of vitamin B₁₂. We, therefore, attribute this deficit to additional irreversible cob(I)alamin−TCE complex formation. Figure 3a,b, moreover, illustrate a pronounced pH dependence of the associated kinetics. Whereas formation of both complexes was fast at high pH, at low pH reversible complexes were also produced, but transformation into irreversible complexes was much slower.

High-Resolution Mass Spectra Give Evidence of Cob(II)alamin Chloroalkyl and Chlorovinyl Complexes. To further explore the chemical nature of these complexes, UV−vis (Figure S3a,b) and high-resolution MS (Figure 3c,d and S4a,b) measurements were conducted. At both pH values, reduction of cob(III)alamin to cob(I)alamin resulted in a shift of the absorption maximum from 370 to 390 nm, consistent with trends previously established by Glod et al.[22] Hence, the reaction of TCE and cob(I)alamin caused the disappearance of the signal at 390 nm and formation of maxima at 310 and 470 nm, which originate from cob(II)alamin complexes[15,22,61] Both pH values triggered these changes indicating that either high pH or low pH enabled the formation of a complex from TCE and cob(I)alamin.

High-resolution mass spectra from direct injection of reaction solutions into an Orbitrap mass spectrometer were obtained in double-negative mode at pH 11 (Figure 3c) and in double-positive mode at pH 2 (Figure 3d). In both cases, TCE labeled with two ^{13}C atoms was brought to reaction with prereduced cob(I)alamine. Mass spectra of chlorinated alkyl, vinyl, and acetylene complexes of cob(II)alamin were observed at both pH values as well as the spectra of chlorinated C₄-complexes at pH 2. Assessing the relative contribution of alkyl vs vinyl vs acetylene complexes in dependence on pH is difficult, because elimination of HCl in the electrospray ion source can convert one type into the other so that the mass spectra do not necessarily reflect proportions of solution chemistry. Even so, strong agreement between experimental and predicted mass spectra at 712.2 m/z and 730.2 m/z (high pH, Figure 3c) and at 713.7 m/z and 731.7 m/z (low pH, Figure 3d) could substantiate the formation of vinyl- and alkyl-cob(II)alamin-complexes from direct addition of cob(I)alamin to TCE (Figure 3a,b). These results are consistent with, but exceed, previous observations by Lesage et al.[25] who employed low instead of high-resolution mass spectrometry, and who detected chlorinated vinyl-complexes, chlorinated C₄-complexes and nonchlorinated alkyl complexes, but not chlorinated alkyl-complexes.

Experimental Evidence Is Not Consistent with Initial Electron Transfer (Either Inner or Outer Sphere). The combined experimental evidence from reactivity trends, isotope effects, product distribution, and detection of intermediates can serve to test and discard current mechanistic hypotheses (Scheme 1). A direct electron transfer (ET) as initial step (either inner or outer sphere) is an unlikely scenario for the following reasons. In the mechanism prevailing with TCE at low pH, an ET would be inconsistent with reactivity trends: whereas the low pH-mechanism was *accelerated* with decreasing pH, an ET would be *slowed* with decreasing pH owing to the decreasing redox potential of Ti(III)citrate.[52] In the mechanism prevailing with TCE at high pH, in turn, an outer sphere-SET can be excluded, because the large chlorine isotope effects of this study (Figure 2) are inconsistent with the small, or even nonexistent chlorine isotope effects observed in outer sphere-SET of chlorinated ethenes in water (gray data in Figure 2).[59] This conclusion is consistent with work of Costentin et al.[62,63]

Scheme 2. Integrating Mechanism for Competing Pathways of Chlorinated Ethene Dehalogenation by Vitamin B$_{12}$[a]
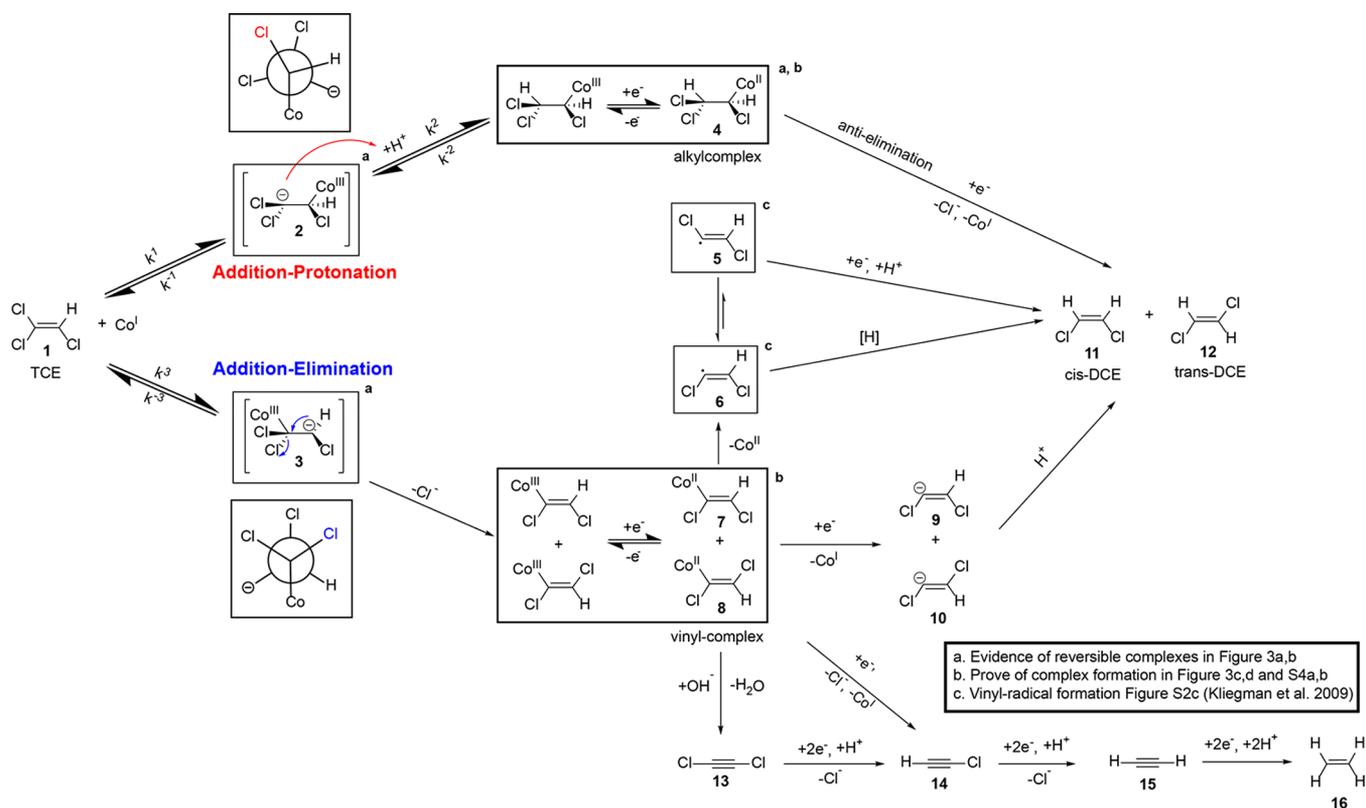
[a] The mechanism accommodates evidence from pH-dependent isotope effects (Figure 1 and 2), chlorinated ethene-vitamin B$_{12}$ complexes (Figure 3) (c), pH- dependent product formation (Figure 2g,h). Here, "-e$^-$, + H$^+$" indicate an electron transfer followed by protonation, whereas "[H]" indicates abstraction of a hydrogen radical. A more detailed scheme illustrating the reaction paths at low and high pH separately is provided in Scheme S1 and S2 in the Supporting Information.

who observed that electrochemical rate constants of PCE and TCE via SET differed strongly from those involving vitamin B$_{12}$ and reductive dehalogenases, respectively, ruling out an SET scenario for these catalysts and calling for more intimate reactant-catalyst interactions. This leaves inner-sphere ET as the remaining possible ET at high pH. An inner-sphere interaction of cob(I)alamin with a halogen atom, as suggested by Payne et al. for bromobenzenes,[8] cannot involve an unoccupied d-orbital of Co^I. Instead, an occupied orbital of cob(I)alamin must attack the antibinding orbital of the Cl−C bond resulting in a homolytic or heterolytic cleavage producing vinyl radicals or vinyl anions, as suggested by recent computational predictions.[20,21] This, however, would be in contradiction to our observation of irreversibly, and even reversibly, formed cob(I)alamin-TCE complexes as key intermediates in reaction with TCE (Figures 3 and S2c). If vinyl radicals and/or anions were formed, they would have to combine quantitatively with free cobalamin to generate such complexes. This is an unlikely scenario considering the difficulty in the synthetic preparation of vinyl-cobalamin complexes through this route[10,16,22,25,26] and considering how readily these radicals or carbanions can abstract H$^•$ or H$^+$, respectively, from surrounding molecules in solution. The corresponding products (*cis*-DCE and *trans*-DCE), however, where not observed in the experiment of Figure 3a,b. Hence, single-electron transfer (either inner- or outer sphere) is not a likely scenario for the initial step of TCE reduction by vitamin B$_{12}$.

The Occurrence of Two Pathways Cannot Be Explained by Alkyl Cob(I)Alamin Complexes as Common Intermediates. Nucleophilic addition of cob(I)alamin leads to alkyl complexes (upper path in Figure 1), which were indeed observed by mass spectrometry (Figure 3c,d) which are consistent with the observation of reversible complex formation—no C−Cl bond is cleaved, all steps are reversible (Figure 3a,b)—and which can explain the absence of chlorine isotope effects at low pH (Figure 2). To explain vinyl complexes, on the other hand, a one-step nucleophilic substitution in analogy to S$_N$2 reactions (Scheme 1) is an unlikely scenario at a sp^2 carbon center so that Rappoport[64] suggested an addition−elimination or addition−protonation with consecutive β-elimination as summarized in Scheme 2. This raises the question whether the mechanistic shift observed in our experiments can be explained by a common addition−protonation mechanism with alkyl complexes (**4** and isomers) as key intermediates (as suggested by Pratt and van der Donk[26]) which may either be further reduced to dichloroethenes (**11**, **12**) or may undergo β-elimination of HCl to form vinyl complexes (**7**, **8** and isomers). Such a role of alkyl complexes at the crossroads of competing pathways, however, can be excluded for the following reasons. On the one hand, this mechanism alone cannot explain the changes in mechanism (and isotope effects) with pH, because both pathways would involve [H$^+$]. On the other hand, it cannot explain the observed product distribution. Reduction of alkyl cob(II)alamin complexes, as brought forward by Pratt and van der Donk[26] would lead to 1,1,-DCE in the case of a 1,1,2-trichloroalkyl-

cobalamin complex (i.e., the intermediate originating from protonation of intermediate **3**, see the pathway at low pH sketched in Scheme S1). This is in disagreement with the observation that 1,1-DCE was hardly observed experimentally (only to about 1%, see Figure S 2b). Alternatively, if a cobalamin vinyl intermediate was formed by elimination of HCl from the cobalamin alkyl complex **4**, this would lead to a 1,1-dichlorovinyl intermediate implying again that 1,1-DCE should be the predominant product. (Note that in comparison, the 1,1,2-trichloroalkyl-cobalamin complex originating from protonation of **3** would be much less reactive toward elimination of HCl.) Hence, we conclude that rather than alkyl cobalamin complexes, the competing pathways observed in this study must involve a different common intermediate.

Cobalamin Carbanions as Key Intermediates Are Consistent with Experimental Observations. Given that we could rule out electron transfer, chlorovinyl complexes and chloroalkyl complexes as crossroads of competing pathways, Scheme 2 leaves carbanion complexes **2** and **3** as the remaining possibility. Nucleophilic addition of cob(I)alamin leads to either **2** or **3**. Both carbanions can either be protonated (addition–protonation), or eliminate Cl⁻ (addition–elimination), and they can explain practically all observations of this and previous studies, as laid out in the following.

Reversible and Irreversible Complex Formation (Figure 3a,b). Scheme 2 uses structural information from Figure 3c,d to interpret the observation of reversible and irreversible complex formation in Figure 3. Formation of cobalamin alkyl complexes (e.g., **4**) only involves reversible steps and is, therefore, overall reversible, whereas C–Cl bond cleavage makes formation of cobalamin chlorovinyl complexes (**7**, **8**) irreversible. Hence, Scheme 2 can explain our experimental observations of Figure 3 if chloroalkyl cobalamines are taken to be the reversibly formed cobalamin complexes, whereas chlorovinyl cobalamins represent irreversibly formed complexes.

Absence of 1,1-DCE. Practically no 1,1-DCE was observed (Figure S2b) implying that the carbanion **2** is almost exclusively protonated, whereas the carbanion **3** eliminates Cl⁻ to form dichlorovinyl complexes, as observed by mass spectrometry (Figure 3c,d). To explain this selectivity on the carbanion level, conformations may be considered in a similar way as computed for alkyl cobalamines in Pratt and Van Der Donk.[26] As sketched in Scheme 2, the energetically favored conformation in intermediate **3** is expected to position the negative charge exactly antiperiplanar to one of the two chlorine atoms facilitating Cl⁻ elimination and leading to cobalamin vinyl complexes **7**, **8**. In contrast, Scheme 2 illustrates that intermediate **2** is expected to maximize the Co–C–Cl angle (115.6°[26]) to minimize interaction of the chlorine atom with the corrin ring. Consequently, the negative charge is no longer positioned antiperiplanar to the C–Cl bond so that elimination is slowed and protonation is favored in comparison. Hence, carbanion **2** may indeed be expected to be protonated to alkyl cobalamine, whereas carbanion **3** would eliminate Cl⁻ to form vinyl cobalamine.

Mechanistic Shift/Change in Chlorine Isotope Effects with pH (Figure 2). According to Scheme 2 and Scheme S1, low pH makes protonation of **2** (k_2) rapid because of high $[H^+]$ concentrations, whereas it makes deprotonation of **4** (k_{-2}) slow because of low $[OH^-]$ concentrations—likely slower than subsequent reduction—so that TCE becomes "trapped" in the alkyl complex **4** (but can, in the absence of a reduction agent, still be recovered by P&T, see Figure 3). This can explain the

small chlorine isotope effects observed at low pH, because no C–Cl bond cleavage is involved in the protonation. At high pH, in contrast, protonation of **2** is slower (by a factor of 10^6 between pH 5 and 11), whereas deprotonation of **4** is faster (also by a factor of 10^6 between pH 5 and 11) so that **2** can dissociate back to TCE (Figure S5). Subsequently, (sterically hindered) attack of cob(I)alamin can take place at the other, geminal (—CCl₂) position of TCE to form **3** in a reversible reaction. Intermediate **3**, in turn, may eliminate chloride forming **7** and **8**, as discussed above. Because all reaction steps prior to Cl⁻ elimination are reversible, the pronounced chlorine isotope effect of this C–Cl bond cleavage can be observed in the substrate TCE at high pH.

Slow Reaction of Chloroalkyl Cobalamine Complexes (Figure 3). The observation that reversibly formed chloroalkyl cobalamine complexes were only slowly further reduced (Figure 3b, Figure S2b) can be explained by the decreased reduction potential of titanium citrate at low pH.[52] Furthermore, Pratt and van der Donk suggested that the alkyl-complex must first convert from the preferred "base on" ($E^0 = -1.78$ V vs NHE) to the "base off" configuration ($E^0 = -0.94$ V vs NHE) to be reduced.[26] In contrast, the observation that irreversibly formed cobalamin vinyl complexes were quickly further transformed (Figure 3a, Figure S2a) is consistent with predictions of Pratt and van der Donk that their homolytic cleavage (0.27 V vs NHE[65]) is rapid leading to vinyl radicals and chlorovinyl anions (Scheme 2). This can explain the higher mass balance deficit at low vs high pH, because more of the TCE was caught up in slowly reacting cobalamin alkyl complexes (Figure 3, Figure S2).

Product Distribution Trends with pH (Figure 2g,h, Figure S2). According to Scheme 2 and Scheme S2, high pH favors the formation of cobalamin vinyl complexes **7** and **8** (Figure 3c,d). Scheme 2 further illustrates that their subsequent reaction can produce both chlorinated and nonchlorinated products as observed in Figure 2h (**11**, **12**, **15**, and **16**). In contrast, low pH favors reversible formation of the alkyl complex, which only reacts to chlorinated products, again consistent with observations of Figure 3b. Scheme 2 also highlights that the same products, that is, *cis*-DCE (**11**) and *trans*-DCE (**12**), are expected to originate from different possible intermediates (alkyl cobalamin complexes vs radicals or carbanions) explaining the pH dependency of *cis*-DCE/*trans*-DCE ratios observed in Figure 2g. Finally, Scheme 2 delineates how chlorovinyl-radicals **5**, **6** or -anions **9**, **10** can be formed from cobalamin chlorovinyl complexes. On the one hand, chlorovinyl-radicals **5**, **6** can react to *cis*- or *trans*-DCE through reduction of a second electron, possibly provided from titanium citrate, and subsequent protonation. On the other hand, they can form *cis*- or *trans*-DCE by abstraction of a hydrogen atom from surrounding molecules. This can explain why radicals could be trapped with d_7-isopropanol in our and previous studies, where they were interpreted as evidence of an initial single-electron transfer mechanism.[18,22,27,65−67] The competing pathways of Scheme 2 can also explain why only a finite proportion of products from TCE carried the deuterium label from radical traps in a previous study;[27] the other part was likely formed via the addition–protonation pathway of Scheme 2 which does not involve radicals. Finally, at lower pH the lifetime of these radicals (and, hence the probability of trapping them) increases with the decreasing reduction potential of titanium citrate[52] explaining why more deuterated dichloroethenes were trapped at pH 6.5 than at pH 9 (Figure S2c). In contrast, negligible

amounts of trapped products—as observed for *cis*-DCE and VC by Glod et al.[18]—would be expected if the reaction was completely dominated by the addition−protonation pathway.

Hydrogen Isotope Effects (Figure 2d, Figure S1). Inverse hydrogen isotope effects were observed indicating that TCE with ^{1}H reacted more slowly than with ^{2}H. Such secondary inverse hydrogen isotope effects were previously observed in biodegradation of TCE[45] and are a hallmark of a change from sp^2 to sp^3 hybridization leading to a more cramped coordination environment around the C−H bond and thus stiffer bending vibrations.[68,69] Our observation that stronger inverse hydrogen isotope effects were observed at pH 6.5 (ε_H = +130‰ ± 14‰) compared with pH 9.0 (ε_H = +98‰ ± 6‰) are indeed consistent with a more cramped coordination environment in the transition state to cobalamin alkyl complexes as opposed to smaller inverse hydrogen isotope effects in the formation of carbanions. Therefore, hydrogen isotope effects also support the mechanistic picture of Scheme 2.

Implications for Reactivity and Product Formation. Our results suggest that the dual-element (C, Cl) isotope plot of TCE (Figure 2) is a sensitive indicator of these competing pathways (addition−elimination at high pH vs addition−protonation at low pH) and may even allow estimating their relative contribution (Figure S6). When transferring this insight to data of PCE and *cis*-DCE, Figure 1c suggests that PCE is mainly degraded via an addition−elimination reaction, whereas *cis*-DCE reacts via the addition−protonation pathway. According to Scheme 2, the differences in reactivity—and hence, the question why *cis*-DCE and VC are less reactive than TCE and PCE—may be traced back to cobalamin carbanions as key intermediates. PCE contains two dichlorovinylidene groups (= CCl$_2$) giving rise to conformations analogous to intermediate **2**, whereas *cis*-DCE contains two chlorovinylidene groups (= CHCl) groups leading to conformations like in intermediate **3**. The addition−protonation pathway is consistent with the mechanism originally brought forward for *cis*-DCE and VC by Glod et al.,[22] whereas for TCE and PCE a single-electron transfer mechanism has been favored until now.[8,9,19] Notably, Scheme 2 can also explain why 1,1-DCE showed a thousand-fold higher reactivity and much higher proportions of trapped radicals than *cis*-DCE or *trans*-DCE in ref 18: unlike *cis*-DCE or *trans*-DCE, the compound 1,1-DCE contains a =CCl$_2$ group, which makes it amenable to the addition−elimination pathway. Hence, in a similar way as recently suggested by Ji et al.,[24] this study could indeed bring forward evidence from multielement isotope analysis to pinpoint different reaction mechanisms of chlorinated ethenes with vitamin B$_{12}$—even though we arrive at slightly different mechanistic picture involving an addition−elimination pathway instead of a concerted nucleophilic substitution.

Relevance for Enzymatic and Bacterial Biodegradation of Chlorinated Ethenes. This raises the question whether the same approach can be used to transfer mechanistic insight into real-world systems like enzymes or dehalogenating organisms. Can reaction mechanisms be compared even though cofactor-catalysis is much slower than with enzymes, although added vitamin B$_{12}$ exceeds environmental concentrations, and despite extreme pH values? Theory predicts that comparable isotope effects are obtained as long as the manner and order of bond changes in the chlorinated ethene is similar—irrespective of reaction rates or reaction partners. In other words, a reaction in solution at extreme pH may mimic protonation/deprotona-

tion in an enzymatic pocket, whereas transformation at neutral pH may mimic an enzyme reaction in the absence of such a functionality. Still, intrinsic isotope effects may potentially be modulated by (i) the diffusion of substrates inside the enzyme core, (ii) interactions with amino acid residues, (iii) the position of the lower ligand of vitamin B$_{12}$ (base-on vs base-off), as well as (iv) the necessity of products (i.e., the less chlorinated product and the chloride ion) to diffuse out of the enzymatic pocket.

First insight on the influence of such complicating factors can be derived from a recent study by Renpenning et al. on carbon and chlorine isotope effects with the reductive dehalogenase (RDase) PceA from *Sulfurospirillum multivorans*.[42] This PceA is one of the best characterized RDases to date. It contains a cave-like enzyme structure which introduces substrates in a specific orientation to the active center hypothesized to facilitate OS-SET.[7,9] Isotope effects in PCE catalysis by the enzyme PceA were indeed much smaller than with vitamin B$_{12}$ suggesting that diffusion into the enzyme channel was partially rate-determining for this highly reactive substrate.[42] Similarly small isotope effects were obtained with whole organisms by Badin et al.,[48] whereas isotope effects with *Desulfitobacterium* sp. strain Viet1[46] were fully expressed and agree perfectly with those of the present study ($\lambda = \Delta\delta^{13}C/\Delta\delta^{37}Cl = 3.8 \pm 0.2$ vs λ = 3.9 to 4.2). Dual-element isotope trends of PCE, finally, were slightly different in reaction with corrinoids that contained an axial base (nor-B$_{12}$; cyano-B$_{12}$, λ = 4.6 to 5.0), compared with corrinoids without (dicyanocobinamid, λ = 7.0).[42]

In contrast to these differences observed with PCE, observed isotope effects of TCE are remarkably consistent in enzyme catalysis by PceA from *Sulfurospirillum multivorans*. Dual-element isotope trends with pure corrinoids (both "nor-B$_{12}$" and "norpseudo-B$_{12}$") were similar to those of the present study and earlier investigations[41,45] with λ values clustering between 3.7 and 4.5, confirming that the axial base does not play a decisive role. The corresponding λ values in enzymatic catalysis by PceA (both "nor-B$_{12}$" and "norpseudo-B$_{12}$"), λ = 5.0 and 5.3, were indistinguishable within error from those of the corrinoids. Figure 4 plots these experimental data together with the trends obtained in this study with vitamin B$_{12}$, and with isotope effect trends for OS-SET model reagents.

Figure 4 illustrates that all isotope effect studies with corrinoids and enzymes share pronounced chlorine isotope effects, in contrast to nonexistent chlorine isotope effects observed with aqueous outer-sphere single-electron transfer agents.[70] On the basis of this evidence, we have recently concluded that outer-sphere electron transfer is an exception rather than the rule in natural and engineered reductive dehalogenation reactions,[70] consistent with independent conclusions brought forward by Saveant and co-workers.[71] This conclusion certainly applies to the mechanism of PCE and TCE with vitamin B$_{12}$ in this study, and Figure 4 suggests it should also apply to the reaction of TCE with PceA from *Sulfurospirillum multivorans*, despite recent evidence for an OS-SET.[7] We, therefore, hypothesize that an OS-SET is not at work in the PceA of *Sulfurospirillum multivorans*, but we favor instead an addition−elimination mechanism as laid out in Scheme 2.

Still, we caution that Figure 4 expresses the degree to which C−Cl bond cleavage occurs in the rate-determining step of the reaction. In a similar way as the steep slope λ with vitamin B$_{12}$ reflects the addition−protonation mechanism with little involvement of C−Cl cleavage, the steep slope in the OS-

Dual Element Isotope Plot of TCE Degradation by Enzymes, Cobalamin and Dicyanocobinamid
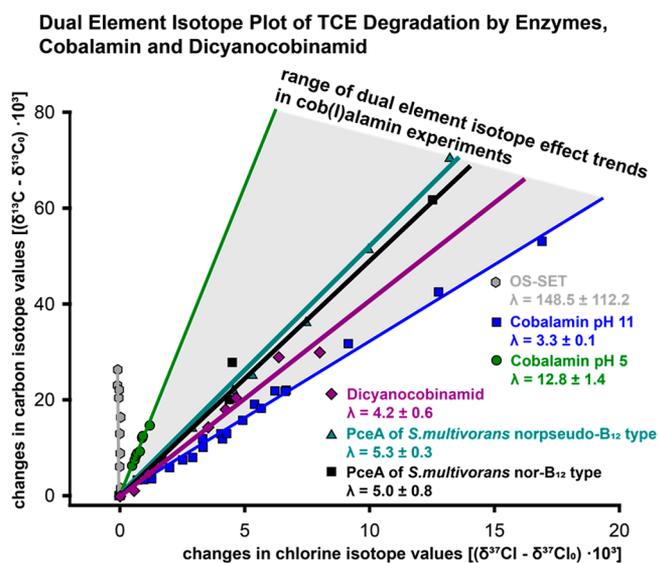
Figure 4. Trends of dual-element isotope effects of TCE in enzymatic catalysis by PceA of *Sulfurospirillum multivorans* (data from Renpenning et al.[42]) compared to reaction with vitamin B_{12} at different pH (this study), dicyanocobinamid (data from Renpenning et al.[42]), and model reagents for outer-sphere single-electron transfer (data from Heckel et al.[70]).

SET mechanism reflects a stepwise mechanism where SET occurs into the π^* orbital of the chlorinated ethene so that subsequent C−Cl bond cleavage is decoupled. While absent in water, we observed that chlorine isotope effects could partly be recovered with OS-SET agents in organic solvents.[70] Hence, if the enzyme environment (i) keeps the reactants long enough together to reverse the ET before C−Cl bond cleavage and (ii) in addition, lets the majority of substrate molecules escape from the enzyme pocket as unreacted substrate *even after the ET has occurred and has been reversed* (otherwise the chlorine isotope effect of the transition state of C−Cl bond cleavage would not be observed!), this would still leave room for an OS-SET mechanism as a hypothetical but rather unlikely scenario.

CONCLUSIONS

Pinpointing the reaction mechanism of reductive dechlorination of chlorinated ethenes in natural transformations is intrinsically difficult. For experiments with the enzymatic cofactor vitamin B_{12}, this study combines evidence from reaction rates, product formation, isotope effects, and trapped intermediates to bring forward a reaction mechanism which supports all available observations to date. Our results indicate that after forming an intermediate complex of vitamin B_{12} and a chlorinated ethene (PCE, TCE, and *cis*-DCE), two reaction pathways are possible: 1. addition−elimination or 2. addition−protonation. To answer the question why reductive degradation of chlorinated ethenes often stops at the stage of *cis*-DCE or VC, the integrating mechanism of Scheme 2, therefore, bears the potential to provide consistent answers. Our experiments with TCE suggest that the chloroalkyl cobalamin intermediates of the addition−protonation pathway are of lower reactivity than the chlorovinyl cobalamin intermediates of the chlorovinyl cobalamin pathway offering a potential explanation for the persistence of the respective substrates (*cis*-DCE, VC) at contaminated sites.

The question remains whether these characteristic trends of competing mechanisms (addition−elimination/addition−pro-

tonation) can also be identified in reductive dehalogenases or real microorganisms. As discussed above, recently reported isotope effects in TCE transformation with the enzyme PceA from *Sulfospirillum multivorans*,[42] and similar trends with whole microorganisms[41,43,45,46] hold promise to bridge this gap, and they suggest an addition−elimination mechanism. Conversely, further studies will be required to investigate whether first data on *cis*-DCE and VC transformation may potentially be indicative of an addition−protonation mechanism.[47] The characteristic dual-element isotope trends of Figures 1 and 2 clearly highlight multiple-element isotope analysis as a new opportunity to bridge the gap and to demonstrate that a mechanism characterized in the reaction flask also occurs on the enzyme level in microorganisms—or at contaminated sites. Labeled compounds are not required since isotope analysis is conducted at natural isotopic abundance. Hence, the stage is set for studies in more complex systems such as whole proteins or microorganisms, to use multielement isotope effect measurements to probe for the mechanistic dichotomy—addition−elimination vs addition−protonation, as brought forward in this contribution.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.7b02945.

Overview of the analytical methods and experimental setups (PDF)

AUTHOR INFORMATION

Corresponding Author

*Phone: +49 89 3187 2565. E-mail: m.elsner@tum.de.

ORCID

Kristopher McNeill: 0000-0002-2981-2227
Martin Elsner: 0000-0003-4746-9052

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Carter, J. M.; Lapham, W. W.; Zogorski, J. S. Occurrence of Volatile Organic Compounds in Aquifers of the United States1. *J. Am. Water Resour. Assoc.* **2008**, *44*, 399−416.
(2) Schwarzenbach, R. P.; Gschwend, P. M.; Imboden, D. M. *Environmental Organic Chemistry*, 2nd ed.; John Wiley & Sons, Inc.: Hoboken, NJ, 2003; p 1313.
(3) Ellis, D. E.; Lutz, E. J.; Odom, J. M.; Buchanan, R. J.; Bartlett, C. L.; Lee, M. D.; Harkness, M. R.; DeWeerd, K. A. Bioaugmentation for Accelerated In Situ Anaerobic Bioremediation. *Environ. Sci. Technol.* **2000**, *34*, 2254−2260.

(4) Löffler, F. E.; Edwards, E. A. Harnessing microbial activities for environmental cleanup. *Curr. Opin. Biotechnol.* **2006**, *17*, 274−284.

(5) Fincker, M.; Spormann, A. M. Biochemistry of Catabolic Reductive Dehalogenation. *Annu. Rev. Biochem.* **2017**, *86*, 357−386.

(6) Yan, J.; Simsir, B.; Farmer, A. T.; Bi, M.; Yang, Y.; Campagna, S. R.; Löffler, F. E. The corrinoid cofactor of reductive dehalogenases affects dechlorination rates and extents in organohalide-respiring *Dehalococcoides mccartyi*. *ISME J.* **2016**, *10*, 1092−1101.

(7) Kunze, C.; Bommer, M.; Hagen, W. R.; Uksa, M.; Dobbek, H.; Schubert, T.; Diekert, G. Cobamide-mediated enzymatic reductive dehalogenation via long-range electron transfer. *Nature Commun.* **2017**, *8*, 15858.

(8) Payne, K. A. P.; Quezada, C. P.; Fisher, K.; Dunstan, M. S.; Collins, F. A.; Sjuts, H.; Levy, C.; Hay, S.; Rigby, S. E. J.; Leys, D. Reductive dehalogenase structure suggests a mechanism for B_{12}-dependent dehalogenation. *Nature* **2015**, *517*, 513−516.

(9) Bommer, M.; Kunze, C.; Fesseler, J.; Schubert, T.; Diekert, G.; Dobbek, H. Structural basis for organohalide respiration. *Science* **2014**, *346*, 455−458.

(10) Kliegman, S.; McNeill, K. Dechlorination of chloroethylenes by cob(i)alamin and cobalamin model complexes. *Dalton Trans.* **2008**, *32*, 4191−4201.

(11) McCauley, K. M.; Wilson, S. R.; van der Donk, W. A. Synthesis and characterization of chlorinated alkenylcobaloximes to probe the mechanism of vitamin B_{12}-catalyzed dechlorination of priority pollutants. *Inorg. Chem.* **2002**, *41*, 393−404.

(12) Fritsch, J. M.; Retka, N. D.; McNeill, K. Synthesis, Structure, and Unusual Reactivity of β-Halovinyl Cobalt Porphyrin Complexes. *Inorg. Chem.* **2006**, *45*, 2288−2295.

(13) Rich, A. E.; DeGreeff, A. D.; McNeill, K. Synthesis of (chlorovinyl)cobaloxime complexes, model complexes of proposed intermediates in the B_{12}-catalyzed dehalogenation of chlorinated ethylenes. *Chem. Commun.* **2002**, *0*, 234−235.

(14) Jones, P. G.; Yang, L.; Steinborn, D. (cis-1,2-Dichlorovinyl)bis-(dimethylglyoximato-N,N')(pyridine-N)cobalt(III) Chloroform Solvate, [Co(dmgH)2(py)(CCl = CHCl)].CHCl3. *Acta Crystallogr., Sect. C: Cryst. Struct. Commun.* **1996**, *52*, 2399−2402.

(15) McCauley, K. M.; Pratt, D. A.; Wilson, S. R.; Shey, J.; Burkey, T. J.; van der Donk, W. A. Properties and reactivity of chlorovinylcobalamin and vinylcobalamin and their implications for vitamin B_{12}-catalyzed reductive dechlorination of chlorinated alkenes. *J. Am. Chem. Soc.* **2005**, *127*, 1126−1136.

(16) McCauley, K. M.; Wilson, S. R.; van der Donk, W. A. Characterization of Chlorovinylcobalamin, A Putative Intermediate in Reductive Degradation of Chlorinated Ethylenes. *J. Am. Chem. Soc.* **2003**, *125*, 4410−4411.

(17) Fritsch, J. M.; McNeill, K. Aqueous Reductive Dechlorination of Chlorinated Ethylenes with Tetrakis(4-carboxyphenyl)porphyrin Cobalt. *Inorg. Chem.* **2005**, *44*, 4852−4861.

(18) Glod, G.; Brodmann, U.; Angst, W.; Holliger, C.; Schwarzenbach, R. P. Cobalamin-mediated reduction of *cis*- and *trans*-dichloroethene, and vinylchloride in homogeneous aqueous solution: reaction kinetics and mechanistic considerations. *Environ. Sci. Technol.* **1997**, *31*, 3154−3160.

(19) Cooper, M.; Wagner, A.; Wondrousch, D.; Sonntag, F.; Sonnabend, A.; Brehm, M.; Schüürmann, G.; Adrian, L. Anaerobic Microbial Transformation of Halogenated Aromatics and Fate Prediction Using Electron Density Modeling. *Environ. Sci. Technol.* **2015**, *49*, 6018−6028.

(20) Liao, R.-Z.; Chen, S.-L.; Siegbahn, P. E. M. Unraveling the Mechanism and Regioselectivity of the B_{12}-Dependent Reductive Dehalogenase PceA. *Chem. - Eur. J.* **2016**, *22*, 12391−12399.

(21) Johannissen, L. O.; Leys, D.; Hay, S. A common mechanism for coenzyme cobalamin-dependent reductive dehalogenases. *Phys. Chem. Chem. Phys.* **2017**, *19*, 6090−6094.

(22) Glod, G.; Angst, W.; Holliger, C.; Schwarzenbach, R. P. Corrinoid-mediated reduction of tetrachloroethylene, trichloroethylene and trichlorofluoroethane in homogeneoues aqueous solution:

reaction kinetics and reaction mechanisms. *Environ. Sci. Technol.* **1997**, *31*, 253−260.

(23) Schmitz, R. P. H.; Wolf, J.; Habel, A.; Neumann, A.; Ploss, K.; Svatos, A.; Boland, W.; Diekert, G. Evidence for a Radical Mechanism of the Dechlorination of Chlorinated Propenes Mediated by the Tetrachloroethene Reductive Dehalogenase of *Sulfurospirillum multivorans*. *Environ. Sci. Technol.* **2007**, *41*, 7370−7375.

(24) Ji, L.; Wang, C.; Ji, S.; Kepp, K. P.; Paneth, P. Mechanism of Cobalamin-Mediated Reductive Dehalogenation of Chloroethylenes. *ACS Catal.* **2017**, *7*, 5294−5307.

(25) Lesage, S.; Brown, S.; Millar, K. A different mechanism for the reductive dechlorination of chlorinated ethenes: Kinetic and spectroscopic evidence. *Environ. Sci. Technol.* **1998**, *32*, 2264−2272.

(26) Pratt, D. A.; Van Der Donk, W. A. On the role of alkylcobalamins in the vitamin B_{12}-catalyzed reductive dehalogenation of perchloroethylene and trichloroethylene. *Chem. Commun.* **2006**, *0*, 558−560.

(27) Kliegman, S.; McNeill, K. Reconciling Disparate Models of the Involvement of Vinyl Radicals in Cobalamin-Mediated Dechlorination Reactions. *Environ. Sci. Technol.* **2009**, *43*, 8961−8967.

(28) Sessions, A. L. Isotope-ratio detection for gas chromatography. *J. Sep. Sci.* **2006**, *29*, 1946−1961.

(29) Hayes, J. M. Fractionation of carbon and hydrogen isotopes in biosynthetic processes. *Rev. Mineral. Geochem.* **2001**, *43*, 225−277.

(30) Shouakar-Stash, O.; Drimmie, R. J.; Zhang, M.; Frape, S. K. Compound-specific chlorine isotope ratios of TCE, PCE and DCE isomers by direct injection using CF-IRMS. *Appl. Geochem.* **2006**, *21*, 766−781.

(31) Bernstein, A.; Shouakar-Stash, O.; Ebert, K.; Laskov, C.; Hunkeler, D.; Jeannottat, S.; Sakaguchi-Soder, K.; Laaks, J.; Jochmann, M. A.; Cretnik, S.; Jager, J.; Haderlein, S. B.; Schmidt, T. C.; Aravena, R.; Elsner, M. Compound-Specific Chlorine Isotope Analysis: A Comparison of Gas Chromatography/Isotope Ratio Mass Spectrometry and Gas Chromatography/Quadrupole Mass Spectrometry Methods in an Interlaboratory Study. *Anal. Chem.* **2011**, *83*, 7624−7634.

(32) Renpenning, J.; Kümmel, S.; Hitzfeld, K. L.; Schimmelmann, A.; Gehre, M. Compound-Specific Hydrogen Isotope Analysis of Heteroatom-Bearing Compounds via Gas Chromatography−Chromium-Based High-Temperature Conversion (Cr/HTC)−Isotope Ratio Mass Spectrometry. *Anal. Chem.* **2015**, *87*, 9443−9450.

(33) Hoefs, J. *Stable Isotope Geochemistry*; Springer-Verlag: Berlin, 1997.

(34) Elsner, M.; Zwank, L.; Hunkeler, D.; Schwarzenbach, R. P. A new concept linking observable stable isotope fractionation to transformation pathways of organic pollutants. *Environ. Sci. Technol.* **2005**, *39*, 6896−6916.

(35) Elsner, M.; Hunkeler, D. Evaluating Chlorine Isotope Effects from Isotope Ratios and Mass Spectra of Polychlorinated Molecules. *Anal. Chem.* **2008**, *80*, 4731−4740.

(36) McKelvie, J. R.; Hyman, M. R.; Elsner, M.; Smith, C.; Aslett, D. M.; Lacrampe-Couloume, G.; Sherwood Lollar, B. Sherwood Lollar, B., Isotopic Fractionation of Methyl tert-Butyl Ether Suggests Different Initial Reaction Mechanisms during Aerobic Biodegradation. *Environ. Sci. Technol.* **2009**, *43*, 2793−2799.

(37) Elsner, M. Stable isotope fractionation to investigate natural transformation mechanisms of organic contaminants: principles, prospects and limitations. *J. Environ. Monit.* **2010**, *12*, 2005−2031.

(38) Hofstetter, T. B.; Berg, M. Assessing transformation processes of organic contaminants by compound-specific stable isotope analysis. *TrAC, Trends Anal. Chem.* **2011**, *30*, 618−627.

(39) Vogt, C.; Dorer, C.; Musat, F.; Richnow, H.-H. Multi-element isotope fractionation concepts to characterize the biodegradation of hydrocarbons — from enzymes to the environment. *Curr. Opin. Biotechnol.* **2016**, *41*, 90−98.

(40) Palau, J.; Shouakar-Stash, O.; Hunkeler, D. Carbon and Chlorine Isotope Analysis to Identify Abiotic Degradation Pathways of 1,1,1-Trichloroethane. *Environ. Sci. Technol.* **2014**, *48*, 14400−14408.

(41) Cretnik, S.; Thoreson, K. A.; Bernstein, A.; Ebert, K.; Buchner, D.; Laskov, C.; Haderlein, S.; Shouakar-Stash, O.; Kliegman, S.; McNeill, K.; Elsner, M. Reductive Dechlorination of TCE by Chemical Model Systems in Comparison to Dehalogenating Bacteria: Insights from Dual Element Isotope Analysis ($^{13}C/^{12}C$, $^{37}Cl/^{35}Cl$). *Environ. Sci. Technol.* **2013**, *47*, 6855−6863.

(42) Renpenning, J.; Keller, S.; Cretnik, S.; Shouakar-Stash, O.; Elsner, M.; Schubert, T.; Nijenhuis, I. Combined C and Cl Isotope Effects Indicate Differences between Corrinoids and Enzyme (*Sulfurospirillum multivorans* PceA) in Reductive Dehalogenation of Tetrachloroethene, But Not Trichloroethene. *Environ. Sci. Technol.* **2014**, *48*, 11837−11845.

(43) Wiegert, C.; Mandalakis, M.; Knowles, T.; Polymenakou, P. N.; Aeppli, C.; Macháčková, J.; Holmstrand, H.; Evershed, R. P.; Pancost, R. D.; Gustafsson, Ö. Carbon and Chlorine Isotope Fractionation During Microbial Degradation of Tetra- and Trichloroethene. *Environ. Sci. Technol.* **2013**, *47*, 6449−6456.

(44) Buchner, D.; Behrens, S.; Laskov, C.; Haderlein, S. B. Resiliency of Stable Isotope Fractionation ($\delta^{13}C$ and $\delta^{37}Cl$) of Trichloroethene to Bacterial Growth Physiology and Expression of Key Enzymes. *Environ. Sci. Technol.* **2015**, *49*, 13230−13237.

(45) Kuder, T.; van Breukelen, B. M.; Vanderford, M.; Philp, P. 3D-CSIA: Carbon, Chlorine, and Hydrogen Isotope Fractionation in Transformation of TCE to Ethene by a Dehalococcoides Culture. *Environ. Sci. Technol.* **2013**, *47*, 9668−9677.

(46) Cretnik, S.; Bernstein, A.; Shouakar-Stash, O.; Löffler, F.; Elsner, M. Chlorine Isotope Effects from Isotope Ratio Mass Spectrometry Suggest Intramolecular C-Cl Bond Competition in Trichloroethene (TCE) Reductive Dehalogenation. *Molecules* **2014**, *19*, 6450−6473.

(47) Abe, Y.; Aravena, R.; Zopfi, J.; Shouakar-Stash, O.; Cox, E.; Roberts, J. D.; Hunkeler, D. Carbon and Chlorine Isotope Fractionation during Aerobic Oxidation and Reductive Dechlorination of Vinyl Chloride and cis-1,2-Dichloroethene. *Environ. Sci. Technol.* **2009**, *43*, 101−107.

(48) Badin, A.; Buttet, G.; Maillard, J.; Holliger, C.; Hunkeler, D. Multiple Dual C−Cl Isotope Patterns Associated with Reductive Dechlorination of Tetrachloroethene. *Environ. Sci. Technol.* **2014**, *48*, 9179−9186.

(49) Matthews, D. E.; Hayes, J. M. Isotope-Ratio-Monitoring Gas Chromatography-Mass Spectrometry. *Anal. Chem.* **1978**, *50*, 1465−1473.

(50) Renpenning, J.; Hitzfeld, K. L.; Gilevska, T.; Nijenhuis, I.; Gehre, M.; Richnow, H. H. Development and Validation of an Universal Interface for Compound-Specific Stable Isotope Analysis of Chlorine (Cl-37/Cl-35) by GC-High-Temperature Conversion (HTC)-MS/IRMS. *Anal. Chem.* **2015**, *87*, 2832−2839.

(51) Bernstein, A.; Shouakar-Stash, O.; Ebert, K.; Laskov, C.; Hunkeler, D.; Jeannottat, S.; Sakaguchi-Söder, K.; Laaks, J.; Jochmann, M. A.; Cretnik, S.; Jager, J.; Haderlein, S. B.; Schmidt, T. C.; Aravena, R.; Elsner, M. Compound-Specific Chlorine Isotope Analysis: A Comparison of Gas Chromatography/Isotope Ratio Mass Spectrometry and Gas Chromatography/Quadrupole Mass Spectrometry Methods in an Interlaboratory Study. *Anal. Chem.* **2011**, *83*, 7624−7634.

(52) Uppal, R.; Incarvito, C. D.; Lakshmi, K. V.; Valentine, A. M. Aqueous Spectroscopy and Redox Properties of Carboxylate-Bound Titanium. *Inorg. Chem.* **2006**, *45*, 1795−1804.

(53) Lexa, D.; Saveant, J. M. The electrochemistry of vitamin B12. *Acc. Chem. Res.* **1983**, *16*, 235−243.

(54) Lexa, D.; Saveant, J. M.; Zickler, J. Electrochemistry of vitamin B12. 2. Redox and acid-base equilibria in the B12a/B12r system. *J. Am. Chem. Soc.* **1977**, *99*, 2786−2790.

(55) Totten, L. A.; Roberts, A. L. Calculated One- and Two-Electron Reduction Potentials and Related Molecular Descriptors for Reduction of Alkyl and Vinyl Halides in Water. *Crit. Rev. Environ. Sci. Technol.* **2001**, *31*, 175−221.

(56) Gan, Y.; Yu, T.; Zhou, A.; Liu, Y.; Yu, K.; Han, L. Variability in the carbon isotope fractionation of trichloroethene on its reductive

dechlorination by vitamin B12. *Environ. Sci.: Processes Impacts* **2014**, *16*, 1882−1888.

(57) Slater, G. F.; Sherwood Lollar, B.; Lesage, S.; Brown, S. Carbon isotope fractionation of PCE and TCE during dechlorination by vitamin B12. *Groundwater Monit. Rem.* **2003**, *23*, 59−67.

(58) Cichocka, D.; Siegert, M.; Imfeld, G.; Andert, J.; Beck, K.; Diekert, G.; Richnow, H.-H.; Nijenhuis, I. Factors controlling the carbon isotope fractionation of tetra- and trichloroethene during reductive dechlorination by Sulfurospirillum ssp. and *Desulfitobacterium* sp. strain PCE-S. *FEMS Microbiol. Ecol.* **2007**, *62*, 98−107.

(59) Nonnenberg, C.; van der Donk, W. A.; Zipse, H. Reductive Dechlorination of Trichloroethylene: A Computational Study. *J. Phys. Chem. A* **2002**, *106*, 8708−8715.

(60) Follett, A. D.; McNeill, K. Reduction of Trichloroethylene by Outer-Sphere Electron-Transfer Agents. *J. Am. Chem. Soc.* **2005**, *127*, 844−845.

(61) Lexa, D.; Sayeant, J. M.; Zickler, J. Electrochemistry of vitamin B12. 5. *J. Am. Chem. Soc.* **1980**, *102*, 2654−2663.

(62) Costentin, C.; Robert, M.; Saveant, J.-M. Does Catalysis of Reductive Dechlorination of Tetra- and Trichloroethylenes by Vitamin B12 and Corrinoid-Based Dehalogenases Follow an Electron Transfer Mechanism? *J. Am. Chem. Soc.* **2005**, *127*, 12154−12155.

(63) Costentin, C.; Robert, M.; Saveant, J. M. Successive removal of chloride ions from organic polychloride pollutants. Mechanisms of reductive electrochemical elimination in aliphatic gem-polychlorides, alpha,beta-polychloroalkenes, and alpha,beta-polychloroalkanes in mildly protic medium. *J. Am. Chem. Soc.* **2003**, *125*, 10729−10739.

(64) Rappoport, Z. Nucleophilic Vinylic Substitution. *Adv. Phys. Org. Chem.* **1969**, *7*, 1−114.

(65) Pratt, D. A.; van der Donk, W. A. Theoretical investigations into the intermediacy of chlorinated vinylcobalamins in the reductive dehalogenation of chlorinated ethylenes. *J. Am. Chem. Soc.* **2005**, *127*, 384−396.

(66) Shey, J.; van der Donk, W. A. Mechanistic studies on the vitamin B-12-catalyzed dechlorination of chlorinated alkenes. *J. Am. Chem. Soc.* **2000**, *122*, 12403−12404.

(67) Shey, J.; McGinley, C. M.; McCauley, K. M.; Dearth, A. S.; Young, B. T.; van der Donk, W. A. Mechanistic Investigation of a Novel Vitamin B12-Catalyzed Carbon-Carbon Bond Forming Reaction, the Reductive Dimerization of Arylalkenes. *J. Org. Chem.* **2002**, *67*, 837−846.

(68) Melander, L.; Saunders, W. H. *Reaction Rates of Isotopic Molecules*; John Wiley: New York, 1980; p 331.

(69) Marlier, J. F. Multiple isotope effects on the acyl group transfer reactions of amides and esters. *Acc. Chem. Res.* **2001**, *34*, 283−290.

(70) Heckel, B.; Cretnik, S.; Kliegman, S.; Shouakar-Stash, O.; McNeill, K.; Elsner, M. Reductive Outer-Sphere Single Electron Transfer Is an Exception Rather than the Rule in Natural and Engineered Chlorinated Ethene Dehalogenation. *Environ. Sci. Technol.* **2017**, *51*, 9663−9673.

(71) Costentin, C.; Robert, M.; Savéant, J.-M. Does Catalysis of Reductive Dechlorination of Tetra- and Trichloroethylenes by Vitamin B12 and Corrinoid-Based Dehalogenases Follow an Electron Transfer Mechanism? *J. Am. Chem. Soc.* **2005**, *127*, 12154−12155.