FINAL REPORT

Identification of Abiotic Degradation Pathways of Chlorinated Ethenes by Compound-specific Stable Isotope Analysis: A Proof-of-Concept Study

SERDP Project ER-2623

JULY 2021

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SERDP
DOD ▪ EPA ▪ DOE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
26/07/2021	SERDP Final Report	3/18/2016 - 9/18/2020

4. TITLE AND SUBTITLE

Identification of Abiotic Degradation Pathways of Chlorinated Ethenes by Compound-specific Stable Isotope Analysis: A Proof-of-Concept Study

5a. CONTRACT NUMBER
16-P-0025

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Tomasz Kuder

5d. PROJECT NUMBER
ER-2623

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Oklahoma
100 E. Boyd Street, Room 710
Norman, OK 73019

8. PERFORMING ORGANIZATION REPORT NUMBER
ER-2623

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Strategic Environmental Research and Development Program (SERDP)
4800 Mark Center Drive, Suite 16F16
Alexandria, VA 22350-3605

10. SPONSOR/MONITOR'S ACRONYM(S)
SERDP

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
ER-2623

12. DISTRIBUTION/AVAILABILITY STATEMENT
DISTRIBUTION STATEMENT A. Approved for public release: distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Legacy spills of chlorinated ethenes (CEs) remain one of the key environmental challenges, at DOD facilities and elsewhere, in the US and worldwide. At certain sites, abiotic degradation of CEs may be an important attenuation mechanism. Unequivocal demonstration and quantitation of abiotic contaminant mass destruction remains difficult, typically, due to the absence of pathway-specific degradation products or due to poor mass balance of such products. To fully benefit from abiotic degradation in contaminant remediation work, the process must be documentable. This study explored the utility of multi-element Compound-specific Isotope Analysis (CSIA) to identify evidence of abiotic degradation in scenarios with concurrent abiotic and biological degradation. The objective was to identify isotope parameters that would be diagnostic of abiotic pathways including in the present of degradation yield from concurrent biodegradation of the same CEs.

15. SUBJECT TERMS
Compound-specific Isotope Analysis, CSIA, Isotopes, Reductive dechlorination, Groundwater contaminants, Abiotic degradation, Hydrogen exchange

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Tomasz Kuder
UNCLASS	UNCLASS	UNCLASS	UNCLASS	48	**19b. TELEPHONE NUMBER** *(Include area code)* 405-325-4453

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List of Acronyms

CEs chlorinate ethenes

CSIA compound-specific isotope analysis

CSM conceptual site model

GC gas chromatograph

IRMS isotope ratio-monitoring mass spectrometer

MBTs molecular biological tools

MNA monitored natural attenuation

MTBE methyl tert-butyl ether

PCE perchloroethylene (tetrachloroethene)

qMS quadrupole mass spectrometer

TCE trichloroethene

ZVI zero-valent iron

Keywords: Compound-specific Isotope Analysis, CSIA, Isotopes, Reductive dechlorination, Groundwater contaminants, Abiotic degradation, Hydrogen exchange.

Acknowledgements.

I would like to thank Dr. Frank Loeffler (The University of Tennessee, Knoxville) for providing anaerobic cultures for this study, and Dr. Lee Krumholz (University of Oklahoma, Norman) for helpful advice on anaerobic microcosm setup is acknowledged. I thank Dr. Anat Bernstein and Dr. Almog Gafni (Ben Gurion University Negev, Israel), for sharing samples from their TCE degradation experiments, for hydrogen isotope analysis of TCE.

ABSTRACT

Introduction and Objectives. Legacy spills of chlorinated ethenes (CEs) remain one of the key environmental challenges, at DOD facilities and elsewhere, in the US and worldwide. At certain sites, abiotic degradation of CEs may be an important attenuation mechanism. Unequivocal demonstration and quantitation of abiotic contaminant mass destruction remains difficult, typically, due to the absence of pathway-specific degradation products or due to poor mass balance of such products. To fully benefit from abiotic degradation in contaminant remediation work, the process must be documentable. This study explored the utility of multi-element Compound-specific Isotope Analysis (CSIA) to identify evidence of abiotic degradation in scenarios with concurrent abiotic and biological degradation. The objective was to identify isotope parameters that would be diagnostic of abiotic pathways including in the present of degradation yield from concurrent biodegradation of the same CEs.

Technical Approach. Degradation experiments were conducted, and published data were evaluated to collect information of multi-element (C, Cl, H) isotope effects in reaction pathways that can be plausible expected to compete in two specific field scenarios: 1) aerobic environments conducive to abiotic degradation on iron mineral surfaces and to aerobic biodegradation; and 2) overlapping biotic and abiotic reductive dechlorination. Data were collected from microcosm experiments, with ZVI degradation serving a s model for abiotic reactions. Isotope effect data were collected from ZVI experiments, and from several aerobic and anaerobic biodegradation experiments. Parent CEs (PCE or TCE) and their dechlorination products (down to cDCE) were analyzed. The data were evaluated by pair-wise comparison of the specific reactions, to identify the characteristic of the isotope record that are most informative in pathway discrimination.

Results. Three highlights from this project are: (1) Hydrogen CSIA characterization of dechlorination products from biotic and abiotic reductive dechlorination appears to yield a highly specific information which clearly delineated biological and abiotic products yields based on their hydrogen isotope signatures. The approach is readily deployable in field site assessment to constrain the relative significance of biotic and abiotic reduction of CEs. (2) Carbon-chlorine CSIA can readily discriminate between aerobic biodegradation TCE and reductive dechlorination and possibly from Fenton-like reactions (more research is necessary to ascertain the latter). The results should be robust for systems with biodegradation dominated by one specific type of oxygenases, but mixed methane and toluene oxygenases can be confounding. (3) A study of CEs susceptibility to hydrogen exchange with water was performed for quality control purposes. The results from that study have broader significance for any application of hydrogen CSIA in the assessment of chlorinated solvents. One significant conclusion is that under certain conditions, hydrogen isotope signatures of TCE are subject to relatively rapid overprinting by the exchange and this should be borne in mind when planning H CSIA activities.

Benefits. More specific answers from CSIA should translate to better quality of contaminated site assessment and to cost savings though more effective site remediation. While this demonstration shows a promise for better characterization of sites with abiotic degradation processes, routine field deployment would benefit from real-world examples of successful applications to overcome reluctance of the remediation community.

EXECUTIVE SUMMARY

Introduction

Chlorinated ethenes (CEs) have been among the most widely used industrial solvents, with numerous large volume users within the structure of US DOD. CE solvents such as trichloroethene (TCE) and tetrachloroethene (PCE) are among the most commonly detected groundwater contaminants. Legacy CEs spills remain one of the key environmental challenges, at DOD facilities and elsewhere, in the US and worldwide.

Monitored natural attenuation (MNA) and miscellaneous active remediation solutions aim to eliminate the environmental impact of contaminants, either though in-situ destruction of the contaminant mass or by physical removal from the impacted aquifer or soil profile. Historically, monitored natural attenuation MNA of CEs relied on documenting the efficacy of biological reductive dechlorination. The challenge of degradation pathway-specific assessment is a limiting factor for incorporating abiotic degradation of CEs into conceptual site models (CSM) and realizing the benefits of such abiotic processes in contaminated sites remediation.

This project investigated several lines of evidence based on compound-specific stable isotope analysis (CSIA) as a prerequisite to applications in the assessment of abiotic degradation at contaminated sites. While CSIA was proven to be very useful in the assessment of biodegradation of CEs (Hunkeler et al., 2008), fewer studies centered on abiotic degradation. In effect, there are data gaps on isotope effects determined in controlled studies of key degradation mechanisms, in respect to hydrogen and for certain aspects of chlorine isotope fractionation. The incomplete reference data make CSIA less informative, in particular for scenarios with mixed biological/abiotic degradation, due to the inability to attribute changes of isotope ratios of contaminants specifically to abiotic degradation vs biological degradation.

Prior results from laboratory studies of abiotic degradation systems demonstrated that abiotic degradation of CEs can be expected to produce measurable carbon isotope fractionation, and for certain degradation pathways, chlorine and hydrogen fractionation. However, biodegradation is likely to contribute to the overall CEs mass destruction in nearly every plausible field scenario involving abiotic degradation. To be useful in the assessment of such mixed mechanism systems, CSIA results must be pathway-specific, so that the abiotic and biological degradation effects can be deconvoluted. Ideally, CSIA would be able to discriminate between alternative biological vs. abiotic mechanisms and permit quantitative allocation of contaminant degradation if more than one pathway were involved. For certain compounds, such as MtBE and benzene, CSIA has been demonstrated to provide such pathway-specific information.

The general objective of this project was to reduce the uncertainty of future contaminated site assessment projects, through exploration of C-Cl-H isotope data from several key CEs degradation pathways (biological and abiotic, to allow for comparing/contrasting between the two categories). The criterion of success for this exploration is to identify isotope characteristics that can be adopted as specific pathway indicators, suitable for differentiation of abiotic and biotic pathways that can plausibly overlap under field conditions. Ultimately, CSIA data would serve to improve the quality of conceptual models and permit more efficient and cost-effective contaminated site management.

Objectives

The present proof-of-concept is a demonstration of multi-element CSIA (including CSIA of chlorinated degradation products if such compounds are present) for discrimination between abiotic degradation and common biodegradation pathways. Isotope data from laboratory degradation experiments were obtained to address two specific field scenarios. The scenarios have been chosen to constrain the selection of degradation pathways to be discriminated from each other.

> Senario 1: Identification/discrimination of reaction mechanisms in oxic/suboxic environments conducive to aerobic biodegradation and to abiotic degradation on iron mineral surfaces. A typical field example of such site is an TCE plume with MNA Tier-1 evidence of TCE attenuation, but without confirmatory evidence of reductive dechlorination by geochemical footprints and degradation product.
>
> Scenario 2: Identification/discrimination of reaction mechanisms in anoxic environments conducive to reductive dechlorination, biological or abiotic. Typical field examples of such sites are locations with ZVI permeable reactive barriers (with biological reductive dechlorination augmenting the yield from ZVI degradation) or sites conducive to precipitation of reactive iron minerals, such as FeS.

Technical Approach

Interpretation of CSIA data from field sites requires sound understanding of isotope effects (isotope fractionations) associated with (bio)chemical transformations of the CEs. This is most important in applications of CSIA in assessment of contaminant transformations by competing degradation pathways, each associated with its own specific pattern of isotope fractionation. In the present case, the assumption is that abiotic degradation is not the sole or perhaps not even the dominant mechanism of CEs mass destruction, so that it is necessary to also consider isotope fractionation in biodegradation pathways that can be plausibly expected to impact the contaminant plumes under assessment.

The following categories of degradation systems were compared/contrasted with each other, using data from laboratory degradation experiment:

- Abiotic degradation – ZVI degradation of TCE as a model for “abiotic degradation. The pros and cons of using ZVI as the model abiotic reaction have been discussed in a following paragraph.
- Biodegradation – dechlorination of PCE and TCE by reductive dechlorination cultures.
- Biodegradation – dechlorination of TCE by cometabolic aerobic cultures (that data set combines carbon and chlorine isotope ratios obtained previously by a partner research group and hydrogen data obtained using archived samples from that former project).

The results from those controlled degradation experiments were evaluated, to specifically answer the question if CSIA can be informative in reaction pathway discrimination for two specific field scenarios.

Results and Discussion

Two promising lines of CSIA evidence for discrimination between competing biotic and abiotic degradation pathways were defined using present experimental data and published reference data. The first line of evidence is provided by hydrogen isotope ratios of reductive dechlorination precursors (TCE) and products (TCE in PCE degradation, cDCE in TCE degradation). Figure 1 illustrates the contrast between the abiotic ZVI reaction and biodegradation. Clearly, the mechanisms of product hydrogenation favor incorporation of ^{1}H over ^{2}H in the abiotic pathway. The difference is in the order of hundreds permil units and can be readily determined by current analytical methodology, for differentiation of abiotic and biological reductive dechlorination (cf. Scenario 2 defined in the preceding section).

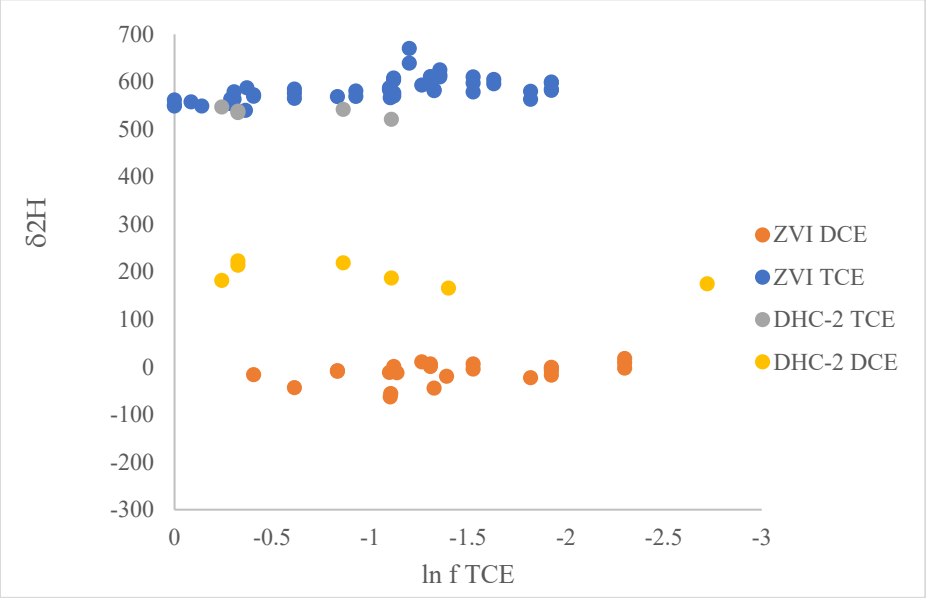

Figure 1. Hydrogen isotope ratios of TCE and cDCE vs remaining mass of TCE, in degradation of TCE on ZVI and in biodegradation of identical TCE by *Dehalococcoides mccartyi* strain 195. ZVI data represent four rounds of degradation experiments. The same lot of TCE was used in both experiments.

Hydrogen isotope ratios of reductive dechlorination products depend in part on the isotope signature of the precursor compound (along the dechlorination chain, new hydrogen atoms are inserted into the degradation products and hydrogen atoms already present in the precursor

compounds are transferred to the products as well). Moreover, the isotope signature of product hydrogenation is most likely anchored to ambient water, which serves as hydrogen donor or equilibrates rapidly with hydrogen produced by fermentation of organic substrates. Common isotope composition (δ^2H) of TCE falls between +600 and -200, depending on the origin of TCE (manufactured TCE solvent is commonly but not always at the positive end of the range) and whether TCE was impacted by isotope exchange with ambient water. Groundwater δ^2H is near zero in coastal areas, trending towards more negative values in the continental interior. Accordingly, numerical values shown in Figure 1 are representative of specific experimental conditions but would not necessarily apply at any specific field site. Figure 2 presents a comprehensive picture of the isotope ratios to be expected for different combinations of TCE precursor and water isotope signatures.

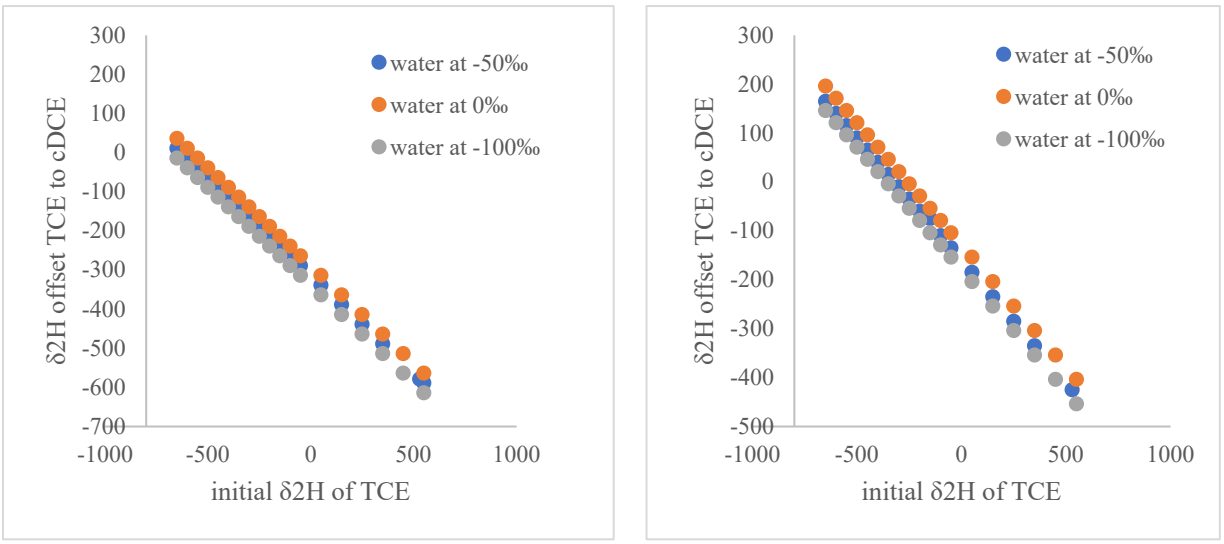

Figure 2. Offsets of δ^2H between TCE and cDCE in abiotic (A) and biological (B) reductive dechlorination. Panel B is based on isotope data in degradation of PCE to cDCE by *Desulfuromonas michiganensis* experiment (see the following section). Net value of δ^2H of cDCE is obtained by subtracting the value of offset (Y axis) form the corresponding δ^2H of TCE (X-axis).

The second line of CSIA evidence is dual element C-Cl CSIA applied to discrimination of aerobic biodegradation from reductive processes (biotic and abiotic alike). C-Cl characterization of aerobic organisms was included in the original proposal, but identical data were made available elsewhere in early stages of the project (A. Gafni, Environmental Sci. & Tech. Letters. 2018, 5, 202-208 and A. Gafni, Chemosphere, 2020, 242, 125130). This line of evidence applies to Scenario 1 defined in the preceding section. C-Cl trends are clearly divergent from reductive dechlorination trends (Figure 3). The role of hydrogen CSIA (dual element C-H) remains

unknown pending future studies, since ZVI may be not an ideal model reaction for abiotic reactivity under oxic/suboxic conditions.

Other lines of CSIA evidence were assessed and while it is possible to define discernible differences for pair-wise comparisons of abiotic and biological pathways, interpretation of those is more ambiguous and at this point they do not offer significant site assessment benefit specifically in biotic-abiotic reaction pathway differentiation. However, comprehensive, multi-element and multi-compound CSIA offers other benefits in site assessment, for example by decreasing the uncertainty of dealing with commingled contaminant sources, where changes of isotope ratios over distance or over time cold result from mixing of divergent source signatures rather than from degradation.

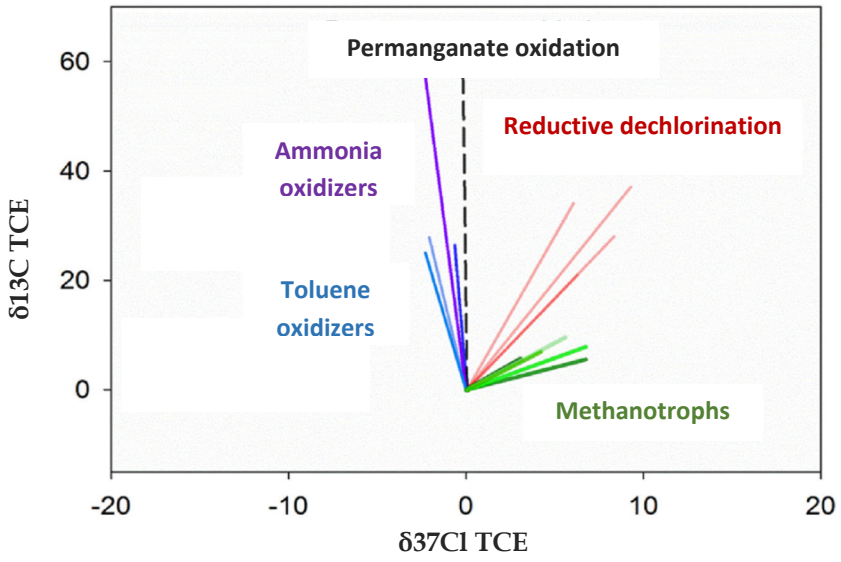

Figure 3. Dual element C-Cl trends in aerobic cometabolic biodegradation of TCE contrasted with reductive dechlorination. Isotope ratios are normalized to zero for the initial TCE isotope composition. Figure modified after Gafni et al. (Chemosphere, Volume 242, March 2020, 125130)

The third and final highlight of this project is the assessment of hydrogen isotope effects resulting from hydrogen exchange between CEs and water. Common perception of hydrogen exchange of carbon-bound hydrogen prevalent in isotope earth and environmental science community is that the process is only significant over geological time scales or at extreme geological conditions in deep subsurface. This turns out to be incorrect for certain chlorinated hydrocarbons, such as chloroform and CEs. The reaction is a simple acid-base proton exchange, with the dominant base being hydroxide ions and chlorinated hydrocarbons acting as weak Brønsted acids. The rate of the exchange process was shown to be dependent on the concentrations of hydroxide in solution (higher rates at higher pH). In this project, exchange experiments were conducted under a range of pH and temperatures similar to those encountered

in groundwater or microcosm media to identify the rates and the attendant changes of TCE and cDCE isotope composition. The overall message from that study is that the process is too slow to impact cDCE data at the laboratory or field settings, but the exchange can impact δ^2H of TCE in certain aquifers over timescales as short as months and certainly over years to decades. In general, hydrogen CSIA data from TCE would be increasingly affected for groundwater pH increasing above 7 and for aquifers situated in warm climates (Figure 4).

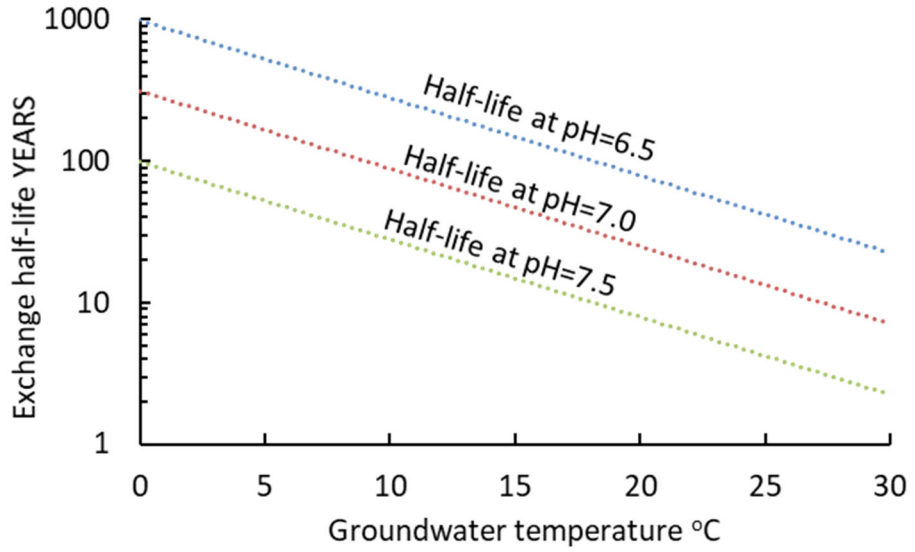

Figure 4. Hydrogen exchange half-lives as a function of aquifer pH and temperature. The rates are based on kinetic experiments performed as part of this project.

Implications for Future Research and Benefits

Higher degree of precision of contaminates site assessment translates to cost savings though more effective site remediation approach.

Two lines of CSIA evidence yielded most conclusive results in the laboratory demonstration. The first one is the discrimination between biotic and abiotic reductive dechlorination based on the differences in the extent of δ^2H depletion in dechlorination products, TCE and cDCE. The approach could be employed to assess the efficacy of ZVI remedies (by deconvolution of mass destruction by ZVI from that of ambient biodegradation) and possibly also to identify overlooked abiotic processes in anoxic aquifers conducive to such abiotic reductive dechlorination with FeS and other ferrous minerals.

The second line of evidence is the dual-element C-Cl, which appears to be a robust discriminative parameter for separation of aerobic degradation of TCE from reductive pathways (biotic and abiotic alike). The approach C-Cl could be implemented at any of the aerobic TCE plumes where aerobic degradation or abiotic degradation is part of or a feasible addition to the

conceptual site model. An additional lab study to confirm the C-Cl trend attendant of the Fenton-like pathway would be advisable to decrease the risk of uncertainty in data interpretation.

The results from this project illustrate the benefits of comprehensive, multi-element characterization of CEs. While the issue has not been part of this project, contributions from multiple of sources of CEs can complicate interpretation of degradation pathways. While certain lines of evidence can have no direct value in the discrimination of biotic vs abiotic pathways (e.g., chlorine in the context of reductive pathways), additional dimensions of isotope composition characterization may help in addressing isotope signature interferences from commingled sources of CEs. While the cost of analysis is proportionally higher for multi-element CSIA, such data sets are most informative for source discrimination and for degradation pathway assessment.

None of the presented lines of CSIA evidence is a silver bullet in terms of providing a specificity of a DNA fingerprint. Ideally, field site assessment should combine CSIA with other technologies, such as molecular biological tools (MBTs). MBTs can constrain the biological processes, to decrease the challenge for pathway discrimination by eliminating certain endmember reactions. For example, C-Cl evidence can be expected to be very robust for identification of dominant reaction process, in pair-wise reaction pathway comparison. For example, degradation of TCE by toluene oxygenase organisms is readily discernible from reductive elimination. On the other hand, C-Cl results can be more ambiguous at sites with significant contributions from multiple pathways. Using the example of cometabolic degradation of TCE, net isotope fractionation caused by mixed aerobic consortia utilizing a combination of toluene and methane oxygenases could in theory overlap with that resulting for reductive processes. Using MBTS to exclude significant contributions from methane monooxygenases would decrease the overall uncertainty.

Another valuable contribution from this project is better understanding of the role of hydrogen isotope exchange between chlorinated ethenes and water. While that line of research was initiated as a side project to assure the quality of microcosm data, the results are of general interest for users of hydrogen CSIA. Reaction kinetic of the exchange will help to avoid data misinterpretations in field site assessment, from sites where exchange processes overprinted contaminant source or degradation signatures.

OBJECTIVE

Chlorinated ethenes (CEs) have been among the most widely used industrial solvents, with numerous large volume users within the structure of US DOD. CE solvents such as trichloroethene (TCE) and tetrachloroethene (PCE) are among the most commonly detected groundwater contaminants. Legacy CEs spills remain one of the key environmental challenges, at DOD facilities and elsewhere, in the US and worldwide.

Monitored natural attenuation (MNA) and miscellaneous active remediation solutions aim to eliminate the environmental impact of contaminants, either though in-situ destruction of the contaminant mass or by physical removal from the impacted aquifer or soil profile. Historically, monitored natural attenuation MNA of CEs relied on documenting the efficacy of biological reductive dechlorination. The challenge of degradation pathway-specific assessment is a limiting factor for incorporating abiotic degradation of CEs into conceptual site models (CSM) and realizing the benefits of such abiotic processes in contaminated sites remediation.

This project investigated several lines of evidence based on compound-specific stable isotope analysis (CSIA) as a prerequisite to applications in the assessment of abiotic degradation at contaminated sites. While CSIA was proven to be very useful in the assessment of biodegradation of CEs (ref), fewer studies centered on abiotic degradation. In effect, there are data gaps on isotope effects determined in controlled studies of key degradation mechanisms, in respect to hydrogen and for certain aspects of chlorine isotope fractionation. The incomplete reference data make CSIA less informative, in particular for scenarios with mixed biological/abiotic degradation, due to the inability to attribute changes of isotope ratios of contaminants specifically to abiotic degradation vs biological degradation.

Prior results from laboratory studies of abiotic degradation systems demonstrated that abiotic degradation of CEs can be expected to produce measurable carbon isotope fractionation, and for certain degradation pathways, chlorine and hydrogen fractionation. However, biodegradation is likely to contribute to the overall CEs mass destruction in nearly every plausible field scenario involving abiotic degradation. To be useful in the assessment of such mixed mechanism systems, CSIA results must be pathway-specific, so that the abiotic and biological degradation effects can be deconvoluted. Ideally, CSIA would be able to discriminate between alternative biological vs. abiotic mechanisms and permit quantitative allocation of contaminant degradation if more than one pathway were involved. For certain compounds, such as MtBE and benzene, CSIA has been demonstrated to provide such pathway-specific information.

The general objective of this project was to reduce the uncertainty of future contaminated site assessment projects, through exploration of C-Cl-H isotope data from several key CEs degradation pathways (biological and abiotic, to allow for comparing/contrasting between the two categories). The criterion of success for this exploration is to identify isotope characteristics that can be adopted as specific pathway indicators, suitable for differentiation of abiotic and biotic pathways that can plausibly overlap under field conditions. Ultimately, CSIA data would serve to improve the quality of conceptual models and permit more efficient and cost-effective contaminated site management.

BACKGROUND

In foreseeable future, remediation of chlorinated ethenes contamination will remain a considerable economic burden to DOD and to other stakeholders, in the US and worldwide. MNA and miscellaneous active remediation solutions aim to decrease the environmental impact of the contaminants, either though in-situ destruction of the contaminant mass or by physical removal from the impacted aquifer or soil profile. At certain sites, naturally occurring degradation processes can be sufficient to assure a timely closure, following established MNA protocols. At other sites, natural or stimulated degradation can be an element of the overall remediation effort. In any case, site assessment and development of a robust conceptual site model (CSM) are key prerequisites of a successful remediation project.

At certain sites, abiotic degradation of chlorinated ethenes may be an important addition to the net attenuation. Unequivocal demonstration and quantitation of abiotic contaminant mass destruction yield remains difficult, typically, due to the absence of detectable pathway-specific degradation products or due to the transient nature of such products and resulting poor mass balance based on such products. Therefore, means to identify the dominant degradation pathway(s) that do not rely on product mass balance and do not involve time-consuming experimental work (such as "abiotic microcosms") would be very valuable. Molecular biological tools and CSIA are relatively recent technologies that have been used extensively over the past couple of decades to collect such evidence in the assessment of biological degradation.

CSIA can be potentially a valuable tool for abiotic degradation assessment, provided that the isotope signal determined by CSIA can be unequivocally attributed to a specific abiotic pathway, as opposed to a generic "degradation process".

CSIA publications relevant to the issue of mechanism discrimination fall into two categories. The first category is characterization of isotope for specific degradation pathway using samples from well-constrained lab degradation experiments. Such basic reference data must be available to permit reaction pathways to be discriminated from each other. Relevant work includes abiotic and biological pathways impacting the contaminants of interest in a specific environment. For example, if the objective is an investigation of a site with redox conditions conducive to reductive dechlorination, reference data on abiotic reductive dechlorination AND on biotic deductive dechlorination of the same target contaminant are necessary to distinguish one from the other. The second category is application of CSIA to identify abiotic degradation evidence in field samples.

Significant body of published work was dedicated to characterization of carbon isotope effects in dechlorination of common CEs, by microorganisms, cell-free enzyme extract, in aerobic cometabolic biodegradation, in abiotic reactions with corrinoid compounds, in abiotic reactions with zero-valent iron (ZVI) and iron minerals, permanganate, radicals, and others (reviews of the literature can be found in Hunkeler et al., 2008; Ojeda et al., 2020). Combined carbon-chlorine isotope effects were reported for most of the reductive dechlorination pathways, for the parent CEs. On the other hand, there are few reports of chlorine isotope compositions of reductive dechlorination products (Wiegert et al., 2012; Kuder et al., 2013; Cretnik et al., 2014) virtually no reports on hydrogen isotope effects in those reactions (except the Kuder et al., 2013 paper limited to a single *Dehalococcoides* culture, and two early studies utilizing bulk isotope methods

with results that are likely biased by methodological limitations of the period; Ertl et al., 1998; Shouakar-Stash et al., 2003).

Since preparation of the project proposal, there have been additional published contributions on characterization of carbon and chlorine isotope effects in degradation pathways of CEs. A comprehensive summary of those recent advances is available in a recent review by Ojeda et al. (2020). One significant addition to the body of knowledge is a study of TCE degradation by aerobic cometabolic organisms, reporting both carbon and chlorine isotope effects (Gafni et al., 2018 and 2020). Since these publications overlapped with some of the objectives of the present project, the scope of experimental work was modified accordingly (see below, Methods, section "Biological cometabolic aerobic dechlorination experiments").

These published results facilitate certain general conclusions. Literature data show overlapping dual-element C-Cl CSIA trends and overlapping net magnitudes of carbon and chlorine fractionation for most studied dechlorination reactions, so that the pathways would be hard to differentiate using CSIA data on the parent CE alone.

Few studies directly addressed the use of CSIA data in discrimination of abiotic and biotic degradation pathways in-situ or for validation of abiotic pathways active at field sites. These studies relied exclusively on C isotope ratios. Early applications of CSIA in the assessment of abiotic degradation (reductive dechlorination mediated by iron mineral surfaces) postulated that abiotic reductive dechlorination can be distinguished from biological reductive dechlorination by the relatively larger magnitudes of C isotope fractionation in the abiotic reactions (Liang et al., 2007). That proposition is no longer accepted due to reports of equally strong isotope effects associated with biodegradation. Nevertheless, carbon CSIA can be still informative in specific contexts, mainly in the applications centering on the assessment of active remedies such as ZVI barriers. This includes an application based on carbon isotope composition of degradation products (C_2 hydrocarbon gases) produced at ZVI treatment sites (Elsner et al., 2008) and applications where samples are collected prior to, during and after in-situ treatment, to evaluate the contribution of the treatment to the overall attenuation budget (Audi Miro et al., 2015). To date, other than the recently published rationale for using dual element C-Cl CSIA to discriminate between aerobic biodegradation of TCE vs reductive dechlorination of TCE (Gafni et al., 2018 and 2020), no CSIA applications offered a clear outlook for validation of abiotic degradation processes at sites with "native" degradation processes.

Proof-of-concept statement. The present proof-of-concept is a demonstration of multi-element CSIA (including CSIA of chlorinated degradation products if such compounds are present) for discrimination between abiotic degradation and common biodegradation pathways. Two specific field scenarios have been chosen to constrain the selection of degradation pathways to be discriminated from each other.

> Senario 1: Identification/discrimination of reaction mechanisms in oxic/suboxic environments conducive to aerobic biodegradation and to abiotic degradation on iron mineral surfaces. A typical field example of such site is an TCE plume with MNA Tier-1 evidence of TCE attenuation, but without confirmatory evidence of reductive dechlorination by geochemical footprints and degradation product.

Scenario 2: Identification/discrimination of reaction mechanisms in anoxic environments conducive to reductive dechlorination, biological or abiotic. Typical field examples of such sites are locations with ZVI permeable reactive barriers (with biological reductive dechlorination augmenting the yield from ZVI degradation) or sites conducive to precipitation of reactive iron minerals, such as FeS.

MATERIALS AND METHODS

Overall design of the study

Interpretation of CSIA data from field sites requires sound understanding of isotope effects (isotope fractionations) associated with (bio)chemical transformations of the CEs. This is most important in applications of CSIA in assessment of contaminant transformations by competing degradation pathways, each associated with its own specific pattern of isotope fractionation. In the present case, the assumption is that abiotic degradation is not the sole or perhaps not even the dominant mechanism of CEs mass destruction, so that it is necessary to also consider isotope fractionation in biodegradation pathways that can be plausibly expected to impact the contaminant plumes under assessment.

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- Biodegradation – dechlorination of PCE and TCE by reductive dechlorination cultures.
- Biodegradation – dechlorination of TCE by cometabolic aerobic cultures.

The results from those controlled degradation experiments were evaluated, to specifically answer the question is CSIA can be informative in reaction pathway discrimination for two specific field scenarios. The Scenario-specific data evaluation has been chosen to constrain the number of reaction pathways to be discriminated from each other.

While the rationale for the chosen biological degradation systems appears to be self-explanatory, the choice of the abiotic component is more complicated. Ideally, isotope effects should be studied using authentic minerals or direct synthetic analogues of the minerals responsible to the reactivity under field conditions. Such studies would be time-consuming and cost-prohibitive for a limited-scope project, due to slow reaction kinetics of such reactions. ZVI offers logistic benefits of fast reaction kinetics and relatively simple microcosm maintenance, but it is not necessarily an ideal or universal model for "abiotic reactions with iron minerals". Reductive dechlorination of CEs by ZVI follows the dominant pathway of β-elimination to produce acetylene, ethene and ethane, with a minor pathway of hydrogenolysis, producing the typical chlorinated intermediates (cDCE in degradation of TCE). The yield of the hydrogenolysis pathways varies, depending on the type of iron and the degradation environment. Based on the shared degradation products, ZVI appears to be a good model of reactions with ferrous minerals in reducing environments, although the relative significance of β-elimination and hydrogenolysis

in different degradation systems could well vary. Moreover, dual-element C-Cl trends among diverse biotic and abiotic reductive dechlorination pathways are similar to each other, supporting broad similarity of the transformations involved in those pathways.

The question of whether ZVI is good model for the isotope effects in abiotic pathways in oxic/suboxic environments is more difficult to answer and will likely require input from future research (cf. ongoing project ER20-1368, Development of Protocols to Quantify Abiotic Transformation Rates and Mechanisms for Chlorinated Ethenes in Water Supply Aquifers). Results made available recently, including those from the SERDP's abiotic degradation program suggest that the classic ZVI reaction may be inappropriate as a generic model of "abiotic reaction pathway" for oxic/suboxic conditions. One possibility for such environments is a Fenton reaction-like pathway, where the reactive species is hydroxyl radical (Schaefer et al., 2018). One report to date, by Liu et al. (2014) shows a dual element C-Cl trend for TCE from a similar reaction type, resulting with a dual element C-Cl trend that is not distinguishable from that of ZVI. The limitation of Liu's study is that the chlorine isotope results were obtained not directly for TCE but for the total volatile chlorine present in the reaction vessel, with the unverified assumption of the absence of volatile chlorine-bearing degradation products. If the Fenton reaction result is confirmed by compound-specific methods, the present conclusions, at least regarding the evidence from dual element C-Cl, remain valid for scenarios with abiotic Fenton reactivity.

Finally, to validate the use of hydrogen CSIA in characterization of the reductive pathways, a study of the effects of hydrogen isotope exchange was conducted. The study highlighted risks attendant to evaluation of sites with conditions conducive to TCE-water exchange (at elevated pH and in warm climates) but confirmed the rates of the exchange should be negligible in short-termed lab experiments and long-term, at sub-neutral pH (Appendix A).

Chemicals

TCE and PCE used for the separate experiments were shared among experiments. The same lot of TCE was used to amend ZVI microcosms, the TCE-degrading anaerobic biological microcosms and hydrogen exchange batch experiments. The same lot of PCE was used to amend all PCE-degrading microcosms.

Degradation experiments with ZVI

The experimental setup was modified after Arnold and Roberts (2000), with container sizes scaled down for sacrificial sampling of multiple vials prepared and incubated under the same conditions. Preliminary trials were performed using CC-1200 Connelly Iron (50-60 mesh) and 100 mesh Iron Electrolytic Powder, Fisher Chemical™. Due to low yield of cDCE from the latter, all experiments harvested for CSIA samples were conducted with the Connelly iron.

Four ZVI experiments were sampled for CSIA. Each experiment consisted of a set of identical 20 ml serum vials sealed with Teflon faced butyl rubber septa, with 3 grams of ZVI each, were incubated on a roller, protected from atmospheric oxygen by sealing in nitrogen-flushed mason jar. Vials were sampled sacrificially, with the time intervals for sampling based on degradation kinetic determined in a preliminary experiment and then the time intervals were adjusted based

on real-time concentration data from consecutive samples. Three sets of samples for CSIA were prepared without TRIS buffer. A fourth set of vials was prepared with TRIS buffer. Initial concentrations of TCE were set at 20-30 ug/l. In the TRIS experiment, pH was stabilized at 7.2.

Biological reductive dechlorination experiments

Samples for CSIA were obtained from degradation experiments with *Desulfuromonas michiganensis* strain BB1, Bio-Dechlor Inoculum, a mixed *Dehalococcoides* culture, *Geobacter lovleyi* strain SZ and *Dehalococcoides mccartyi* strain 195. The cultures were kindly provided by Dr. Frank Loeffler, University of Tennessee. Incubations were conducted at University of Oklahoma with help from of Dr. Lee Krumholz (Microbiology Dept). Experimental procedures were similar to those adopted previously by the same laboratory (Kuder et al., 2013), with a key difference of the serum bottles (160 ml) filled with 120 ml of medium, resulting with a considerable headspace volume. Headpace was avoided in the 2013 study to improve the quality of concentration data by eliminating liquid-headspace partitioning.

All four cultures were amended with PCE, with two replicate bottles incubated and sampled to replicate the isotope data. Additionally, incubations were repeated with *G lovleyi*. A separate TCE degradation experiment, also using two replicate bottles, was conducted with *D. mccartyi*. Serum bottles were sampled sacrificially and analyzed immediately for CEs concentrations. Sampling intervals were estimated based on those real time data. Aliquots of the microcosm media scheduled for CSIA were transferred to standard VOA vials prefilled with VOC-free water and acidified to pH 1 using HCl and refrigerated or frozen prior to analysis. For selected degradation experiments, splits of the CSIA samples were collected into 20 ml serum vials with gray Teflon-lined septa and frozen for long term storage. The frozen vials contained approx.1/3 of headspace to prevent glass damage by expanding ice and the vials were inverted to create an ice plug at the vial outlet. Freezing of samples for CSIA was validated elsewhere. (Elsner et al., 2006)

Biological cometabolic aerobic dechlorination experiments

Samples from experiments on aerobic degradation of TCE by cometabolic organisms (*Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase, *Methylosinus trichosporium* OB3b expressing particulate MMO, *Methylococcus capsulatus* Bath expressing soluble methane monooxygenase, and *Pseudomonas putida* F1 expressing toluene dioxygenase) were shared by the Israeli partner. The samples were produced in biodegradation experiments performed for a project specifically focused on the assessment of carbon and chlorine isotope effects in cometabolic degradation of TCE. The Israeli group obtained and published an extensive data set on those two elements. Archived (frozen) samples from that study were shipped to the laboratory at the University of Oklahoma for hydrogen CSIA. Carbon and chlorine results discussed below are the results from the Israeli study. Experimental procedures pertaining to aerobic degradation of the samples analyzed f or hydrogen isotope ratios of TCE are available in two publications of the Israeli group. (Gafni et al., 2018 and 2020)

Hydrogen Exchange Experiments

A standalone description of the experimental procedures is included in Appendix A.

Analytical chemistry

Concentrations of the parent TCE or PCE and the concentrations of degradation products in reductive dechlorination experiments (TCE and cDCE in degradation of PCE; cDCE in degradation of TCE) were determined by purge and trap/GC/MS following the procedures described previously (Kuder et al., 2013). For degradation experiments performed in the later part of the project, concentrations were determined by sampling of headspace from the microcosm bottles, followed by analysis by GC with a flame ionization detector.

Carbon, chlorine and hydrogen isotope ratios were determined using the instrumentation and methodology described previously. (Kuder et al., 2013) In brief, samples of microcosm medium with target CE compounds were analyzed by purge and trap/GC/IRMS for their carbon and hydrogen isotope ratios, and by purge and trap/GC/qMS for their chlorine isotope ratios. Concepts pertaining to data presentation (Rayleigh-type plots and dual element CSIA plots) and isotope terminology have been extensively described elsewhere (Hunkeler et al., 2008; Elsner, 2010; Elsner and Imfeld, 2016). Detailed presentation of the recent iteration of the hydrogen CSIA methodology is included in the Methods section of Appendix A.

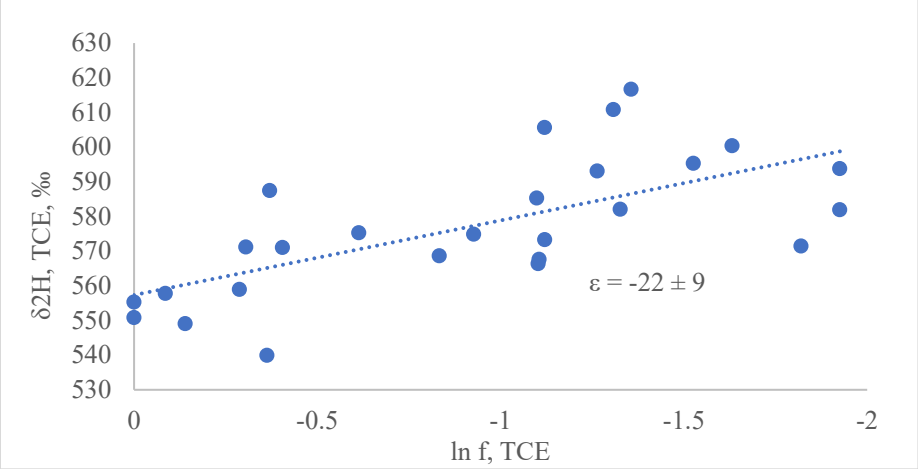

Figure 1. Rayleigh-type plot of TCE degradation on ZVI. X axis represents the natural log of TCE fraction remaining. Data represent four rounds of degradation experiments.

RESULTS AND DISCUSSION

Isotope Effects in Abiotic (ZVI) Reductive Dechlorination

Reaction kinetics and carbon isotope results in the ZVI experiments were consistent with expectations based on previously published work. Similarly, dual element C-Cl trends of TCE were like those reported previously for ZVI degradation. Together, these results confirm the present ZVI data set is representative of former reports on ZVI degradation. These results are peripheral to the present proof-of-concept.

Two elements of the ZVI data set are of primary interest as potential novel lines of CSIA evidence: 1) the hydrogen isotope data from CSIA of TCE (parent CE) and cDCE (daughter CE); and 2) chlorine isotope data for the parent -daughter pair of CEs (TCE and cDCE).

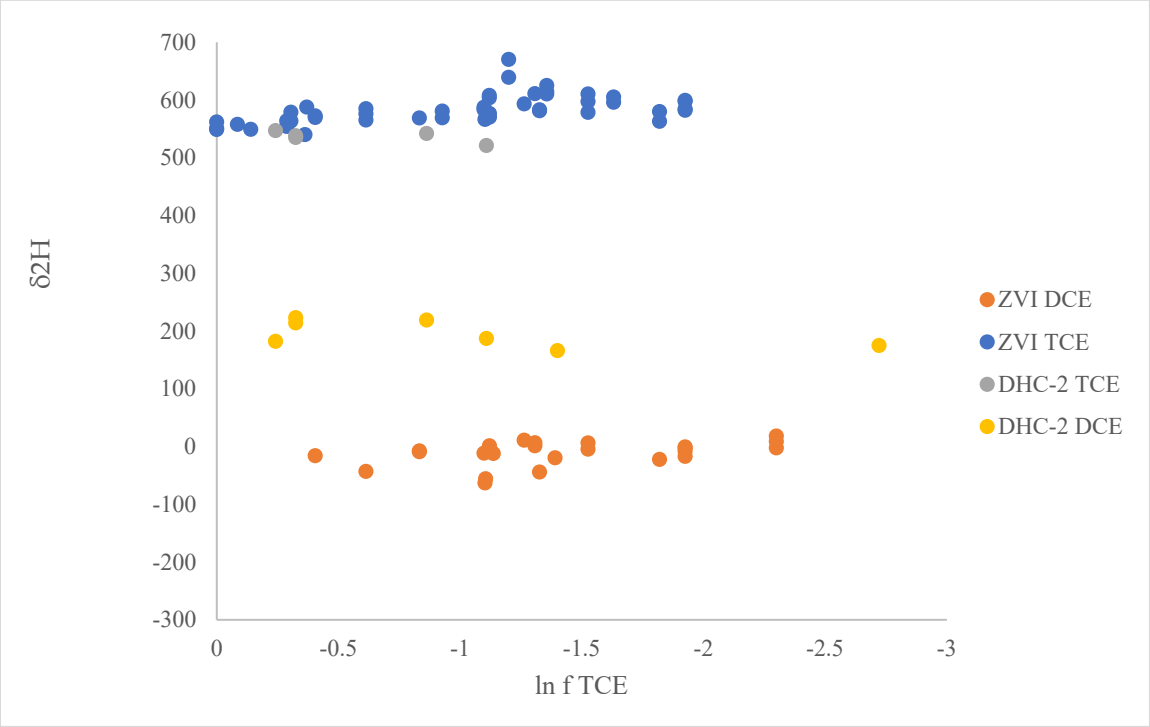

Figure 2. Hydrogen isotope ratios of TCE and cDCE vs remaining mass of TCE, in degradation of TCE on ZVI and in biodegradation of identical TCE by *Dehalococcoides mccartyi* strain 195. ZVI data represent four rounds of degradation experiments.

Hydrogen isotope ratios of TCE in all four degradation experiments (three unbuffered, one with TRIS buffer) show a moderate enrichment of ^{2}H progressing over the course of TCE degradation

(Figure 1). The Rayleigh-type regression of the isotope ratios against a log scale of concentrations of TCE is consistent with the isotope enrichment resulting from preferential degradation of ^{1}H-TCE. Since no C-H bond is broken in the reaction, the enrichment represents a secondary kinetic effect, with ^{2}H stabilizing the adjacent C-Cl bonds. Enrichment factor (ε) determined using data pooled from the four degradation experiments is -22 ± 9‰.

Hydrogen isotope ratios of cDCE in all four degradation experiments show a significant depletion of ^{2}H relatively to the precursor TCE (Figure 2), seemingly followed by a moderate enrichment trailing that of TCE (the regression slope is not statistically significant). If further degradation of cDCE resulted with a kinetic effect of its own, the magnitude of such effect was negligible.

The offset of $\delta^{2}H$ between TCE and cDCE is approximately 510‰, in water at $\delta^{2}H$ of -42‰. One of the two H atoms of cDCE is inherited from the precursor TCE, the second H atom is inserted during the transformation step. The isotope signature of the newly added hydrogen ($\delta^{2}H_{H\text{-}new}$) can be determined using Equation 1, modified after Kuder et al. (2013) to account for the kinetic isotope effect in TCE degradation, yielding the value of $\delta^{2}H_{H\text{-}new}$ = -610‰.

Equation 1. $$\delta^{2}H_{H\text{-}new} = 2 \times \delta^{2}H_{DCE} - (\delta^{2}H_{TCE} + \varepsilon_{TCE})$$

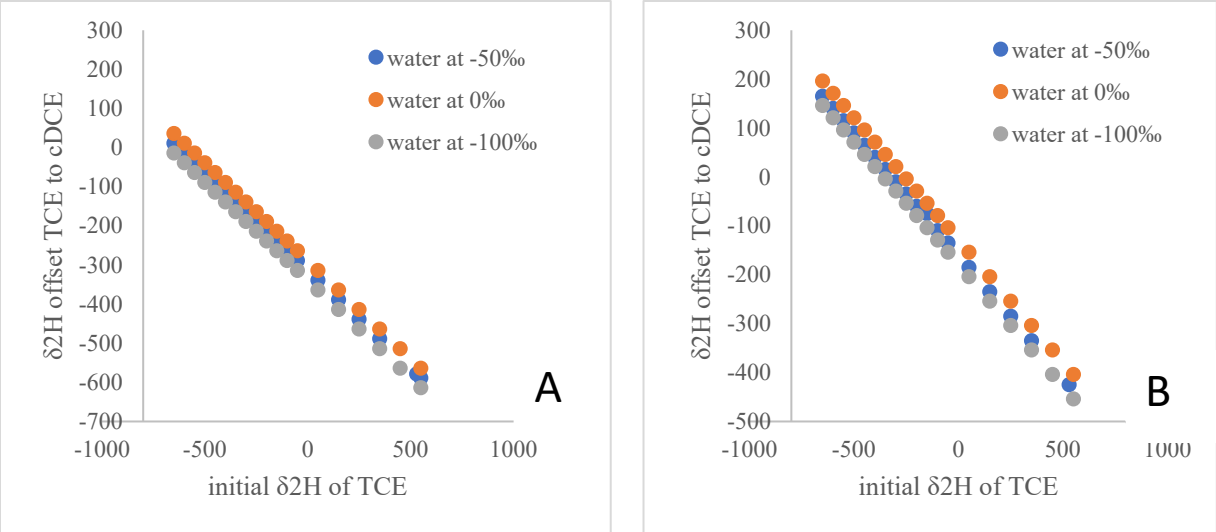

Figure 3. Offsets of $\delta^{2}H$ between TCE and cDCE in abiotic (A) and biological (B) reductive dechlorination. Panel B is based on isotope data in degradation of PCE to cDCE by *Desulfuromonas michiganensis* experiment (see the following section).

Since the hydrogen atom it ultimately derived from ambient water, it can be assumed that $\delta^2H_{H\text{-}new}$ is benchmarked to the isotope composition of microcosm water. Accordingly, the value of $\delta^2H_{H\text{-}new}$ would change proportionally to the δ^2H of water. In the present case, the newly added hydrogen is depleted relative to microcosm medium water by approximately -570 permil. Assuming the measured secondary isotope effect is representative of reductive dechlorination on ZVI (see the following paragraphs for discussion of potential uncertainties), Figure 3A illustrates the magnitude of the TCE-cDCE offset as a function of the isotope composition of ambient water and the isotope signature of the precursor TCE. The magnitude of the offset can serve as a robust pathway discrimination criterion since the offsets in biological reductive dechlorination are much lower (see the following section and Figure 3B).

There are few reports of hydrogen isotope effects in ZVI reactions. The sole peer-reviewed contribution is a 2003 paper by Shouakar-Stash et al. (2003), showing a much lower -120 permil depletion of δ^2H (relative to ambient water) in TCE produced by dechlorination of PCE. It is more than likely that the reported -120 permil is grossly underestimated. Shouakar-Stash obtained his result from a bulk purge and trap effluent where TCE was commingled with water vapor. In effect, bulk isotope signatures comprised a mix TCE and water, the latter diluting the overall hydrogen signature towards less depleted values.

The second hydrogen isotope data set is a 2016 master thesis (Ribeiro, 2016) and it appears that these data were never published in a peer-reviewed publication. Interestingly, hydrogen isotope fractionation in degradation of TCE reported by Ribeiro was very different from that observed in the present project even if the experimental conditions (other than the type of iron) were similar. Instead of a moderate enrichment of δ^2H over the course of degradation, Ribeiro observed a strong trend of depletion of δ^2H. Unfortunately, the description of H CSIA methodology in that thesis is scarce and the laboratory performing the analyses never published any other applications of purge and trap-H CSIA, so that it is difficult to determine if the presented analytical data can be taken at face value. If the observed depletion of TCE were real, the difference does not have a simple explanation. One possibility is that the ^2H depletion trend reported by Ribeiro resulted from a relatively rapid hydrogen isotope exchange, occurring at faster rate that that predicted by the concentrations of hydroxide in water at near-neutral pH (cf. Appendix A). Unlike this study, Ribeiro used iron nanoparticles (40-60 nm) rather than the much coarser particulate ZVI, and hydroxide yield at iron particle surface could be dependent on the iron type, potentially creating a boundary layer susceptible to fast hydrogen exchange even in a buffered solution with bulk water close to neutral pH. Future studies should clarify if the hydrogen isotope fractionation is indeed significantly affected by the properties of ZVI or reactive iron minerals and whether iron solids can promote rapid hydrogen exchange.

Chlorine isotope ratios of TCE and cDCE in the early stages of the reaction show a moderate offset (depletion of $\delta^{37}Cl$ of cDCE by ~1.4 ‰, Figure 4) consistent among the four degradation experiments. In the later stages of degradation, the values of $\delta^{37}Cl$ of cDCE showed a progressive increase and eventually exceeded the time zero value of the precursor TCE. The trend of increasing $\delta^{37}Cl$ of cDCE is consistent with progressive degradation of cDCE, which is to be expected in a ZVI reaction.

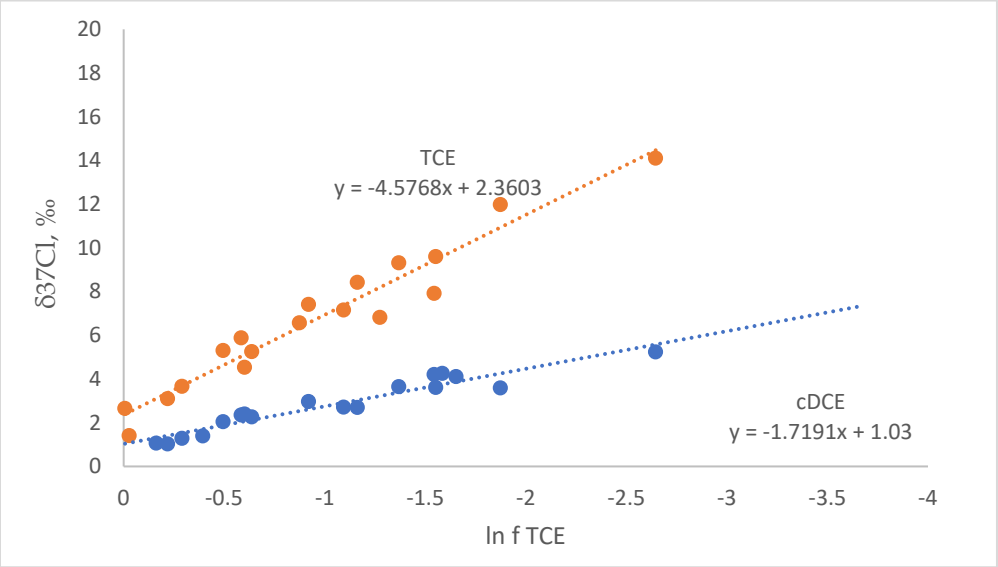

Figure 4. Rayleigh-type plot of chlorine isotope data in ZVI degradation of TCE. The early product depletion of cDCE can be read from the regression intercepts. TCE reactant is isotopically identical as in the data set shown in Figure 7.

Isotope Effects in Biological Reductive Dechlorination

Carbon and hydrogen isotope ratios in biological reductive dechlorination were determined for degradation of PCE by three of the studied cultures: *Desulfuromonas michiganensis* strain BB1, Bio-Dechlor Inoculum (BDI), a mixed *Dehalococcoides* culture, and *Geobacter lovleyi* strain SZ. Carbon and hydrogen isotope ratios were also determined for degradation of TCE by *Dehalococcoides mccartyi* strain 195. Chlorine isotope ratios for PCE and daughter compounds, TCE and cDCE were determined for all four cultures.

As in the previous section on ZVI results, two elements of the biological reductive dechlorination data set are of primary interest as potential novel lines of CSIA evidence: 1) the hydrogen isotope data from CSIA of TCE (parent CE) and cDCE (daughter CE); and 2) chlorine isotope data for the parent-daughter pair of CEs (PCE-TCE and TCE-cDCE).

The values of carbon enrichment factors for individual transformations (PCE to TCE and TCE to cDCE) are consistent with previous reports for the same cultures, except of the lower-than-expected enrichment factor for PCE-TCE transformation by the BDI culture (enrichment factor of only -1‰). BDI is a mixed *Dehalococcoides* culture, and it is possible that the change represents a difference in expression of member species. All dual element C-Cl trends were consistent with previous reports from reductive dechlorination experiments (not shown).

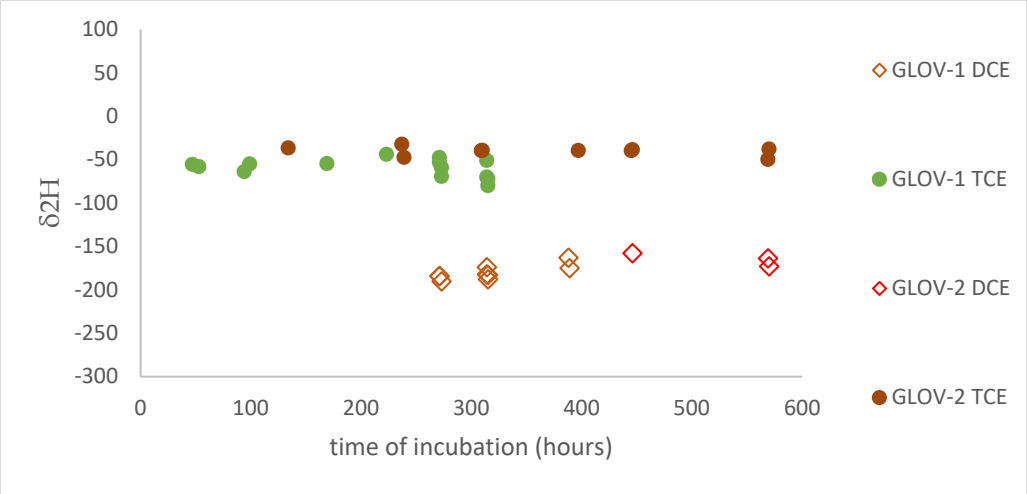

Figure 5A. Parent-daughter (TCE-cDCE) hydrogen isotope ratios in two separate rounds of PCE degradation by *Geobacter lovleyi.*

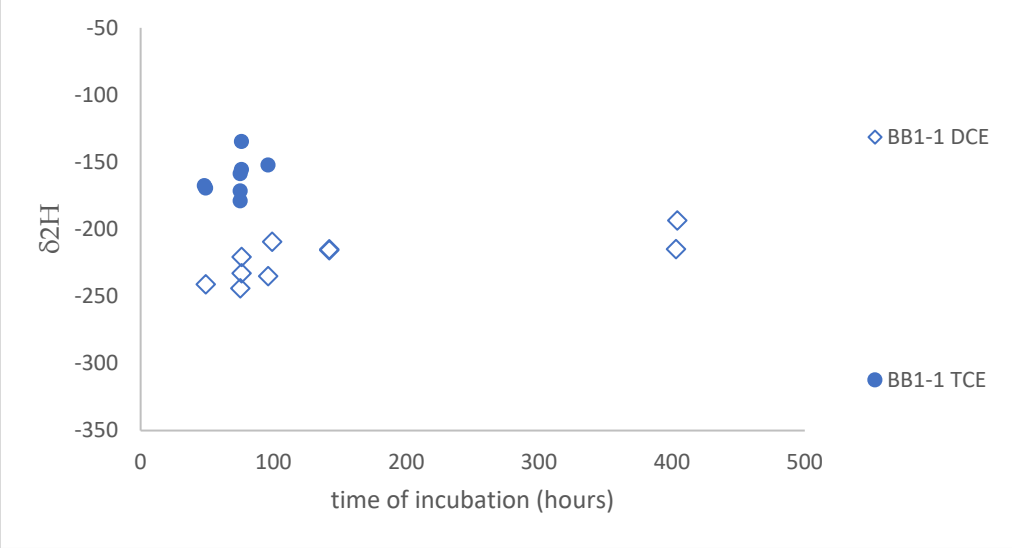

Figure 5B. Parent-daughter (TCE-cDCE) hydrogen isotope ratios in PCE degradation by *Desulfuromonas michiganensis*. Note the depletion in TCE is larger than those in Figures 5A and 5C.

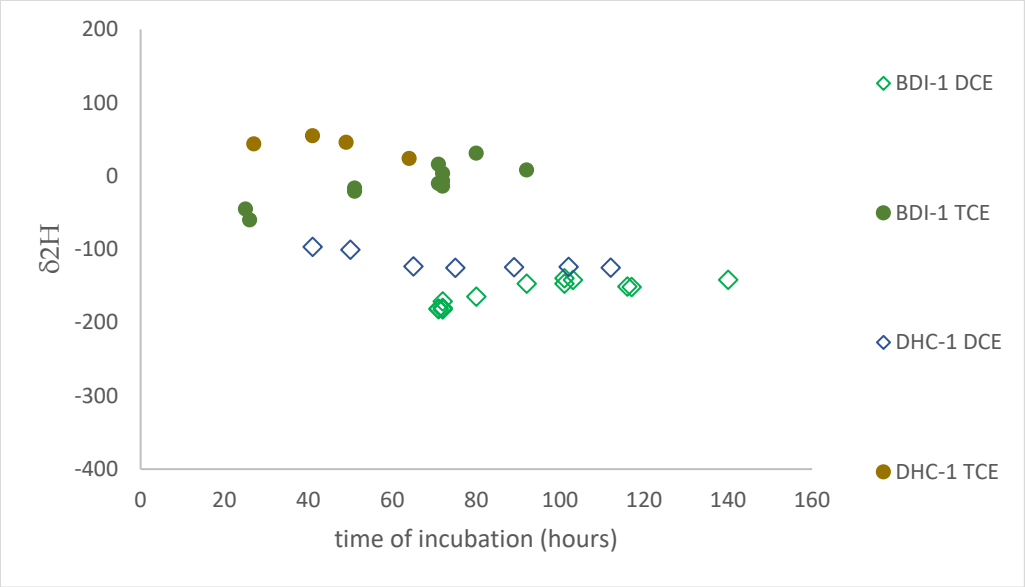

Figure 5C. Parent-daughter (TCE-cDCE) hydrogen isotope ratios in PCE degradation by Bio-Dechlor Inoculum (BDI), a mixed *Dehalococcoides* culture, and *Dehalococcoides mccartyi* (DHC).

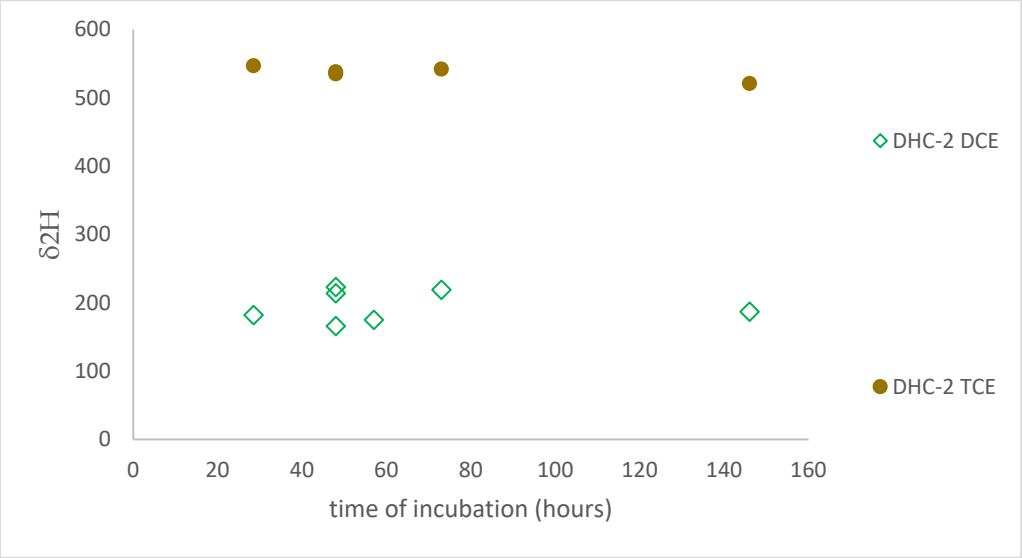

Figure 5D. Parent-daughter (TCE-cDCE) hydrogen isotope ratios in TCE degradation by *Dehalococcoides mccartyi* (DHC). Note the larger offset between TCE and cDCE than that in Figure 5C. The difference is due to a more positive δ^2H of the TCE precursor.

Hydrogen isotope ratios of TCE in all but one PCE-degrading microcosms show δ^2H values falling between -50 and +50‰ (Figures 5A-D). This is surprising, since the values are relatively close or even more ^2H-enriched vs microcosm water (-42‰). TCE product in *D. michiganensis* microcosms was more depleted, with δ^2H of approximately -170‰. The values of cDCE were relatively depleted vs TCE in the same microcosms, by approximately 120‰ (80‰ in *D. michiganensis* microcosms). The depletions converted to the isotope signatures of hydrogen added to the product compounds by hydrogenolysis (Equation 1 with the secondary kinetic effect for TCE degradation set at zero for the cultures where TCE was the reaction intermediate) show a very consistent distribution of the results, with $\delta^2H_{H\text{-new}}$ of TCE-cDCE transformation clustering around near -300‰ for the cultures with PCE precursor and -200‰ for *D. mccartyi* with TCE precursor and in the historical data on the BDI culture with TCE precursor (Kuder et al., 2013). The present values of $\delta^2H_{H\text{-new}}$ are strongly contrasting with the results from the ZVI experiment (Figure 2).

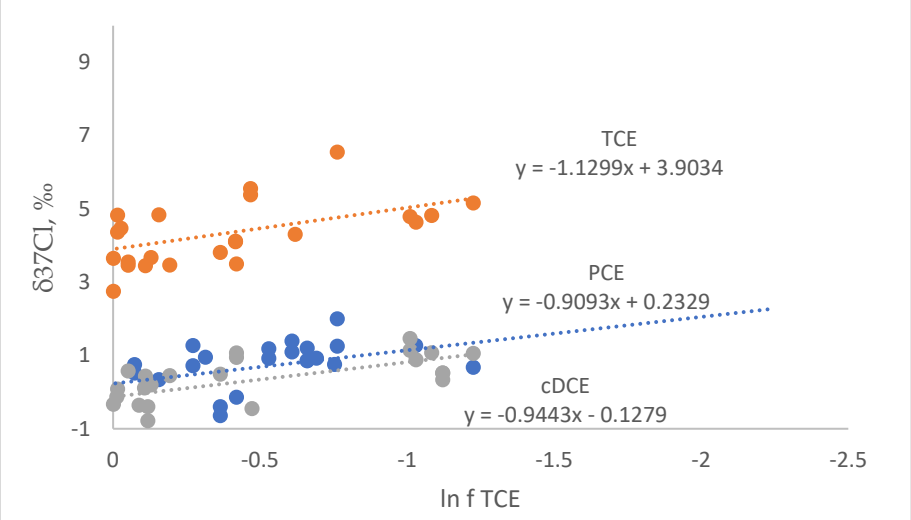

Figure 6. Rayleigh-type plot of chlorine isotope data degradation of PCE by *Desulfuromonas michiganensi*, Bio-Dechlor Inoculum (BDI), *Geobacter lovleyi*. The early product depletion of cDCE and the early product enrichment of TCE can be read from the regression intercepts. The same PCE reactant was used in all experiments.

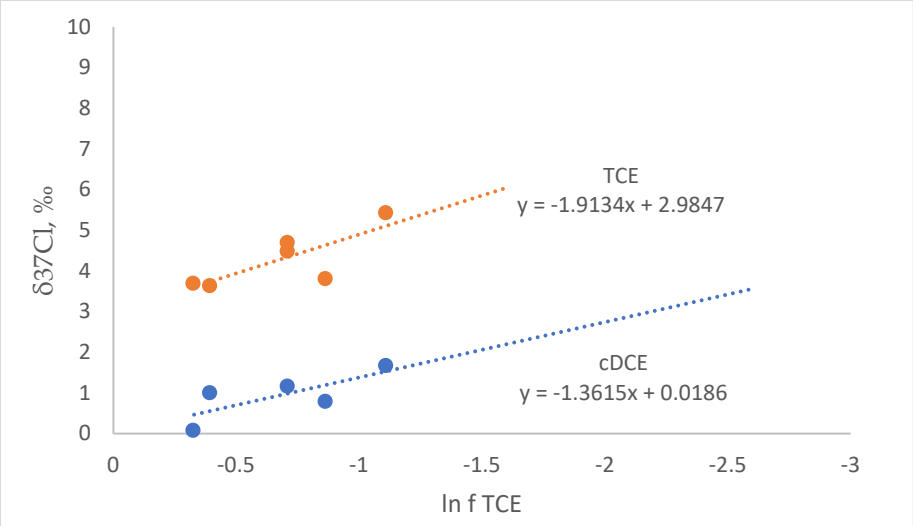

Figure 7. Rayleigh-type plot of chlorine isotope data in degradation of TCE by *Dehalococcoides mccartyi.* The early product depletion of cDCE can be read from the regression intercepts. TCE reactant is isotopically identical as in the data set shown in Figure 4.

In the early stages of the degradation progress, all PCE degradation experiments displayed a distinct offset of $\delta^{37}Cl$ for the parent-daughter CEs, with $\delta^{37}Cl$ of TCE enriched by ~4‰ vs that of PCE and with $\delta^{37}Cl$ of cDCE depleted by ~3.7‰ vs that of TCE (Figure 6). Chlorine isotope ratios of TCE and cDCE in the early stages of the *D. mccartyi* experiment show a somewhat larger offset than that in the ZVI experiments but smaller than that for the cultures fed with PCE rather than TCE (depletion of $\delta^{37}Cl$ of cDCE estimated at ~3.0‰, Figure 7). The observed pattern of depletions and enrichments of the $\delta^{37}Cl$ values among the dechlorination products cannot be confidently explained at this point. One possibility is a conceptual flaw in the current principles scheme of data calibration in Cl CSIA, resulting with problems in accuracy if $\delta^{37}Cl$ data are compared among different compounds. Another possibility is that the observed effects represent genuine kinetic isotope effects associated with CE transformations (so-called secondary effects, cf. Kuder et al., 2013). Both explanations can be partially true.

Isotope Effects in Biological Aerobic Dechlorination of TCE

As indicated previously, the discussion of isotope effects in aerobic biodegradation of TCE is based in large part on results obtained and published outside this project and summarized in recent papers by Gafni et al. (2018 and 2020). Hydrogen isotope effects were determined as part of this study, using degradation experiments samples provided by the Israeli group. Figure 8 (modified after Gafni et al., 2020) illustrates the distribution of C-Cl trends among different cometabolic oxygenases. The bimodal distribution with proportionally weak chlorine isotope effects for toluene and ammonia oxygenases vs proportionally strong chlorine effects for methane oxygenases contrasts with a divergent C-Cl trend of reductive dechlorination.

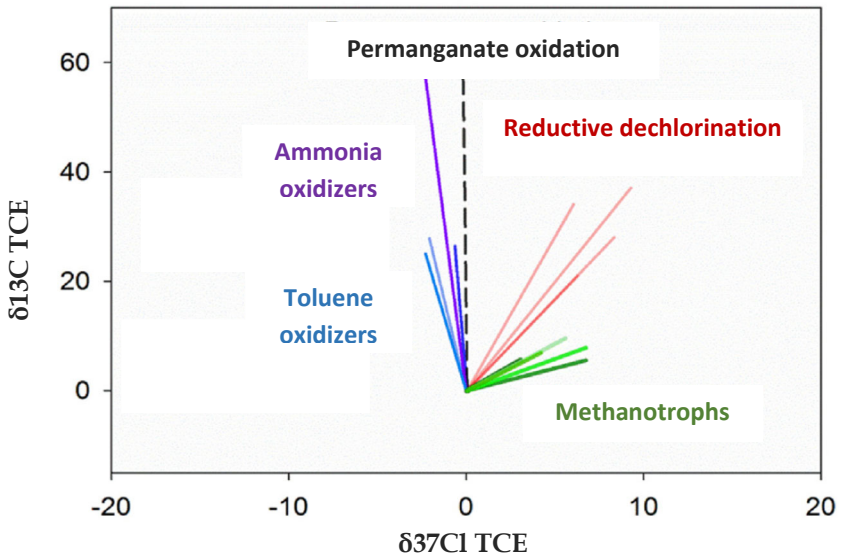

Figure 8. Dual element C-Cl trends in aerobic cometabolic biodegradation of TCE contrasted with reductive dechlorination. Isotope ratios are normalized to zero for the initial TCE isotope composition. Figure modified after Gafni et al., 2020.

Figure 9 shows hydrogen CSIA data for *P. putida* F1 (toluene dioxygenase), two *M. trichosporium* OB3b data sets (expressing soluble and particulate methane monooxygenase, respectively), and *M. capsulatus* Bath expressing soluble methane monooxygenase. None of the cultures produced a strong hydrogen isotope effect and net fractionation of δ^2H exceeded analytical precision of H CSIA only at relatively advanced stages of TCE mass destruction.

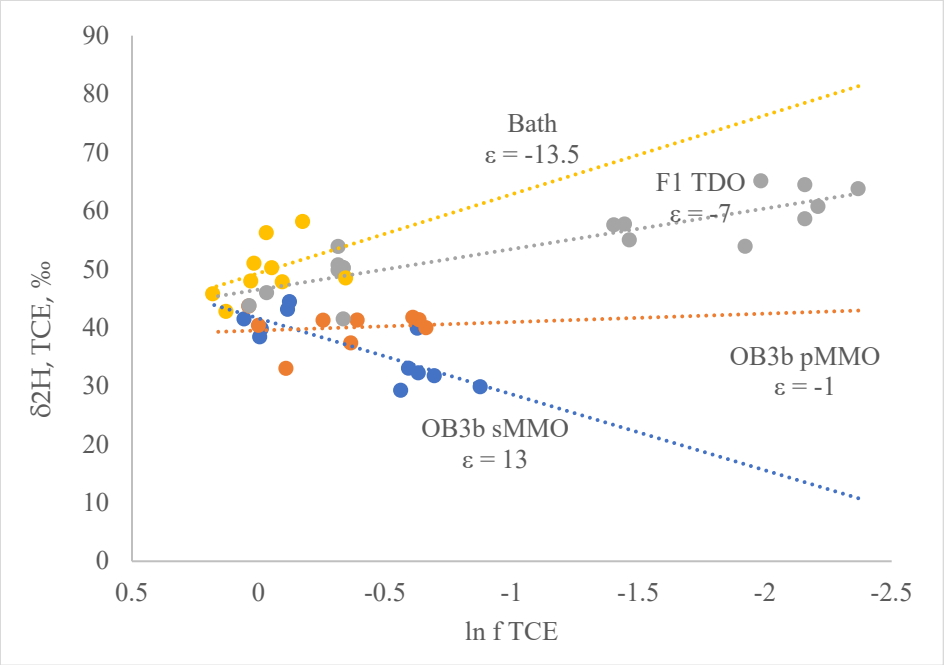

Figure 9. Rayleigh-type plot of hydrogen data from aerobic biodegradation. Note that the regression slope except in OB3b sMMO and F1 TDO are not statistically significant.

Reaction Pathways Discrimination in Application Scenario 1

CSIA evidence for Scenario 1 (CE plume degrading without diagnostic product in oxic or suboxic environment) comes from analysis of the primary compound of interest, TCE. To evaluate the utility of CSIA for this Scenario, patterns of isotope fractionations have been compared between those in abiotic reaction with ZVI and those in biodegradation. The nature of the abiotic degradation in oxic or suboxic aquifers remains elusive. As explained above (Methods, section "Overall design of the study"), the principle of application of C-Cl may still apply if the abiotic degradation mechanism is a Fenton-like reaction rather than β-elimination.

Degradation mechanism discrimination based on dual-element CSIA plots: Figure 8 contrasts the dual element C-Cl trends from the alternative pathways. The ZVI trend is distinct from the aerobic alternatives, suggesting that application of dual-element CSIA should readily differentiate between the TCE degrading abiotically and TCE degraded by any specific type of cometabolic oxygenases. Discrimination of abiotic degradation from by mixed aerobic biodegradation a mix of methane and toluene oxygenases could be more problematic since net isotope effects resulting in a system with contributions from methane oxygenases (proportionally large Cl fractionation) and toluene oxygenases (proportionally small Cl fractionation) could overlap with the ZV reaction-like C-Cl trends.

The significance of hydrogen CSIA data in discrimination cometabolic biodegradation and Fenton-like abiotic degradation is uncertain, pending the availability of H CSIA characterization of the latter pathway. Discrimination of aerobic biodegradation pathways from ZVI-like trends using dual element C-H plots appears implausible without improvement of analytical precision

of hydrogen CSIA and even then, the approach would only apply at advanced stages of TCE mass destruction (>>50% of the initial mass destruction).

Reaction Pathways Discrimination in Application Scenario 2

Scenario 2 focuses on discrimination between abiotic and biological reductive dechlorination. Examples of relevant environmental conditions include ZVI permeable reactive barriers and anoxic aquifers conductive to precipitation of reactive ferrous minerals. Unlike in the previous section, evidence for this Scenario comes from analysis of the primary solvent compound of interest, TCE or PCE, and of the chlorinated degradation products from reductive dechlorination.

Degradation mechanism discrimination based on hydrogen isotope ratios of parent-daughter chlorinated ethenes. The characteristic distribution of δ^2H of TCE and cDCE provides the most persuasive criterion identified in this study for discrimination between abiotic and biological reductive dechlorination mechanisms (Figures 2, 3, and 5). Field scenarios dominated by either biological or abiotic reductive dechlorination processes should be readily identifiable, given the large contract of the respective isotope signature. Field scenarios with contributions from both processes could be addressed by simple mass balance modeling, to assess the relative significance of the biological and abiotic elements. One limitation of the applicability of the approach would be the absence of degradation products attributable to the hydrogenolysis mechanism or very significant predominance of products from biodegradation (or vice versa, but the predominance of abiotic hydrogenolysis products is unlikely). Based on current analytical precision and the scale of biotic-abiotic offset in δ^2H, biotic-abiotic product mixing ratios between 1:10 and 2:10 should be readily accessible to interpretation. Another limitation is the potential for hydrogen isotope signatures of degradation to be superimposed by hydrogen exchange, a distinct possibility if pH exceeds 7 (see Appendix A).

Degradation mechanism discrimination based on dual-element CSIA plots. As indicated in the Background section, the C-Cl has no practical value in discrimination of biotic and abiotic reductive dechlorination. The potential of dual element C-H plots (data obtained in this study, Figure 10) appears to be somewhat more relevant, in that the ZVI reactions seem to show a consistent C-H trend that seems to be divergent from at least some of the individual biological experiments. However, most of the biodegradation data shown in Figures 6 and 7 come from microcosms where TCE was a transient degradation product of PCE. The apparent negative and positive regression slopes for various data subsets may or may not represent kinetic isotope effects of TCE degradation. Instead, the trends may reflect changes in the ^1H vs ^2H selectivity of hydrogenolysis of PCE. The limitations do not apply to scenarios with TCE as the precursor of the dechlorination chain. C-H trends in two degradation experiments meeting this criterion are divergent from those of ZVI (*D. mccartyi* in this project and BDI in Kuder et al., 2013). More confident assessment of the utility of the dual element C-H approach would require a larger reference data set.

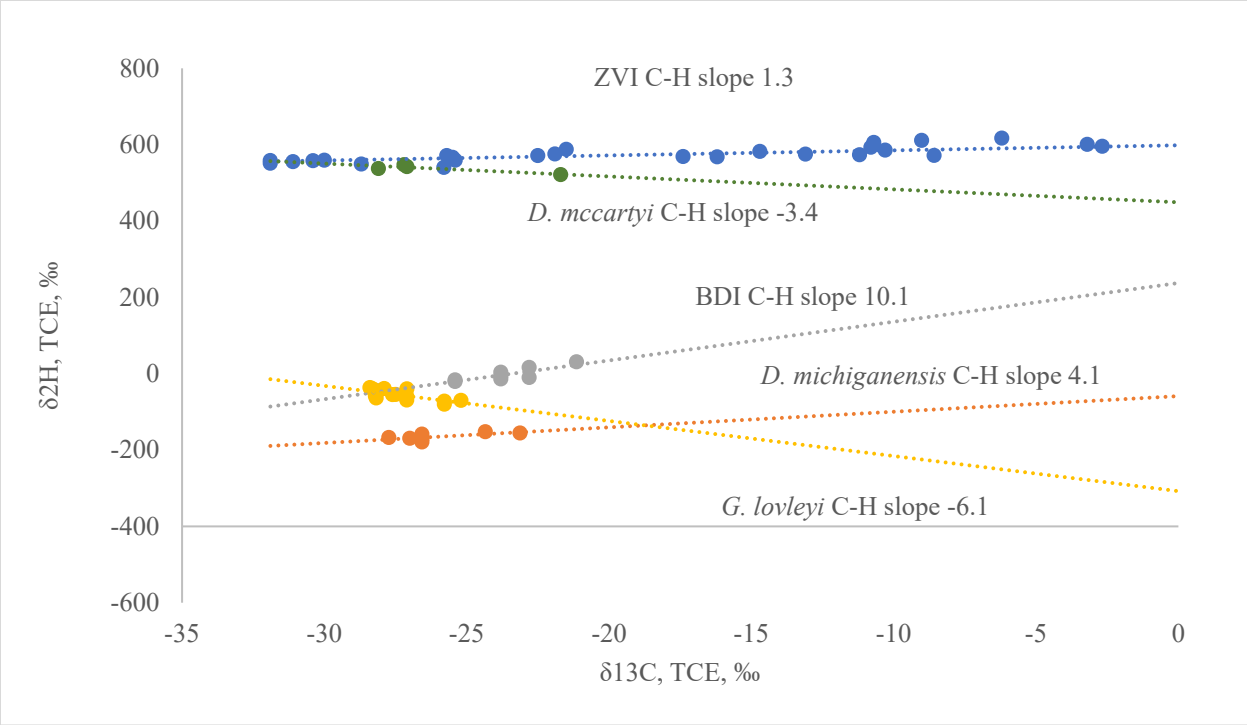

Figure 10. Dual element C-H trends for TCE in biotic and abiotic reductive dechlorination. Note that TCE is the parent compound in ZVI data set and in *D. mccartyi*. Other trends represent TCE being a reaction intermediate. For reference, the slope of the trend reported for degradation of TCE by DBI in 2013 was -2 (Kuder et al., 2013). Based on current analytical precision, none of these slopes (possibly, excepting BDI) would be readily discernible from each other unless degradation progressed significantly to build sufficient net magnitude of fractionation.

Degradation mechanism discrimination based on chlorine isotope ratios of chlorinated degradation products. While chlorine isotope ratios seem to show differences in the parent-daughter CEs offsets among the reactions, the difference between the ZVI reaction and biodegradation is not dramatic. It is clearly discernible only in the earliest phase of degradation. Chlorine CSIA data may be of more significance to constrain the role of commingled contaminant sources, where mixing of contaminant streams with different isotope ratios can complicate deciphering degradation signal (Audi Miro et al., 2015).

Assessment of the Potential for Alteration of Hydrogen Isotope Signatures of Chlorinated Ethenes by Exchange with Water

Evidence based on hydrogen CSIA must be carefully evaluated to eliminate the potential for isotope exchange to distort the degradation signal. Common perception of hydrogen exchange of carbon-bound hydrogen prevalent in isotope earth and environmental science community is that the process is only significant over geological time scales or at extreme geological conditions in deep subsurface. This has been well documented for hydrocarbons components of crude oils

(Sessions et al., 2004). Some early hydrogen isotope publication stated, without adequate experimental data, that the same applies to compounds such as TCE (Shouakar-Stash et al., 2003). In fact, a careful survey of chemical literature yielded reports of rapid TCE-water hydrogen exchange in experiments conducted is relatively harsh conditions of high pH and temperature (Gabricevic et al., 2015). The reaction is a simple acid-base proton exchange, with the dominant base being hydroxide ions and CEs acting as very weak Brønsted acids. The rate of the exchange process was shown to be dependent on the concentrations of hydroxide in solution (higher rates at higher pH), whereas the temperature effect on the rates remained unknown. To validate the conclusions from degradation experiments conducted for this project, hydrogen exchange experiments were conducted to confirm the reaction rates at pH similar to those encountered in groundwater or microcosm media and to determine the temperature. These results are presented in Appendix A as a standalone pre-submission manuscript. The overall message from that study is that abiotic exchange driven by hydroxide concentrations is too slow to impact the present laboratory experiment data. However, hydrogen exchange can be significantly impacting δ^2H of TCE in certain aquifers over timescales of years or decades, which will significantly affect the approach to incorporating H CSIA into field site assessment. In general, hydrogen CSIA data from TCE would be increasingly less reliable as evidence of degradation processes for groundwater pH increasing above 7 and for aquifers situated in warm climates. The present study did not address the hypothetical potential of hydrogen exchange rates augmentation by enzymatic reactions or by mineral surface-associated Brønsted bases (the speculative explanation of the data set presented by Ribeiro, 2016, see Results/Discussion, section "Isotope Effects in Abiotic (ZVI) Reductive Dechlorination"). Further work will be needed to assess such limitations.

CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH

Two lines of evidence are promising and apparently ready for field applications. One is the discrimination between biotic and abiotic reductive dechlorination based on the differences in the extent of δ^2H depletion in dechlorination products, TCE and cDCE. The approach could be employed to assess the efficacy of ZVI remedies and possibly also to identify overlooked abiotic processes in anoxic aquifers conducive to abiotic reactivity with FeS and other ferrous minerals.

The second is the dual-element C-Cl that was proposed as part of this project as promising approach for discrimination of reductive dechlorination (biotic or abiotic) from aerobic cometabolic biodegradation. Note that the C-Cl results discussed above have been obtained by another group (Gafni et al., 2018 and 2020) prior to initiation of aerobic experiments planned for this project. The approach could be implemented at any of the aerobic TCE plumes where aerobic degradation or abiotic degradation is part of or a feasible addition to the conceptual site model. Laboratory validation of the C-Cl trend of the Fenton-like pathway (similar to that described by Schaefer et al., 2018) would be advisable to limit the uncertainty of interpretation. Another possible limitation for the aerobic/abiotic pathway discrimination in the small magnitude of the isotope effect. While the C-Cl scopes attributable to specific pathways may be clearly divergent from each other, low-fractionating processes (such as MMO organisms and likely abiotic Fenton-like reactions) must progress significantly to accumulate enough net isotope fractionation to make them detectable in field samples. If it turns out that the abiotic

pathway active in oxic aquifers are low-fractionating, low yields from such reactions may be hard to catch by CSIA applications. Results from the ongoing study ER20-1368 should help to answer this question.

The results from this project illustrate the benefits of comprehensive, multi-element characterization of CEs. While the issue has not been part of this project, contributions from multiple of sources of CEs can complicate interpretation of degradation pathways. While certain lines of evidence can have no direct value in the discrimination of biotic vs abiotic pathways (e.g., chlorine in the context of reductive pathways), additional dimensions of isotope composition characterization may turn out to be helpful in addressing isotope signature interferences from commingled sources of CEs (Audi Miro et al., 2015). While the cost of analysis is proportionally higher for multi-element CSIA, such data sets are most informative for source discrimination and for degradation pathway assessment.

None of the presented lines of CSIA evidence is a silver bullet in terms of providing a DNA fingerprint equivalent for identification of a specific degradation pathway. Ideally, field site assessment should combine CSIA with other technologies, such as molecular biological tools (MBTs). MBTs can constrain the biological processes, to decrease the challenge for pathway discrimination by eliminating certain endmember reactions. For example, C-Cl separates aerobic biodegradation of TCE by toluene oxygenase organisms from abiotic elimination. C-Cl results can be more ambiguous at sites with significant contributions from the activities of methane monooxygenases. Using MBTS to exclude significant contributions from methane monooxygenases would decrease the overall uncertainty.

Arguably, present results will be significant outside the area of abiotic degradation assessment, mainly by clarifying poorly understood aspects of hydrogen isotope chemistry. Present results falsified certain prevalent environmental forensics assumptions, including the overly simplistic assumption regarding the bimodal distribution of hydrogen isotope ratios (^{2}H-enriched values indicative of TCE origin as manufactured solvent, ^{2}H-depleted values indicative of TCE origin as PCE degradation product). Moreover, better understanding of the role of hydrogen isotope exchange will help avoid misinterpretations in field site assessment, from sites where exchange processes overprinted contaminant source or degradation signatures.

Results to date open an avenue for fundamental and applied research on mechanisms of hydrogenation of reductive dechlorination products. Implementation of hydrogen isotope analysis could yield valuable insight into reductive dechlorination enzymes involved in the biological transformations and answer fundamental questions on the mechanism of product hydrogenation in ZVI and reduced iron species in general.

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APPENDIX A

HYDROGEN ISOTOPE EXCHANGE BETWEEN TRICHLOROETHENE AND WATER UNDER MILD ENVIRONMENTAL CONDITIONS – IMPLICATIONS FOR THE USE OF HYDROGEN CSIA IN CONTAMINATED SITE ASSESSMENT

(Intended to be submitted to Env. Sci. Tech Letters)

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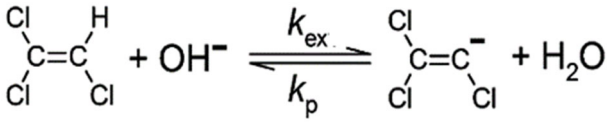
Introduction

Hydrogen isotope exchange between halogenated hydrocarbon (methanes and ethanes) and water is well-documented in chemical literature, with initial reports dating to 1940s.[1, 2] The reaction is an acid-base proton exchange, with hydroxide ion (HO^-) acting as a Brønsted base, and the halogenated compound acting as a Brønsted acid (Scheme 1). All published reaction rates were obtained at high pH and/or at high temperature. Assuming the reaction rates scale down with decreasing HO^- concentrations, extrapolation of the reported rates to typical groundwater pH suggests that even with net rate decreasing by several orders of magnitude, the process could be impacting isotope composition of compounds such as Trichloroethene (TCE), at the time scales of residence in groundwater (years to decades).

Scheme 1



Given the significant interest in the use of stable isotope techniques in the assessment of the environmental fate of halocarbons, it is surprising that the role of hydrogen exchange has not been recognized in the context of environmental contaminant research. Stable isotope data have been utilized in environmental contaminant assessment projects for over two decades.[3] While the advent of analytical techniques for hydrogen compound-specific isotope analysis (H CSIA) of halocarbons is more recent,[4-7] $^2H/^1H$ data have been utilized by environmental forensic consultants and remediation industry for nearly a decade, primarily as a line of evidence for apportionment of TCE sources.[8] The scientific basis for those H CSIA applications is limited, with few peer-reviewed studies to constrain the distribution of hydrogen isotope compositions of manufactured TCE, characterize hydrogen isotope effects applicable to TCE transformation or production, or characterize isotope compositions of TCE at field sites.[9, 10 11-13 14, 15] None of the existing papers adequately addressed the data quality pitfalls caused by hydrogen exchange.

The present study is to clarify the limitations of H CSIA data in environmental forensic application. While the potential of the exchange impacting certain H CSIA applications appears to be well-supported by the existing literature, it is important to obtain more direct data, to illustrate the reaction rates under pH and temperature conditions typical of those with those prevalent in contaminated aquifers. Accordingly, the primary objectives of the present study were: 1) to characterize the rates of hydrogen exchange of TCE under laboratory conditions permitting direct extrapolation of the results to groundwater conditions; and 2) to evaluate the applicability of H CSIA applications in the assessment of the sources and the fate of chlorinated ethenes at contaminated sites.

Experimental

Experimental design. Literature data highlight the rates of isotope exchange depend on HO^- concentrations. Moreover, rates can be expected to respond to solution temperature, based on the postulated high activation energy barrier. On the other hand, TCE concentrations and co-solutes affecting ionic strength of the medium were shown to have no effect. Accordingly, incubations were set for a range of pH values and temperatures, to isolate the respective reaction kinetic effects (Table 1).

Incubations. Table 1 lists pH-temperature-water isotope composition treatments. 10 ml aliquots of aqueous TCE (270 mg/l) were incubated in replicate 20 ml serum vials, with PTFE-lined gray butyl rubber septa sealed with crimp caps. The solutions were buffered with TRIS and CAPS buffers, respectively. For most of the pH-temperature treatments, two isotope compositions of water were used, deionized water at δ^2H = -50 ‰, and water labeled by ^2H_2O at δ^2H = +26500 ‰. Vials were incubated in the dark, upside-down, using a GC oven (Agilent 6890) to control the temperature. The 20ºC experiment was conducted at the ambient temperature of the laboratory. Samples were harvested over time by repeated sampling of the vials, using a disposable syringe to withdraw 0.5 ml of medium through a septum, followed by dilution and acidification in VOA vials for refrigerated storage. Vials incubated at high temperatures were chilled prior to sampling and recapped after sampling. This resulted with certain losses of TCE over time, but the effect was inconsequential due to the approach chosen to quantify the extent of the exchange (see below).

Analytical chemistry. Aqueous samples were analyzed after purge and trap extraction (model O&I 4660). The analysis used a modified low temperature chromium reduction approach, described previously.[4] Detailed description of the method is included in the Supporting Information.

Calculations. Numerical values of $^2H/^1H$ are reported as δ^2H (delta notation), following the conventions of environmental contaminant literature. The isotope ratios served to calculate the exchange fractions (f) over time, following Equation 1 with δ_0 representing the initial isotope composition of TCE, δ_{EQ} representing the isotope composition of TCE in equilibrium with water, and δ_t representing the isotope composition of TCE at time t. Rate constants (k) were obtained following a standard first-order kinetic model, using f rather than absolute concentrations of TCE isotopologues. This approach simplified the experimental logistics, since the same vials could be sampled repeatedly, with no adverse effect of absolute TCE concentrations decreasing over time.

Eq. 1 $$f = (\delta_t - \delta_{EQ})/(\delta_0 - \delta_{EQ})$$

Results and Discussion

Reaction kinetics. All experiments yielded readily measurable changes of $^{2}H/^{1}H$ of TCE, with the isotope composition of TCE trending towards the isotope signature of water (Figure 1). Equilibrium of the exchange was observed only for the faster reaction at higher pH and temperature, with $\delta^{2}H$ of TCE at -140 ‰, vs. -50 ‰ of water.

Reaction rates were calculated for the six data of obtained for specific combinations of pH and temperature, after conversion of the TCE isotope ratios to the fraction of isotope exchange (cf. Eq. 1 and Figure 2). For specific pH and temperature, rate constants in ^{2}H-labeled and in unlabeled water were similar (data from both setups were pooled in Figure 2). Pseudo first-order kinetics was apparent for all data sets, as expected for the postulated reaction (Scheme 1). Table 1 summarizes the rate constants obtained (k_{obs}). Based on the premise of first-order relationship between the rate and the HO^{-} concentration, the values of k_{obs} were normalized to pH=7 to investigate the so far unknown temperature dependence. Figure 3 illustrates a strong Arrhenius-type correlation between the rate and the temperature, and a significant activation energy barrier (E_a 109 kJ/mol), which is higher than that postulated previously. A very good agreement of the present results with those obtained by Gabričević et al.[2] suggests a continuum for pH range of 7.2 to 14, which can be reasonably extrapolated towards somewhat lower pH values encountered in certain groundwaters.

An auxiliary data set was also obtained for cis-1,2-Dichloroethene (cDCE). The observed rates of the exchange were significantly below those of TCE under matching pH and temperature (see Supporting Information).

Fate of $^{2}H/^{1}H$ of TCE residing in groundwater. In a hypothetical scenario of a spill of ^{2}H-depleted TCE, where the respective $\delta^{2}H$ values of TCE and water are close to the exchange equilibrium, there could be no measurable TCE effect. However, since most TCE products are ^{2}H-enriched ($\delta^{2}H$ > +400 ‰), whereas groundwater $\delta^{2}H$ varies from close to zero (coastal areas) to well below -100 ‰ (continental interior), the net changes of TCE $\delta^{2}H$ upon equilibration with water to would be comparable to those in the present experiments with unlabeled water (Figure 1A). Local rates of the hydrogen isotope exchange would be highly variable. Assuming HO^{-} is the only significant strong Brønsted base in solution, available to drive the reaction, the exchange half-life can be predicted using local pH and temperature (Figure 4). Four half-life intervals allow for near-complete exchange, with the measurable isotope composition of TCE becoming similar to that of water, with the equilibrium reached after five intervals. Less than two half-lives are required to mask the strongly positive $\delta^{2}H$ signatures of typical manufactured TCE. Notably, even a minor fraction of exchange, at 10%, can be sufficient to shift $\delta^{2}H$ of TCE by more than several tens of ‰, provided the contrast of isotope composition of TCE vs that of water is similar to that in the present study (Figure S1).

Generally, sites with groundwater temperatures typical of those in most of the Southern US and with pH above neutral would be subject to very rapid equilibration of hydrogen signatures, with exchange half-lives of potentially well under 10 years, and with significant changes of TCE $\delta^{2}H$ occurring in well under a year of residence in groundwater. On the opposite end of the spectrum, the exchange would be much slower at sites with acidic groundwater. For example, the half-life at pH=6 and temperature of 5ºC would be slightly over 1,700 years. Even under such unfavorable conditions, measurable changes of if $\delta^{2}H$ would be expected to occur after several

decades of residence in groundwater. On the other hand, slow reaction rates for cDCE suggest the compound in groundwater would not be significantly affected .

Isotope exchange affects the scope of interpretation of hydrogen TCE data. Textbook scenario for the use of H CSIA data relies on the δ^2H contrast between manufactured TCE products and the ^2H-depleted TCE formed as a product of dechlorination of Tetrachloroethene. [8] Accordingly, ^2H-enriched TCE detected at a site is the evidence of a spill of TCE solvent, whereas ^2H-depleted TCE indicates TCE is a degradation product. Sample-to-sample differences in δ^2H of tens to hundreds ‰ are interpreted as end-member mixing, suggesting multiple sources of TCE dissolved in groundwater at the site. Once the hydrogen isotope exchange is considered, this interpretation template is clearly too simplistic. Detections of the highly ^2H-enriched TCE signatures that are consistent with the typical manufactured TCE remain informative. However, significant shifts of δ^2H due to the exchange alone can be relatively rapid, causing depletions of δ^2H of tens or hundreds of ‰. Spatial differences in δ^2H may simply reflect minor pH fluctuations over distance or variable duration of TCE residence in groundwater, not different source signatures. Specific conclusions on end-member mixing appear problematic, unless the local groundwater environment is clearly inconducive to the exchange (tentatively, with groundwater pH well below 6.0-6.5, depending on local temperatures; cf. Figure 4) or unless the site history is well known, to constrain the time of residence of TCE in groundwater. In contrast, the isotope signatures of cDCE appear to be immune to the exchange phenomena, at least at the time scales of interest of contaminant research.

Published work on hydrogen isotope ratios of TCE should be reevaluated to address the impact of hydrogen isotope exchange. This applies to all field data sets published to date, [13-15] and to laboratory experiment results, where long-time incubations were performed without adequate control of pH. [16]

Potential for novel applications of hydrogen TCE data. The positive spin on the role of the hydrogen exchange is that it can potentially provide means to age-date TCE contamination. While accurate dating could be challenging, it could be feasible to project conservative maximum residence time in groundwater, for specific temperature and pH of ambient water. The application would estimate the time of residence in groundwater rather than the date actual spill of TCE solvents. HO^- activity within dense non-aqueous phase liquid (DNAPL) is restricted by low solubility of water into DNAPL. We speculate that TCE would undergo negligible exchange prior to dissolution from DNAPL into ambient water. Under favorable hydrogeological conditions, such results could help to detect subsurface DNAPL pools or to age-date the residence of TCE in specific compartments of a contaminant plume. More research would be required to advance the concept to contaminated site assessment applications. The present study does not answer several important questions, including on the behavior of TCE hosted in low permeability sediment (does sorption of TCE exert steric protection from contact with HO^-?) and on reactivity with other Brønsted bases (such is HS-, which is a weaker base, but locally present at significant concentrations), including the potential for enzymatic catalysis of the exchange.

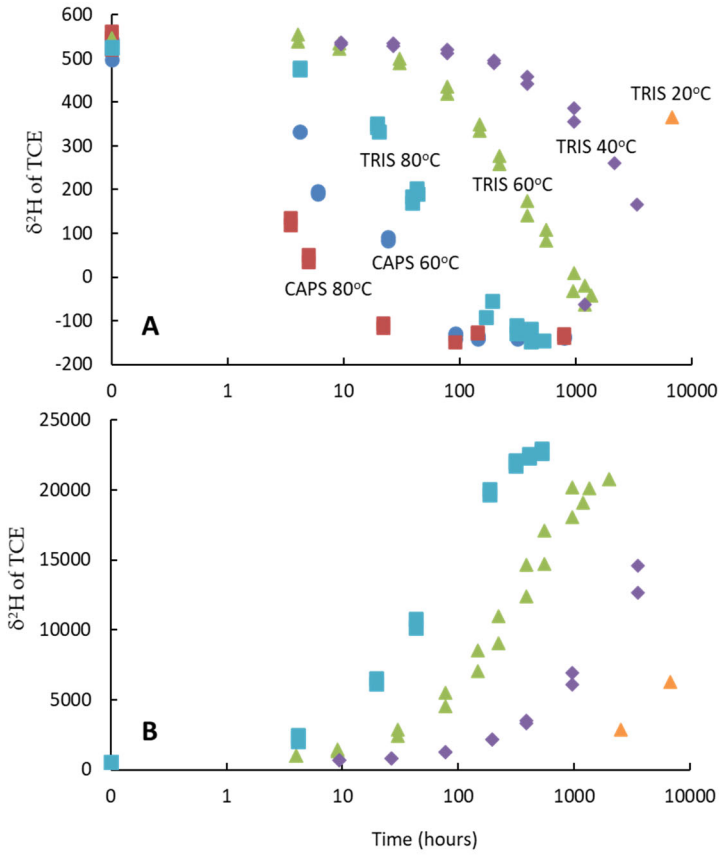


Figure 1. Evolution of δ^2H of TCE over equilibration with (A) water at d2H = -50‰; and (B) water at δ^2H = +26500‰. Time T=0 hrs is recorded as 0.1 hrs, to permit the use of the log scale of the time axis. Data subsets are labeled by the respective pH buffer and temperature.

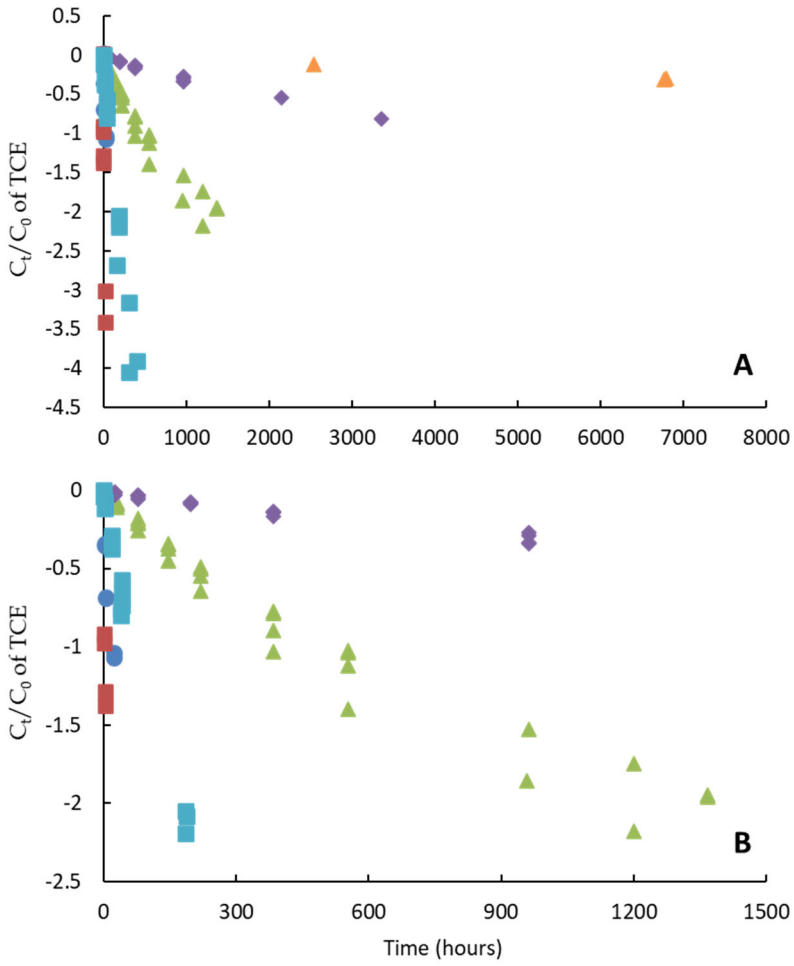


Figure 2. Fractional progress of the exchange over time (A), and the same rescaled for better visibility of the data at the early stages of the exchange (B), with C_t/C_0 calculated following Eq. 1. See Figure 1 for data subsets identification. Incubations with the same temperature and pH but using different isotope composition of water are not separated. Deviations from the ideal regression lines, specifically for sample sets obtained from high temperature incubations, could be rationalized by minor inaccuracies of the temperature control over time and by increasing impact of headspace TCE losses for samples taken in the later stages of the incubation due to septum leaks.

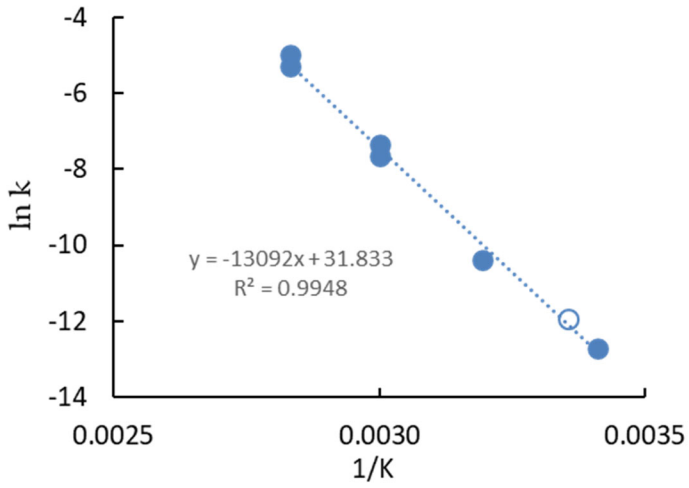


Figure 3. Arrhenius plot of data normalized to pH=7, including those reported by Gabričević et al., indicated by the hollow marker. See Table 1 for numerical data.

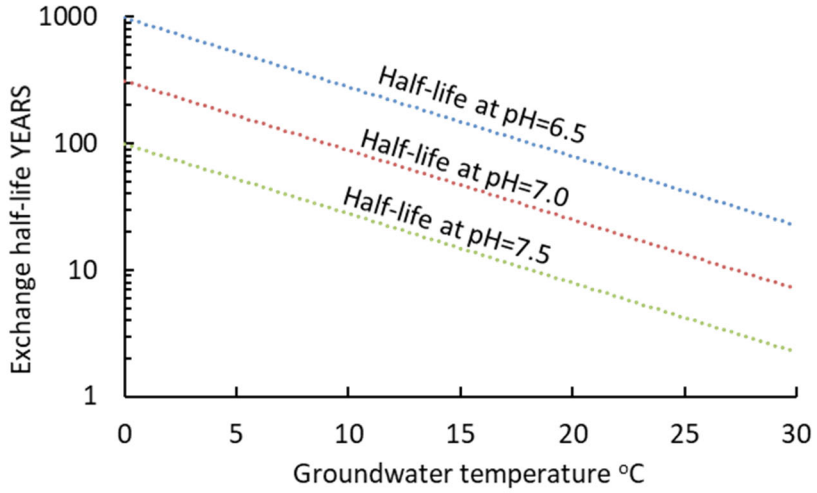


Figure 4. Half-lives of the exchange as a function of groundwater pH and temperature.

Supporting Information

Determination of δ^2H of TCE. Aliquots of the harvested samples were diluted to 100-200 ug/l for analysis and TCE was extracted from water using a purge and trap model O&I 4660, with He purge of 12 ml x 40 ml/min at 40ºC. VOCs were deposited on K-type adsorbent rap (Supelco). Complete sample transfer from the purge and trap to the GC was achieved, using the inlet configuration described previously.[4] The complete sample recovery permitted minimizing the primary sample volumes and thus it permitted the repeated sampling of individual incubation vials. GC (Agilent 7890) was set up with a 60 m x 0.32 mm DB-MtBE column. The following program omits initial 22 minutes (the purge and trap cycle and sample transfer into the cryogenic focuser. Initial oven temperature was 40ºC, held for 8 minutes, and then ramped at 6ºC/min until the end of the data acquisition, followed by rapid increase to 230ºC and 15 min bakeout. Ramped carried gas flow was applied, with the flow rate set at 1.5 ml/min, followed by a decrease to 0.8 ml for the time interval coinciding with deactivation of the backflush valve immediately prior to and after the transit of TCE through a reduction reactor tube towards the MAT 253 detector. Thermal conversion followed a modified analytical protocol described previously. [4] The horizontal reactor oven was held at 850ºC. The reactor tube was a standard hollow ceramic tube with a stainless-steel capillary extension at the tube inlet (the "hydrogen CSIA" tube from Thermo Scientific), with 18 cm bed of Goodfellow chromium granules positioned at the tube inlet and held in place by a quartz glass plug with Tungsten wire v-shaped clip. Reactor tube effluent was passed through several coils of adhesive particle trap capillary (Restek) and then though a loop of inert capillary immersed in liquid nitrogen. The setup served as a fail-safe to protect the GC/IRMS interface from HCl and/or particulates that were potentially released from the reactor tube.

Data processing. Raw δ^2H calculated by the Isodat software and normalized to the instrumental reference H_2 were reprocessed, using the bracketing analyses of Control Samples (CS). The CS were prepared with TCE of known δ^2H of +550‰ (the same lot of TCE was used to prepare the incubation vials). CS were included into each analytical sequence, to bracket the results from the unknowns. The bracketing interval was before and after not more than three unknows. Additional CS prepared using a lot of ^2H-depleted TCE (δ^2H of -85‰ obtained using the +550‰ anchor) were included to validate the consistency of δ^2H linearity among data subsets obtained over extended period (Figure S1). As the objective δ^2H value of the ^2H-depleted is not known, the depleted standard was not used for 2-point calibration.

Data precision and accuracy. Precision of the purge and trap-CSIA with low-temperature chromium reduction method varies, mainly depending on the target compound, the condition of the reactor tube and to lesser extent, of the purge and trap adsorbent. For TCE, the typical range of precision of δ^2H is ±10-20‰, defined as maximum offset of individual measurements from the known reference value. Note that this definition does not rely on standard deviation of n replicates. The precision for the present data set was validated using the CS results. Figure S1 shows the distribution of the CS data. Out of the 171 analyses, 151 fell within ±10‰, and only one exceeded ±15‰. Several CS analyzed following the highly enriched samples from ^2H-labeled incubations exceeded the typical precision. Since the cause was identifiable (apparent memory effect, see below) the affected CS and certain exchange experiment samples analyzed following the highly enriched samples were excluded from the data set. Accuracy of the results for the highly ^2H-enriched samples has not been determined. While water medium was enriched

to δ^2H of +26500‰, the results from TCE at equilibrium leveled at ~ 22500‰. The labeled water analyzed via direct injection, using the same Cr reactor, showed a similar result rather than the expected value of +26500‰. One possibility of the apparent discrepancy is a compression of the isotope scale due to non-linear detector responses. From practical point of view, the issue is not significant since the bias is does not impact the result from data processing following Eq. 1. The lack of 2-point calibration is more problematic regarding the δ^2H value of TCE in with water. TCE at isotope equilibrium is at approximately -140‰ (with the calibration anchor at +550‰). In theory, the use of a single calibration anchor allows for bias propagation if the linearity of the detector deviates from the ideal. However, the same instrument was also calibrated using USGS C16 standards, without showing an indication of a detector bias between -166 to +381‰. ^{2}H/^{1}H-dependent bias propagation by the thermal conversion is unlikely, since such bias should follow the typical Raileigh-type fractionation principles, where the net isotope effect is not affected by the initial isotope composition of the substrate. Such TCE-specific bias would be corrected by means of the single anchor point.

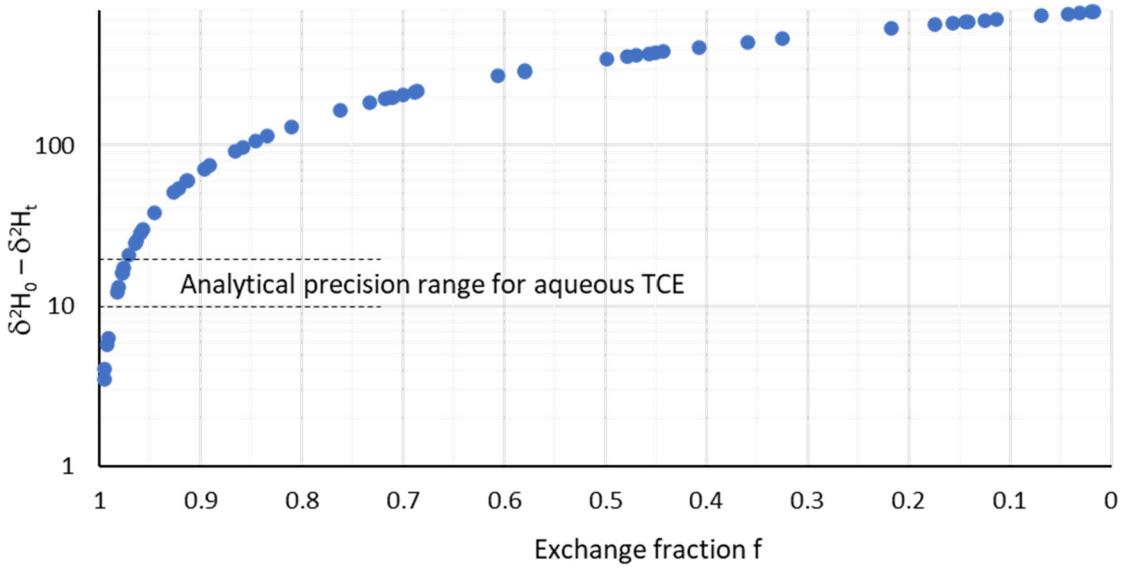


Figure S1. Net change of δ^2H of TCE as a function of exchange fraction, based on the results from exchange with non-labeled water, at δ^2H difference between water and TCE of 600 ‰. The results shown are for all experiments with TRIS buffer. The precision range is based on the low-temperature chromium method applied to relatively dilute samples with purge and trap preconcentration.

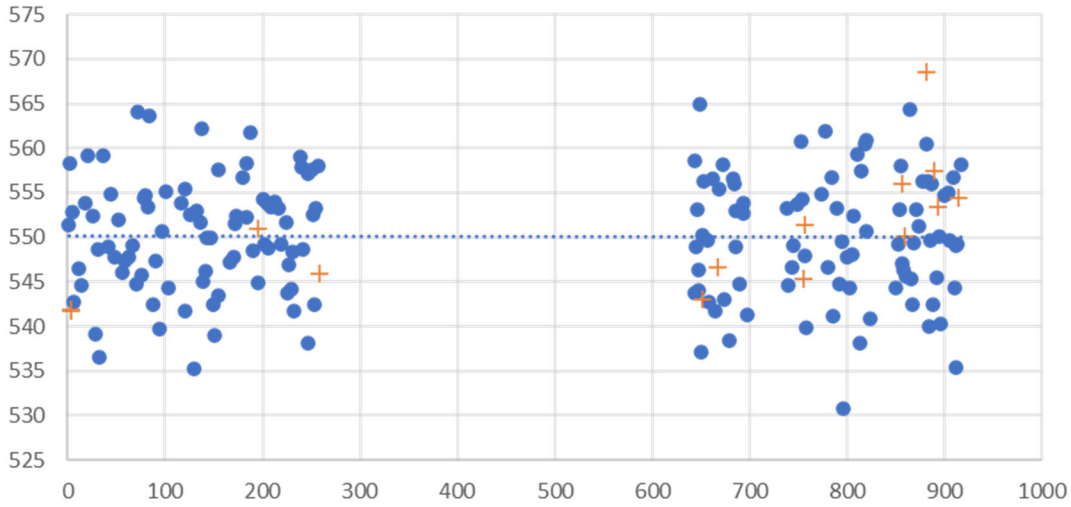


Figure S2. TCE control samples, d2H in consecutive analyses, ; + symbols indicate the depleted TCE controls, with their d2H values adjusted by +635 to match the average of the primary control sample dataset.

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